HARNESSING PATIENT-DERIVED ORGANOIDs AND MICROFLUIDICs TO INVESTIGATE CHOLINERGIC REGULATION OF THE EPITHELIAL BARRIER

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By

Sanjin Hosic

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ABSTRACT

Two decades ago, it was demonstrated that electrical vagal nerve stimulation (VNS) inhibits gastrointestinal (GI) inflammation. In-vivo studies concluded that VNS inhibits GI inflammation by releasing neurotransmitter acetylcholine (ACh) from efferent vagus nerve fibers which binds intestinal macrophage nicotinic acetylcholine receptors (nAChRs), inhibiting pro-inflammatory cytokine tumor necrosis factor alpha (TNF-\(\alpha\)) production. The next decade of research demonstrated that ACh activated intestinal epithelial cell (IEC) muscarinic acetylcholine receptors (mAChRs) to ameliorate epithelial barrier integrity. However, several contradictory studies were published. One plausible explanation for the discrepancies is differential mAChR expression and/or biological function between GI cell lines. Evidently, our cellular understanding of cholinergic regulation of the intestinal epithelium is in its infancy. Nevertheless, bioelectric medicine may potentially augment inflammatory bowel disease (IBD) treatment, warranting further investigation of cholinergic regulation of the intestinal epithelium.

This research leveraged primary human organoids and microfluidics to develop physiologically relevant models for studying cholinergic regulation of intestinal epithelial barrier integrity. Primary human intestinal organoids were dissociated and seeded on Transwell inserts. The primary epithelium exhibited functional differences as compared to immortalized epithelium: increased barrier integrity, increased and polarized interleukin 8 (IL-8) secretion, and the presence of both absorptive enterocytes and secretory goblet cells. TNF-\(\alpha\) was used to model an inflamed state exhibiting increased paracellular permeability, apoptosis, and basal IL-8 production. Quantitative
immunofluorescent image segmentation and analysis demonstrated that TNF-α reduced epithelial integrity through both apoptosis and tight junction (TJ) rearrangement while epithelial cells adopted a distinct phenotype that sealed TJs. Though the primary epithelium expressed the previously implicated subtype M₃ muscarinic receptor, neither nAChR nor mAChR activation inhibited TNF-α induced inflammation as previously described. However, mAChR activation decreased transcellular transport of a 70 kDa dextran. Temporally resolved experiments confirmed that mAChR activation of primary epithelium does not augment the shedding of TNF receptor 1 (TNFR1) to attenuate TNF-α signaling as previously described. The results demonstrated a first application of primary human organoid technology toward studying cholinergic regulation of intestinal epithelial barrier integrity.

Microfluidic cell culture devices, termed organ chips, lend advantages to traditional static Transwell culture. An organ chip manufacturing technique was developed, circumventing cost, throughput, and scalability disadvantages of traditional microfabrication. The technique was validated by culturing Caco-2 monolayers in a bilayer architecture and by integrating primary monolayers and organoids in a novel tri-layer architecture. The results represent a novel toolbox for studying intestinal epithelium and fabricating organ chips.
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LIST OF ABBREVIATIONS AND NOMENCLATURE

1. ENS: Enteric nervous system
2. IEC: Intestinal epithelial cells
3. EGC: Enteric glial cells
4. VNS: Vagus nerve stimulation
5. ACh: Acetylcholine
6. α7nAChR: alpha-7-nicotinic acetylcholine receptor protein
7. TJ: tight junction
8. 3D: 3-dimensional
9. ECM: extracellular matrix
10. EGF: epidermal growth factor
11. 2D: 2-dimensional
12. PDMS: polydimethylsiloxane
13. IBD: inflammatory bowel disease
14. GI: gastrointestinal
15. LPS: lipopolysaccharide
16. TNF: tumor necrosis factor
17. mRNA: messenger RNA
18. IL-1β: interleukin 1 beta
19. IL-6: interleukin 6
20. IL-18: interleukin 18
21. MLCK: myosin light-chain kinase
22. NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells
23. GFAP: glial fibrillary acidic protein
24. GSNO: S-nitroso glutathione
25. CCh: carbachol
26. VIP: Vasoactive intestinal peptide
27. ZO-1: Tight junction protein 1
28. TEER: Transepithelial electrical resistance
29. TGF-β: Transforming growth factor beta
30. IFN-γ: Interferon gamma
31. GPCR: G-protein coupled receptor
32. BET: betahanechol
33. HRP: horseradish peroxidase
34. ATR: atropine
35. TTX: tetrodotoxin
36. CRF: corticotropin-releasing factor
37. UC: ulcerative colitis
38. FCS: fetal calf serum
39. HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
40. FAK: focal adhesion kinase (FAK)
41. ERK: extracellular signal-regulated kinase (ERK)
42. siRNA: small interfering RNA
43. COX-2: Cyclooxygenase-2
44. TNFR1: TNF receptor 1
45. TACE: TNF-α converting enzyme
46. MAPK: mitogen activating protein kinase
47. EGFR: epidermal growth factor receptor
48. MLC: myosin light chain
49. LGR5: Leucine-rich repeat-containing G-protein coupled receptor 5
50. NGF: Nerve growth factor
51. TGF-β1: transforming growth factor beta 1
52. NCC: neural crest cell
53. CAD: computer aided design
54. UV: ultraviolet
55. PET: polyethylene terephthalate
56. CYP3A4: cytochrome P450 3A4
57. LGG: Lactobacillus rhamnosus GG
58. RT-qPCR: real-time quantitative polymerase chain reaction
59. ADME: absorption, distribution, metabolism, and excretion
60. CNC: computer numerical control
61. PC: polycarbonate
62. PBMC: peripheral blood mononuclear cell
63. PMMA: poly(methyl methacrylate)
64. ATCC: American Type Culture Collection
65. DMEM: Dulbecco’s Modified Eagle Medium
66. FBS: fetal bovine serum
67. PBS: phosphate buffered saline
68. EDTA: ethylenediaminetetraacetic acid
69. ROCK: Rho-associated, coiled-coil containing protein kinase
70. AP: Alkaline phosphatase
71. ELISA: enzyme-linked immunosorbent assay
72. TRITCH: tetramethylrhodamine
73. DAPI: 4′,6-diamidino-2-phenylindole
74. MUC2: mucin 2
75. SEM: standard error of the mean
76. ANOVA: analysis of variance
77. HIMEC: human intestinal microvascular endothelial cell
1. INTRODUCTION

The human small intestine is a truly multi-tasking tissue. The small intestine’s basic functions are digestion, absorption, secretion, motility, and protection from external pathogens. Many of these tasks are executed by a single layer epithelium that is stratified on a crypt-villus architecture. The single layer epithelium consists of a variety of specialized cells that perform discrete tasks: enterocytes, goblet cells, Paneth cells, enteroendocrine cells, transit amplifying cells, and stem cells. Enterocytes are the most prominent cell lineage of the small intestine and regulate the digestion and transport of molecules at the intestinal lumen. Goblet cells are the second most prominent cell lineage in the small intestine and secrete mucins, large proteins that form a 10^2 µm thick mucus layer that lubricates and protects the epithelium. The remaining IEC lineages also perform highly specialized functions, but it is their coordinated function that enables intestinal homeostasis.

Beyond these basic functions, the intestine is also responsible for bidirectional gut-brain communication via the enteric nervous system (ENS), a vast network of 200-600 million neurons and enteric glial cells (EGCs). The majority of ENS neurons and glial cells are found in the myenteric and submucosal plexus though cellular extensions protrude to the single layer epithelium. The importance of neural regulation of the human intestine has been studied in various contexts, including inflammation. Using primarily murine models, several studies demonstrated anti-inflammatory properties of the vagus nerve in the small intestine. Researchers agree that electrical vagus nerve stimulation (VNS) induces a local anti-inflammatory response by inducing the ENS to secrete acetylcholine (ACh) which subsequently targets intestinal macrophage associated α7-
nicotinic acetylcholine receptor protein (α7nAChR) via the α7nAChR cholinergic anti-inflammatory pathway. However, other studies established that α7nAChR is widely expressed in non-neuronal cells such endothelial and epithelial cells. Given that IECs are critical regulators of intestinal homeostasis, VNS induced activation of IEC ACh receptors may critically affect gut health but is unexplored. The reliance on animal models precludes the systematic study of VNS and epithelial specific mechanisms due to the complexity of the multicellular environment in the intestine. Furthermore, biological differences between human and murine species limit study relevance to humans. Thus, human tissue derived in-vitro or ex-vivo models of the intestine that enable heterogeneous cell co-culture would greatly facilitate the study of cholinergic modulation of intestinal inflammation.

The most common in-vitro models of the intestine are human colorectal derived cells, typically Caco-2 or HT-29, cultured on extracellular matrix (ECM) coated porous membranes. Caco-2 and HT-29 cells spontaneously differentiate after extended culture on porous membranes. Differentiated Caco-2 express some morphological and biological characteristics of small intestinal enterocytes such as monolayer formation, polarization, tight junction (TJ) expression, apical microvilli, and small intestinal enzyme activities on the apical cell surface. The differentiated HT-29 phenotype also models small intestinal enterocytes, except HT-29 cells produce mucin like intestinal goblet cells. Nevertheless, both Caco-2 and HT-29 are malignant cell types and it is unclear whether these cells faithfully represent native intestinal epithelium. Ex-vivo models utilizing human intestinal explants are high fidelity models of in-vivo tissue but this approach is hindered by a short lifespan and tissue availability, particularly for experimental replicates.
Furthermore, whole tissue explants are relatively complex due to multicellularity. Primary human intestinal epithelial tissue culture remained elusive, until the recent advent of 3-dimensional (3) organoid cultures, which have revolutionized the field.

Primary human intestinal organoid cultures are derived by embedding intestinal stem cells, found in intestinal crypts, within a 3D extracellular matrix (ECM) Matrigel. The cell culture medium is supplemented with particular growth factors that enable in-vitro stem cell maintenance and expansion: EGF, Wnt, R-spondin, and noggin. Intestinal organoids have numerous advantages. Organoids contain all of the cell lineages found in the native intestine, can be indefinitely passaged, and can be cryogenically stored for later use. Thus, primary human organoids are both scalable and more physiologically relevant compared to traditional immortalized cell models. However, organoids form closed lumen cystic structures surrounded by ECM which complicates luminal and serosal analysis. Thus, organoids are limited for studying epithelial barrier integrity. This limitation has been recently overcome by the development of 2-dimensional (2D) monolayers derived from primary human organoids.

Primary human organoid monolayers are formed as 2D monolayers on porous membrane inserts, termed Transwells, with distinct apical and basal culture medium compartments. This culture mode enables the facile assay of epithelial barrier integrity and compartmentalized co-culture of varying cell populations. Microfluidic cell culture devices, termed organ chips, lend additional advantages to traditional Transwell culture. Organ chips contain continuously perfused chambers which can directly contribute to intestinal function and allow for the co-culture of cell types requiring different medium or oxygen tension. Microfluidic organ chips are predominantly manufactured via
polydimethylsiloxane (PDMS) soft lithography, which has some functional advantages but also some functional and significant manufacturability disadvantages. Today’s 3D printers, CNC mills, cutter plotters, and laser cutters are competitive techniques for microfluidics manufacturing, albeit with reduced resolution. But in the future, these rapid prototyping techniques may enable scalable manufacturing of organ chips, driving both commercialization and technological development.

This work seeks to leverage primary human organoids and microfluidics to develop more physiologically relevant models of the intestinal epithelium for studying cholinergic regulation of epithelial barrier integrity. This dissertation discusses research with the following 3 aims: (1) develop a primary organoid derived model of small intestinal epithelium in healthy and diseased state, (2) investigate cholinergic amelioration of epithelial barrier integrity in a diseased state, and (3) develop a low cost, rapid, and scalable microfluidic organ chip platform that supports primary organoid derived epithelium for future work.
2. LITERATURE REVIEW

Our current understanding of cholinergic modulation of epithelial barrier integrity and/or inflammation is primarily based on murine models and immortalized cell derived in-vitro models (Section 2.1). Due to multicellular complexity, in-vivo models preclude the systematic study of epithelial specific processes. Biological differences between primary and malignant cells limit in-vitro study relevance. Recently developed primary human intestinal organoids (Section 2.2) are well suited for high fidelity modeling of the intestinal epithelium. Furthermore, microfluidic organs-on-chips (Section 2.3) confer additional advantages to modeling the intestinal epithelium.

2.1 Cholinergic Modulation of Gastrointestinal Inflammation and Epithelial Barrier Integrity

The current treatments for inflammatory bowel disease (IBD) are primarily biological and target the body’s immune cells; corticosteroids, thiopurines, methotrexate, anti-tumor necrosis factor inhibitors, and monoclonal antibodies target the body’s B cells and T lymphocytes to reduce inflammation\(^8,9\). These current therapies can have side effects or limited efficacy\(^8,9\). For example, patients undergoing corticosteroid treatment may develop diabetes, osteoporosis, steroid-induced psychosis, steroid dependence, and/or opportunistic infections\(^9,10\). Similarly, thiopurines may be toxic to the liver and lead to lymphoma\(^9,11\). Side effects of methotrexate include nausea, anorexia, diarrhea, bone marrow suppression hepatic fibrosis, and/or hypersensitivity pneumonia\(^9,12\). Furthermore, methotrexate is a teratogen thereby limiting its use in the pregnant population\(^13\). The prevalence of side effects associated with current treatment options motivate the need for alternative IBD therapy.
The parasympathetic nervous system contains an anti-inflammatory pathway which inhibits the release of pro-inflammatory cytokines. Direct vagus nerve stimulation (VNS) activates this anti-inflammatory pathway in the gastrointestinal (GI) tract via the ENS. Therefore, it is plausible that VNS may be a viable treatment for IBD. In this section, we discuss: (1) in-vivo studies demonstrating that VNS reduced intestinal inflammation and/or permeability via α7nAChR, and (2) in-vitro studies implicating epithelial barrier integrity amelioration via non-epithelial nAChR and epithelial mAChR.

2.1.1 Nicotinic modulation of inflammation and epithelial barrier integrity in-vivo

In 2000, Borovikova et al. hypothesized that the cholinergic parasympathetic nervous system can modulate a systemic inflammatory response. The researchers cultured primary human macrophages, conditioned them via exposure to lipopolysaccharide (LPS), and exposed the cultured macrophages to acetylcholine (ACh). The researchers observed a dose dependent inhibition of tumor necrosis factor (TNF) release. By measuring TNF messenger RNA (mRNA), the authors confirmed that ACh inhibits TNF production via a post-transcriptional mechanism. Furthermore, the study found that ACh inhibited TNF production via interaction with nicotinic receptors, rather than muscarinic receptors. ACh was found to inhibit release of additional pro-inflammatory IL-1β, IL-6, and IL-18 cytokines. To investigate whether direct VNS has an anti-inflammatory effect similar to ACh, the authors performed VNS on adult male rats for 10 minutes before and after a lethal LPS dose. Reduced TNF concentrations were measured in both the serum and liver compared to sham surgery, thereby implicating efferent vagus nerve signaling in pro-inflammatory cytokine production.
In 2003, Wang et al.\textsuperscript{15} followed up on Borovikova’s work to identify that a α7 subunit of the nicotinic acetylcholine receptor (nAChR) is required for cholinergic inhibition of TNF production by macrophages cultured in vitro. The researchers measured TNF, IL-1β, and IL-6 concentrations of mice deficient in the α7 subunit gene and found that cytokine production was significantly higher following endotoxin exposure, thereby indicating that the α7 subunit is essential for anti-inflammation in vivo. Furthermore, by measuring TNF concentrations in wild-type or α7-deficient mice following endotoxin exposure and VNS, the group found that the α7 subunit is required for VNS induced TNF suppression.

Similar to these previous works De Jonge et al.\textsuperscript{16} tested the effect of VNS on inflammation following intestinal surgery using a mouse model. The researchers performed VNS during intestinal surgery and monitored muscular inflammation and gastric emptying 24 hours later. Mice that underwent intestinal surgery without VNS experienced delayed gastric emptying, indicative of postoperative ileus. However, mice that underwent intestinal surgery alongside VNS did not show delayed gastric emptying. Furthermore, mice that underwent intestinal surgery alongside VNS displayed less granulocytic infiltrates compared to intestinal surgery alone. Incubating the intestinal segments with nicotinic receptor blocker hexamethonium before surgery and VNS failed to prevent granulocytic infiltration, demonstrating that the anti-inflammatory effect of VNS acts via local nicotinic receptor activation. Furthermore, De Jonge et al. demonstrated that anti-inflammatory VNS acts through a JAK-STAT pathway in which vagal efferents release ACh and activate STAT3 following VNS. STAT3 is a transcription factor responsible for negatively regulating inflammatory response. Finally,
the results suggested that VNS should be performed before or during the inflammatory process for optimal anti-inflammatory effect. In 2007, a follow up study led by De Jonge\textsuperscript{17} showed that the anti-inflammatory effect of VNS can be replicated by a nAChR agonist, AR-R17779. In a mouse model, pretreatment with AR-R17779 reduced delayed gastric emptying and leukocyte influx into the small intestine 24 hours after surgery. Furthermore, by removing the vagal nerve before AR-R17779 pretreatment and surgery, the researchers showed that AR-R17779 independently acts on nAChR instead of indirectly increasing efferent vagal nerve activity. Finally, the researchers showed that AR-R17779 agonist reduces LPS induced macrophage TNF production, but not IL-6 production. Given these results, the authors confirmed that AR-R17779 reduced pro-inflammatory cytokine production via modest inhibition of nuclear factor-κB (NF-κB), a pro-inflammatory transcription factor. Additional studies have also implicated the downregulation of NF-κB or halting NF-κB nuclear translocation in anti-inflammatory VNS\textsuperscript{18}.

In 2010, Costantini et al.\textsuperscript{19} used a mouse model of severe burn injury to show that VNS prior to burn injury prevented histological gut injury and reduced burn-induced intestinal permeability as measured by a dextran permeability assay and assay of tight junction protein myosin light-chain kinase (MLCK). Furthermore, the group also demonstrated that post injury VNS protected against burn induced intestinal permeability comparable to pre injury VNS. The authors found an increase in activated enteric glia as measured by increased glial fibrillary acidic protein (GFAP) expression and potentially attributed the protective effect of VNS to glia cell derived S-nitrosoglutathione (GSNO). By performing a splenectomy prior to burn injury and VNS, the researchers eliminated
the effects of spleen produced TNF and confirmed that the protective effect of VNS was attributed to local enteric glia and not modulation of systemic TNF production. More recently, Matteoli et al.\textsuperscript{20} also used a mouse model devoid of splenic innervation to confirm that VNS reduces intestinal inflammation via an anti-inflammatory mechanism in direct contact with the intestine and independent of the spleen. Furthermore, using a $\alpha7$nAChR knockout mouse model, the group demonstrated that VNS is ineffective in mice devoid of $\alpha7$nAChR suggesting that the muscularis resident macrophages express $\alpha7$nAChR and are the target of the GI cholinergic anti-inflammatory pathway.

Zhang et al. used a rat model to demonstrate that LPS induced epithelial barrier dysfunction could be ameliorated via carbachol (CCh), a muscarinic and nicotinic agonist\textsuperscript{21}. LPS increased mucosal permeability, induced intestinal injury, and down-regulated ZO-1 and claudin-2 expression. CCh treatment decreased all of the aforementioned hallmarks of LPS induced injury. Pretreatment with a specific antagonist, $\alpha$-bungarotoxin, reduced the positive effects of CCh, suggesting that $\alpha7$nACh activation was involved.

Based on the above studies, it can be concluded that VNS locally modulates the inflammatory process in the GI tract with limited systemic effect. This suggests that a neuromodulation therapy may be a viable option for treating IBD, without the systemic side effects of current biologics. Furthermore, it can be concluded that VNS is dependent on the $\alpha7$ subtype nAChR. The previous studies implicated two mechanisms: (1) $\alpha7$nAChR activation reduces nuclear translocation of NF-κB which leads to reduced expression of MLCK\textsuperscript{21,22}, and (2) Jak2 recruitment to $\alpha7$nAChR and STAT3 activation\textsuperscript{16}. Several studies attributed anti-inflammatory $\alpha7$nAChR activity to intestinal macrophages,
though MCLK mediated epithelial barrier integrity is attributed to epithelial cells\textsuperscript{23} and one study observed EGC activation following VNS\textsuperscript{24}. In-vitro studies would facilitate elucidating cellular mechanisms of VNS or nicotinic amelioration of epithelial barrier integrity.

\subsection{2.1.2 Cholinergic modulation of inflammation and epithelial barrier integrity in-vitro}

In the previous section, we discussed research regarding VNS and $\alpha_7nAChR$ activation for treating gut inflammation. It should be noted that all of the highlighted studies used rodent models. Despite advancing our understanding of VNS and $\alpha_7nAChR$ activation toward modulating gut inflammation, the precise contribution of distinct cellular components such as macrophages, IECs, and EGCs remain unclear. An in-vitro model of the intestinal epithelium would enable easier and more accurate analysis at the cellular level. Although rodent models are predominantly used, a few research groups have studied electrical stimulation of the ENS and its impact on gut health in-vitro. The following discussion details the models that were used and the findings.

In 2003, Neunlist et al.\textsuperscript{25} described an in-vitro model of the gut-ENS interface. Human submucosa containing the three layers of the submucosal plexus were isolated from patients undergoing surgery for colon carcinoma. The isolated submucosa was pinned to a petri dish while commercial Transwell inserts seeded with human colonic polarized cell lines, Caco-2 or HT-29, were placed directly over the pinned submucosa. Note that this early study was a fully human in-vitro model given the patient derived mucosa and human derived cell lines. The enteric neurons in the pinned submucosa were electrically stimulated via a pair of platinum electrodes embedded in the bottom of the
petri dish. Following co-culture without electrical stimulation of the ENS, paracellular permeability of dextran and inulin increased across both Caco-2 and HT-29 cell monolayers. Upon electrical stimulation the ENS, the permeability increase was partly reduced but not abolished. This effect was eliminated by treating the ENS with tetrodotoxin prior to stimulation. Therefore, the authors attributed the permeability decrease to the ENS. By noting differences in monolayer permeability when stimulating the ENS in the presence of a vasoactive intestinal peptide (VIP) agonist or monoclonal VIP antibody, the group also reported that VIPergic pathways have a role in modulating gut permeability. Specifically, the release of VIP following ENS stimulation reduced paracellular permeability. Finally, the group showed that ENS stimulation increased tight junction protein 1 (ZO-1) expression at both the mRNA and protein level.

Four years after the above work, Neunlist et al. followed up with a similar study in which they sought to determine the role of EGCs on intestinal barrier function. Given that EGCs outnumber enteric neurons by 4x, EGCs may play a critical role in the gut-ENS interface. However, electrical stimulation was not used. EGC cultures were isolated and purified from enzymatically dissociated rat longitudinal muscle-myenteric plexus and four different human intestinal cell lines were used: Caco-2, T84, HT-29, and HT-29-C1.16E. To study EGCs within the context of the gut, the group used a commercial Transwell insert with EGCs seeded in the bottom of 12-well plates and IECs seeded onto the Transwell filters. When Caco-2 cells were co-cultured with EGCs, the transepithelial electrical resistance (TEER) values across the epithelial monolayer were higher and increased faster compared to control cultures. Furthermore, EGC conditioned media had the same effect of increasing TEER values across a Caco-2 monolayer. Interestingly, the
study found that EGCs decreased Caco-2 cell density on Transwell filters while increasing TEER values. The authors attributed these results to a 2.2-fold increase in Caco-2 cell area when co-cultured with EGCs. The authors showed that the anti-proliferative effect of EGCs was specific to EGCs. Since, transforming growth factor beta (TGF-β) is known to inhibit epithelial cell proliferation, the authors measured and confirmed TGF-β1 expression in EGCs cultured alone. TGF-β1 had a dose dependent effect on Caco-2 cell density and these effects were negated using an anti-TGF-β antibody. Along with their previous study, Neunlist\textsuperscript{25,26} showed that the ENS is a major regulator of intestinal barrier function. Although electrical stimulation was not used, this study is worth noting because of the in-vitro gut-ENS interface model and the fact that future in vivo studies confirmed EGC activation via VNS and a subsequent intestinal anti-inflammatory effect\textsuperscript{19}.

Similarly, Savidge et al.\textsuperscript{27} co-cultured Caco-2 cells alongside primary murine or rat EGCs using a filter to separate the two cell types. This group reported similar findings: a 2-fold increase in TEER values across Caco-2 cell monolayers co-cultured with EGCs, a decrease in paracellular permeability of dextran across Caco-2 monolayers, and an up-regulation of ZO-1 expression. Interestingly, Savidge et al. fractionated EGC conditioned media via ultrafiltration and found that the <1 kDa fraction increased TEER values across Caco-2 monolayers whereas the >1 kDa fraction did not. The <1 kDa fraction increased TEER values 3-fold when applied to the basolateral side of Caco-2 monolayers but not the apical side indicating that epithelial cells are basolaterally affected. By further purifying the <1 kDa fraction via size exclusion chromatography, the group determined that s-nitrosothiol (GSNO) induced the increased TEER, though
not in a dose-dependent manner and these effects were also present in vivo at low GSNO doses (10 µM) whereas high doses (250 µM) had the opposite effect. Several other groups studied gut-ENS interactions, though not in the context of VNS, via commercial Transwell inserts, intestinal cell lines (Caco-2, IEC-6) and either primary rat ENS or transformed ENS cell lines: SH-SY5Y (human), JUG2 (rat), CRL-2690 (rat).

After a 2010 in vivo study demonstrated that VNS protects the gut from burn induced intestinal inflammation, Constantini et al. sought to further define the role of α7nAChR via an in-vitro model of Caco-2 cells cultured on a commercial Transwell insert and a rat derived EGC cell line cultured on 12-well plates. The group demonstrated that both Caco-2 cells and EGCs expressed α7nAChR. In order to demonstrate that EGC derived α7nAChR induced protective effects on the epithelial barrier, the group stimulated their co-cultured model with a cocktail of pro-inflammatory cytokines (Cytomix containing interferon gamma (IFN-γ), TNF-α, and IL-1β) in the presence of absence of nicotine. Cells co-stimulated with Cytomix and nicotine maintained epithelial permeability similar to control values whereas cells stimulated with solely Cytomix demonstrated an increased epithelial permeability. Furthermore, when the experiment was repeated without co-culturing EGCs, nicotine did not have a barrier protective effect, thereby demonstrating that the protective effect of nicotine was due to EGCs. One year later, the same group published another study utilizing the same model that demonstrated that EGC secreted GSNO prevented Cytomix induced changes in epithelial barrier permeability and in tight junction protein expression and localization.

Based on these studies discussed thus far in Section 2.1.2, it can be concluded that the ENS contributes to nicotinic and VNS induced amelioration of epithelial
inflammation and/or barrier integrity. Several mechanisms were noted. One study discovered that electrical stimulation of the ENS results in release of VIP which reduces epithelial permeability and increases TJ expression\textsuperscript{25}. Two other studies implicated EGC derived GSNO in maintenance of epithelial barrier integrity and increased TJ expression\textsuperscript{27,33,34}. Zero studies indicated that nicotinic activation regulates barrier integrity via IECs. Though VNS induced ACh released may regulate epithelial barrier integrity via non-IEC associated mechanisms, ACh also binds to muscarinic G-protein coupled receptors (GPCRs). Several in-vitro studies, discussed below, implicated epithelial muscarinic receptors in regulating epithelial barrier integrity.

In 2004, Lotz et al.\textsuperscript{35} investigated intestinal epithelial wound closure in-vitro using T84, a human colon carcinoma derived cell line, monolayers on Transwell supports. The study revealed that stimulation of T84 monolayers with carbachol (CCh), a non-specific muscarinic and nicotinic agonist, inhibited wound closure. The group hypothesized that CCh results in Ca\textsuperscript{2+} release from the endoplasmic reticulum, and activation of Ca\textsuperscript{2+} sensitive K\textsuperscript{+} channels modulate wound closure via actin polymerization mechanisms.

In 2006, Cameron et al.\textsuperscript{36} investigated transcellular macromolecule transport across mouse jejunum and T84 monolayers. Stimulation of mouse jejunum with bethanechol (BET), a muscarinic agonist, increased the flux of horseradish peroxidase (HRP) and the number of HRP containing endosomes. Pre incubation with atropine (ATR), a muscarinic antagonist, but not neuron specific tetrodotoxin (TTX), abrogated the increased transcellular transport, demonstrating that mAChR was IEC specific. These results were corroborated using T84 epithelial cells on Transwell inserts. Furthermore,
using a specific antagonist, it was demonstrated that mAChR subtype M₃ mediated increased transcellular transport post BET stimulation. The involvement of additional mAChR subtypes is possible because baseline transcellular transport was reduced with non-specific antagonist ATR. The group hypothesized that cholinergic stimulation via the M₃ receptor on IECs generates phospholipase A₂ and cyclooxygenase metabolites that increase apical endocytosis with concomitant changes in the apical cell cytoskeleton. Similarly, Bijlsma et al.³⁷ reported a 2.5x increased flux of HRP across rat ileum and enterocyte vesicles filled with HRP post CCh stimulation. Furthermore, the result was also suppressed by ATR. However, Bijlsma attributed the increased flux to both transcellular and paracellular transport.

The two aforementioned studies utilized human T84 monolayers to demonstrate IEC specific muscarinic (via CCh, ATR, or BET) modulation of the epithelial barrier (inhibited wound closure or HRP permeability). In contrast, Wallon et al.³⁸ demonstrated that T84 monolayers did not respond to CCh stimulation in regard to HRP permeability or TEER. Using in-vitro co-culture studies, Wallon et al. identified a neuroimmune circuit (from ENS to eosinophils to mast cells) that mediates epithelial barrier integrity. Specifically, CCh stimulated eosinophils to produce corticotropin-releasing factor (CRF) which activate mast cells resulting in increased HRP permeability and reduced TEER in T84 epithelial monolayers. Neither CCh, eosinophil conditioned media, nor CCh stimulated eosinophil conditioned media elicited this response by T84 monolayers, suggesting that mast cells were necessary. Only co-cultured mast cells activated by conditioned media from CCh stimulated eosinophils produced a T84 response. These in-vitro results were corroborated using biopsies from ulcerative colitis (UC) patients. HRP
permeability across tissue decreased when tissues were treated with ATR, a CRF antagonist, or a mast cell stabilizer. Furthermore, immunostained eosinophils were found localized close to cholinergic neurons and stained positive for M₂ and M₃ mAChRs. The discrepancy between these T84 studies is striking. One plausible explanation is that culture conditions affect the expression of T84 mAChRs; Wallon et al.³⁸ reported M₁-M₃ gene expression by T84 cells, yet no functional response to CCh.

Toward a more definitive metric of mAChR expression, Khan et al.³⁹ used binding methods and western blot to demonstrate that T84 cells express M₁ and M₃ mAChRs with 35% and 65% proportions, respectively. CCh stimulation of T84 cell monolayers resulted in increased phosphorylation of focal adhesion kinase (FAK) and extracellular signal-regulated kinase (ERK), which was inhibited by nonspecific mAChR antagonist ATR or M₁ specific antagonist MT-7. FAK and ERK are key regulators of barrier repair and cellular proliferation, respectively. Furthermore, small interfering RNA (siRNA) silencing of M₁ mAChR reduced the CCh induced FAK and ERK phosphorylation in T84 cells. Towards investigating mAChR modulation of epithelial barrier integrity, CCh treatment augmented the recovery of ethanol induced epithelial injury on T84 cell monolayers, which was abrogated by pretreatment with ATR, confirming mAChR involvement. Interestingly, Khan et al.³⁹ reported that treatment of T84 monolayers with cytokine IFN-γ both decreased TEER and decreased M₁ and M₃ mAChRs expression, with higher reduction in M₁ as compared to M₃. Together, the data suggests that M₁ mAChR activation ameliorates ethanol induced epithelial barrier injury via ERK/FAK pathways, but this anti-inflammatory pathway is inhibited under systemic inflammatory states such as IBD which typically exhibit increased mucosal cytokine
concentrations.

The previous works all utilized T84 cells to model the intestinal epithelium in-vitro. But in 2015, Khan et al.\textsuperscript{40} performed another study utilizing HT-29/B6 cells to study muscarinic suppression of TNF-\(\alpha\) induced NF-\(\kappa\)B signaling and paracellular barrier disruption. HT-29/B6 cells are derived from a glucose-free culture of HT-29 cells. The study demonstrated that HT-29/B6 cells largely express M\(_3\) mAChR, in contrast to T84 cells which expressed M\(_1\) and M\(_3\) subtypes. CCh exposure prior to TNF-\(\alpha\) reduced phosphorylation of NF-\(\kappa\)B and IL-8 production at both the protein and mRNA level. Cyclooxygenase-2 (COX-2) gene expression was similarly reduced from TNF-\(\alpha\) elevated levels. Interestingly, the group reported a distinct mechanism for CCh suppression of TNF-\(\alpha\): CCh results in shedding of TNF receptor 1 (TNFR1) via TNF-\(\alpha\) converting enzyme (TACE). This reduces the potency of TNF-\(\alpha\) and also neutralizes TNF-\(\alpha\) via soluble shed TNFR1. Two years later, the same research group further investigated the molecular mechanism of M\(_3\) receptor mediated epithelial barrier integrity on HT-29/B6 cells\textsuperscript{41}. The research demonstrated that M\(_3\) activates GPCR G\(_{\alpha q}\), leading to p38 mitogen activating protein kinase (MAPK) phosphorylation to suppress TNF-\(\alpha\) signaling. A secondary effect of M\(_3\) induced TACE activity is ERK phosphorylation through increased expression of EGF receptors (EGFR). ERK phosphorylation is in agreement with a previous study of T84 monolayers\textsuperscript{42}, though M\(_1\) was implicated rather than M\(_3\). Finally, time course experiments demonstrated that mAChR induced p38 MAPK phosphorylation precedes and contributes to ERK phosphorylation.

Around the same time as the previous study, Dhawan et al.\textsuperscript{43} studied mAChR suppression of IL-1\(\beta\) induced barrier disruption of Caco-2 cells. Dhawan noted that
pretreatment with BET or ACh, a decreased the IL-1β induced permeability of a 4 kDa dextran whereas nicotine pretreatment did not. Furthermore, this effect was abrogated by ATR, suggesting mAChR involvement. Low voltage electrical impedance corroborated the effect of IL-1β and BET on paracellular permeability. However, high voltage impedance suggested that mAChR does not modulate endocytosis or transcytosis processes, which is in both agreement\textsuperscript{38} and disagreement\textsuperscript{37} with previous T84 studies. Dhawan also demonstrated increased occludin and claudin-3 expression and decreased myosin light chain (MLC) phosphorylation. In contrast to the HT-29/B6 study by Khan et al.\textsuperscript{44}, Dhawan reported that mAChR activation did not alter IL-8 production or NF-κB activity, both of which were strongly induced by IL-1β. Together, this data suggests that mAChR mediates paracellular Caco-2 permeability through MLCK activity independent of NF-κB. This discrepancy is indeed strange because MLC is phosphorylated by MLCK which is a target gene of transcription factor NF-κB.

Given the preceding discussion, it can be concluded that mAChR stimulation modulates IECs in-vitro, oftentimes with a protective effect against epithelial barrier injury. However, the type and extent of effect vary from study to study. Furthermore, the mAChR subtypes and downstream mechanisms vary. Table 1 below highlights several key factors from each of the IEC mAChR studies discussed so far. One plausible explanation for the discrepancies is that the various GI cell lines (T84, Caco-2, HT-29/B6) differentially express mAChR and/or respond to mAChR stimulation. Several studies highlight functional differences between common GI cell lines\textsuperscript{45-47}. Furthermore, it is plausible that differing tissue culture protocols for one cell line may produce different cell phenotypes\textsuperscript{48-51}, resulting in differential mAChR expression. In fact, a
recent study demonstrated that all five mAChR subtypes were present in mouse duodenum, jejunum, and ileum at ages 2-8 weeks, but M1-M5 was detected in mature enterocytes while M2 co-localized with crypt Paneth cells\textsuperscript{52,53}. This finding indicates that cell differentiation may impact mAChR expression, thus complicating experimental design and data interpretation. Furthermore, it suggests that some mAChR subtypes, such as M2, may not be recapitulated via immortalized GI cell lines: the previously discussed studies only specified M1 and M3 subtype detection. Together, these factors motivate the need for a robust humanized in-vitro model of the GI epithelium that faithfully recapitulates in-vivo IEC phenotypes.

Table 1: A summary of the literature investigating mAChR modulation of GI epithelium.

<table>
<thead>
<tr>
<th>Cell</th>
<th>Culture Details</th>
<th>Subtype</th>
<th>Observation</th>
<th>Mechanism</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>T84</td>
<td>Collagen coated</td>
<td>Maturity</td>
<td>DMEM/F-12 + 6% FCS</td>
<td>Not specified</td>
<td>CCh inhibited wound healing</td>
</tr>
<tr>
<td>Rat ileum</td>
<td>Ussing chamber</td>
<td>TEER ≥ 1000</td>
<td>DMEM/F-12 + 6% FCS</td>
<td>M₁-M₃, others</td>
<td>M₁-M₃ mRNA</td>
</tr>
<tr>
<td>T84</td>
<td>3.0µm Transwell</td>
<td>TEER &gt; 1000</td>
<td>DMEM/F-12 + 10% FBS</td>
<td>Not specified</td>
<td>M₁-M₃ mRNA</td>
</tr>
<tr>
<td>HT29/B6</td>
<td>400nm Transwell</td>
<td>9-1 days</td>
<td>RMPI 1640 + 10% FBS</td>
<td>M₁</td>
<td>CCh suppressed TNF-α barrier injury, NF-κB phosphorylation &amp; IL-8 production</td>
</tr>
<tr>
<td>Caco2</td>
<td>Transwell</td>
<td>3-4 weeks</td>
<td>DMEM + 10% FBS + NEAA</td>
<td>Not specified</td>
<td>CCh suppressed IL-1β barrier injury, increased TJ expression, unaltered IL-8 production &amp; NF-κB activity</td>
</tr>
</tbody>
</table>
2.1.3 Primary Human Intestinal Cells In-vitro and Cholinergic Regulation

Up to this point, all of the GI epithelium in-vitro models used to study nAChR or mAChR in regards to epithelial barrier integrity or inflammation (Section 2.1.2) were based on an immortalized GI cell line cultured on a Transwell membrane or rat tissue mounted in Ussing chambers. Discrepancies in the observed results and mechanisms (Table 1) may be due to innate differences between GI cell lines such as T84, HT-29, and Caco-2 and their protocol dependent phenotype. Thus, an in-vitro model that faithfully recapitulates in-vivo human GI biology is highly valuable.

Recently developed human intestinal organoids have largely addressed scalable culture of primary intestinal tissue. Three dimensional intestinal organoids are established by embedding biopsy or resection derived leucine-rich-containing G-protein coupled receptor 5 positive (LGR5) intestinal stem cells in a complex ECM, Matrigel, and supplementing the medium with essential growth factors and small molecule inhibitors: Wnt-3A, EGF, noggin, R-spondin, A83-01 (Alk4/5/7 inhibitor), and SB202190 (p38 inhibitor). Organoids form cystic structures, contain all of the intestinal epithelial cell lineages found in the native intestine, and can be indefinitely passaged to generate enough tissue for experimental replication. Using RNA-sequencing for 23,615 genes, it was demonstrated that primary intestinal organoids in-vitro exhibit broad similarity between both fetal and adult human GI tissue, suggesting similar global transcriptional activity. Thus, in-vitro human intestinal organoids are an excellent platform for studying mAChR and nAChR signaling with higher fidelity to human biology in-vivo.
A handful of studies have utilized intestinal organoids to study GI nAChRs and mAChRs. Takashi et al.\textsuperscript{58} demonstrated that mouse intestinal organoids endogenously synthesize ACh to evoke organoid growth and differentiation via mAChRs. It was shown that murine organoids express the gene for M1-M5 mAChR subtypes and the $\beta_7$ and $\alpha_7$ nAChR subtypes. M1-M4 expression was considerably greater than M5 and nAChR subtype $\beta_7$ was significantly greater than $\alpha_7$. Immunostaining of organoids revealed M1-M5 localization to IEC cell membranes throughout the entire organoid. Organoid treatment with 100 $\mu$M CCh downregulated organoid growth and gene expression. In contrast, organoid treatment with 10 $\mu$M ATR, a mAChR antagonist, enhanced growth, increased gene expression, and increased the number of LGR5$^+$ cells, suggesting mAChRs regulate organoid growth and differentiation rather than nAChRs. Using subtype specific mAChR antagonist, it was demonstrated that M1-M4 regulate organoid growth and differentiation.

Collectively, the former study\textsuperscript{58} provided evidence that mAChRs regulate proliferation and differentiation of LGR5$^+$ stem cells via epithelial derived ACh. However, in 2018, Takashi published a related study demonstrating that mouse organoid treatment with nicotine enhanced organoid growth and differentiation and nAChR antagonist mecamylamine suppressed these effects\textsuperscript{59}. mRNA expression of $\alpha_2$, $\alpha_3$, $\alpha_4$, $\alpha_5$, $\alpha_9$, $\alpha_{10}$, $\beta_1$, $\beta_2$, and $\beta_4$ nAChR subtypes was detected in mouse organoids. Immunostaining revealed only $\alpha_2$ and $\beta_4$ nAChR subtypes, which were among the most abundant mRNA, localized on IEC membranes in the crypt domain, but not the villi domain. Organoid growth was enhanced by 10 $\mu$M nicotine and suppressed by 100 $\mu$M mecamylamine. RNA-sequencing indicated that the Wnt5a gene was upregulated via nicotine and
downregulated via mecamylamine, suggesting that Wnt5a expression is coordinated with nAChR signaling. This was further corroborated by rescue of mecamylamine stunted organoid growth using recombinant Wnt5a. Finally, it was demonstrated that α₂ and β₄ nAChRs localize in Paneth cells. These data suggest epithelial derived ACh binds to 2 and β₄ nAChRs in Paneth cells, inducing Wnt5a expression which bind to Frizzled receptors, activating Wnt signaling, and ultimately regulating proliferation and differentiation of LGR5⁺ stem cells (Figure 1).

**Figure 1**: A schematic of the proposed mechanism of nAChR signaling in intestinal stem cell function. ACh activates Paneth cell associated nAChR α₂/β₄, modulating the expression levels of Wnt5a and/or Wnt9b. The Wnts then mediate Wnt pathway activity through frizzled receptors, enhancing stem cell proliferation and differentiation. Figure adapted from Takahashi et al.⁵⁹

Given that the two studies by Takashi⁵⁸,⁵⁹ addressed mAChR and nAChR in discrete experiments, it may be useful to consider the findings together. mAChR activation stunts organoid growth and proliferation while mAChR suppression increases organoid growth and proliferation, via control of LGR5⁺ stem cells. Various subtypes are involved, M₁-M₄, which are expressed across the entire organoid. In contrast, nAChR activation enhances organoid growth and proliferation while nAChR suppression
decreases organoid growth and proliferation, via Paneth cell associated α2 and β4 nAChRs that regulate Wnt5a production and thus LGR5+ stem cell proliferation. Interestingly, Liu et al.\textsuperscript{60} noted CCh induced Paneth cell degranulation in mouse organoids which may coincide with the stunted organoid growth observed by Takashi\textsuperscript{58}.

The three organoid studies discussed so far studied cholinergic regulation in a traditional manner: using synthetic agonists and antagonists of nAChRs or mAChRs. However, a few recent studies have described the integration of ENS components with 3D intestinal organoids. Although these groups did not study cholinergic modulation of inflammation, they are noteworthy because 3D primary organoid systems are an important milestone for the field of intestinal biology. In 2016, Pastula et al.\textsuperscript{61} co-cultured murine intestinal organoids with murine myenteric neurons in a 3D Matrigel droplet. Interestingly, the results showed that co-cultured neurons are able to replace exogenous Wnt necessary to maintain organoid growth. It was inconclusive which factors led to enhanced organoid growth in the absence of Wnt, but neurons are capable of producing various neurotrophic factors such as nerve growth factor (NGF) and transforming growth factor beta 1 (TGF-β1). It may very well be that the neuron derived ACh stimulated Paneth cell associated nAChRs, increasing Wnt5a production, as postulated by Takashi two years later\textsuperscript{59}.

Another example published in 2017 demonstrated a 3D organoid model composed of human pluripotent stem cell derived intestinal tissue with a functional ENS\textsuperscript{62}. The 3D model was built by mechanically aggregating intestinal organoids with pluripotent stem cell derived neural crest cells (NCCs) via centrifugation and culturing the aggregates in 3D. This example is particularly noteworthy because the model was fully humanized.
Both of these 3D in-vitro gut-ENS models may be powerful tools for systematically investigating the contribution of distinct cellular populations (IECs, EGCs, and neurons) toward regulating GI inflammation, or proliferation via cholinergic pathways.

Though organoids are both scalable genetically similar to the human intestinal epithelium in-vivo, organoids are closed cystic structures (Figure 2). Thus, the lumen is relatively inaccessible which complicates permeability measurements and studying epithelial barrier integrity. A recently described organoid microinjection platform may enable luminal perturbation but is technologically complex. Two dimensional monolayers derived from dissociated organoids enable access to both apical and basal media compartments, greatly facilitating epithelial perturbation and analysis. However, mAChR or nAChR regulation of epithelial barrier integrity has not been studied using 2D organoid derived monolayers.

In summary, human intestinal organoids have enabled scalable and more physiologically relevant in-vitro tissue culture of GI epithelial cells in comparison to immortalized cell lines. Human intestinal organoids have scarcely been used to probe cholinergic regulation but have already facilitated novel findings: activation of Paneth cell associated nAChR and/or mAChR regulates GI proliferation and differentiation. This finding accentuates the need for an in-vitro GI system that contains multiple IEC
lineages, which cannot be accomplished via immortalized cell lines. Finally, 2D in-vitro models of GI epithelium derived from primary human organoids may clarify the discrepancies that have been observed (Section 2.1.2 and Table 1) when using immortalized cell lines to study cholinergic regulation of epithelial barrier integrity. In fact, the Paneth cell localized expression of nAChR versus the widespread expression of mAChR may explain why strictly mAChR activation modified epithelial barrier function on immortalized IEC monolayers.

2.2 Microfluidic Organ Chip Small Intestine Models

The two preceding sections (Section 2.1 and Section 2.2) discussed the current scientific literature regarding cholinergic regulation of IECs. Immortalized IECs cultured on commercial Transwell inserts were predominantly used. Primary intestinal organoids are an emergent technology in the field. Organoids embedded in 3D ECM have been used to study cholinergic IEC regulation. Adaptation of 3D organoids to 2D monolayers on commercial Transwell inserts has enabled easier investigation of epithelial barrier integrity. Microfluidic cell culture devices, termed organ chips, lend additional advantages to traditional Transwell culture. Organ chips contain continuously perfused chambers which can directly contribute to intestinal function and allow for the co-culture of cell types requiring different medium or oxygen tension. In this section, we: (1) introduce PDMS soft lithography organ chip manufacturing, (2) discuss current microfluidic organ chip models of the small intestine, and (3) discuss the cell sources for microfluidic organ chip small intestine models.
2.2.1 Polydimethylsiloxane Soft Lithography

The term “organs chips” was coined because of the manufacturing process to make the cell culture platforms. Currently, practically all intestine chip systems are manufactured via soft lithography. Lithography is traditionally used to manufacture computer microchips with nanometer or micrometer sized features. Therefore, lithography enables fabrication of cell and tissue sized features spanning a 10-100 µm. In soft lithography, a UV sensitive photoresist is first coated onto a flat substrate. The most frequently used substrates are silicon or glass wafers which are readily available in 3, 4, or 6 inch diameters. Standardized 3, 4, or 6 inch substrates enable use of semiconductor wafer fabs equipped with required and compatible equipment such as spin coaters and mask aligners. SU-8 is the most frequently used photoresist and its principal components are a polymer resin, a photoinitiator, and a solvent. Other photoresists have been used but contain the same three principal components, although their chemistry and ratios may vary. Additionally, commercial SU-8 formulations with differing viscosities enable coating distinct thicknesses in a single coating step. The SU-8 photoresist is coated onto the substrate at a predetermined thickness, governed by the spin coat speed. Most commercial manufacturers supply guidance regarding the spin coating speed and the resulting resist thickness. Post coating, the substrate is baked on a hot plate or in an oven in order to remove excess solvent; the coated layer partially solidifies on the substrate. Next, a mask aligner is used to expose the substrate to ultraviolet (UV) light through a transparency mask. The transparency mask is designed by the researcher, typically using a computer aided design (CAD) software.
Transparency masks are printed by third party manufacturers using high resolution printing. Two types of transparency masks are common: a flexible, plastic transparency mask, typically printed on a Mylar film or a rigid transparency mask, usually printed on a soda lime or quartz glass substrate. Rigid transparency masks typically feature 1-2 µm resolution, while the flexible transparency typically have a 7-10 µm resolution. However, glass transparency masks cost approximately 3 times more.

Upon exposure to UV light, the photoinitiator catalyzes a chain reaction which crosslinks the exposed photoresist. A post exposure bake on a hot plate or in an oven further propels the reaction and increases the cross link density. Finally, the entire substrate is soaked in a photoresist developer which removes the unexposed photoresist. The final result is a glass or silicon substrate with patterned photoresist features. An optional step is a wafer hard bake at a relatively high temperature (100-150 °C) which further crosslinks the photoresist and imparts chemical and mechanical stability. The patterned substrate serves as the “master” mold for creating soft, polymeric microfluidic devices using the commercial elastomer PDMS, hence the name soft lithography.

Liquid PDMS is poured over the master mold and cured in an oven or hot plate, which crosslinks the PDMS into a rubberlike solid. The solid PDMS is cut

Figure 3: A schematic of the soft lithography process to create microfluidic devices. Figure adapted from Mazutis et al.1 with permission from Springer Nature.
and peeled off of the master mold, resulting in a negative replica of the patterned features. A hydrophobic silane coating such as hexamethyldisilazane can facilitate peeling and prolong the master mold’s lifetime. Next, fluidic access holes are punched into the PDMS replica. Finally, the PDMS replica with inlet and outlet holes is bonded to a flat substrate. The resulting PDMS microfluidic device can be loaded with cells, media, and connected to tubing via the punched holes. The above discussion introduces the process for creating a microfluidic device via PDMS soft lithography (Figure 3). In the next section, we discuss current microfluidic organ chip models of the small intestine. Additionally, we discuss the advantages and disadvantages of soft lithography and emerging organ chip manufacturing techniques.

2.2.2 Microfluidic Models of the Small Intestine

The first description of a microfluidic small intestine model was published in 2009. At this time, the term “organ chip” was not in use. Nevertheless, Imura et al. developed a PDMS based chip for evaluating intestinal drug adsorption. The chip used upper and lower PDMS sheets which were fabricated via soft lithography as previously described (Section 2.3.1). The sheets were the size of a standard glass slide (25 mm by 75 mm) and 3 mm thick. The channels molded within the sheets were 1.5 mm in width and 200 μm high. Rather than punching holes into the casted PDMS, PDMS was molded around freestanding Teflon tubes to create access holes. A 1.0 μm pore, polyethylene terephthalate (PET) membrane was manually cut from Transwell inserts and sandwiched between the upper and lower PDMS sheets to separate apical and basal fluid compartments. However, bonding of the membrane to the PDMS layers was not detailed. The authors noted frequent leaking due to poor bonding between the upper PDMS layer
and the PET membrane until the channel geometry was redesigned. The system described in this work introduced the major features of all intestine on a chip devices: two microscale fluidic compartments are vertically interfaced using a porous medium in between the compartments. While a commercial membrane eliminated manufacturing a custom porous material, manually cutting Transwell inserts is not scalable or cost effective. Additionally, rapid and leak free bonding of PDMS and non-PDMS membranes remains a challenge as illustrated in this work and is actively researched\textsuperscript{72}. Caco-2 cells were seeded on chip to study permeability across an intestinal epithelium.

Similarly, Gao et al.\textsuperscript{73} fabricated a two layer PDMS chip separated by a commercially available polycarbonate membrane with 0.4 \textmu m diameter pores. The apical and basal microchannels were 1 cm long, 3 mm wide, and 100 \textmu m tall. Bonding was achieved by stamping the PDMS microchannel and the PC membrane against a thin film of PDMS prepolymer, curing agent, and toluene followed by contacting the parts and curing in an 80\textdegree oven. Caco-2 cells were seeded on chip to study the permeability of curcumin across an intestinal epithelium.

In 2012, Kim et al.\textsuperscript{3} designed a similar PDMS chip, albeit with several additional features and called it “gut-on-a-chip” (Figure 4). The upper and lower PDMS layers were also fabricated via soft lithography. However, the group fabricated a custom 30 \textmu m thick PDMS membrane containing 10 \textmu m diameter circular pores with a 15 \textmu m edge to edge

![Figure 4: A schematic of the gut-on-a-chip device. Adapted from Kim et al.\textsuperscript{3} with permission from Royal Society of Chemistry.](Image)
pore spacing. The custom membrane was made by casting liquid PDMS onto a microfabricated silicon wafer containing an array of posts which were 10 µm in diameter and 30 µm high with a 15 µm edge to edge spacing. The custom membrane is more economical to produce compared to commercial Transwell inserts. However, the fabrication procedure was complex and required manual intervention, thereby limiting its scalability. Furthermore, given PDMS’ weak mechanical strength, one has to assume a frequent failure rate due to handling a 30 µm thick, porous PDMS film. All of the PDMS layers (top, bottom, and membrane) were bonded using oxygen plasma. Upon exposure to oxygen plasma, PDMS surfaces form free silanol groups and two PDMS surfaces are covalently bonded via contact to form associated silanols. The most notable feature of the chip design were empty channels which flanked the central, vertically stacked, fluidic compartments. Using an automated control system, the pressure inside the empty channels was oscillated. The oscillating pressure cyclically stretched the central fluidic compartments which the authors described as a simulated peristalsis. This feature required a custom elastomeric membrane whereas more conventional polymeric membranes would most likely tear under the oscillating stretch. This group published three more studies which used the same microfluidic platform and manufacturing process but investigated various biological phenomena.

These former 4 studies from the Ingber lab were particularly interesting as they demonstrated functional differences between IECs cultured on static Transwell inserts versus on continuously perfused microfluidic platforms. For example, Kim et al. demonstrated that continuously perfused Caco-2 exhibit an approximately 4-fold increase in cell height as compared to Transwell cultured Caco-2. Caco-2 cultured on chip for 7
days formed undulations and/or folds which exhibited the crypt-villus morphology found in-vivo\textsuperscript{3,74-76}. Pocock et al. corroborated this observation noted by the former 4 studies: Caco-2 cultured on PDMS chips under perfusion formed 3D morphology\textsuperscript{77}. The basal adjacent “crypt” domain was found to contain EdU\textsuperscript{+} proliferative cells which migrate up the “villi” and the surface area of “villi” displaying cultures was approximately 2-fold greater than true monolayer cultures\textsuperscript{74}. Caco-2 cells cultured on chip under only perfusion or perfusion with mechanical peristalsis exhibited higher TEER than static cutlures\textsuperscript{3}. However, it was unclear whether or not mechanical peristalsis individually contributed to higher TEER values. Interestingly, Caco-2 on chip under perfusion with mechanical peristalsis exhibited higher permeability to a dextran tracer, thus confounding the TEER results\textsuperscript{3}. Caco-2 on chip under 5 day perfusion exhibited aminopeptidase activity higher than 5 day static Caco-2 cultures and equal activity to 21 day static Caco-2 cultures, demonstrating accelerated differentiation under perfusion\textsuperscript{3}. Similarly, Caco-2 on chip under perfusion exhibited drug metalizing cytochrome P450 3A4 (CYP3A4) enzyme activity whereas static cultures did not\textsuperscript{74}. Increased CYP3A4 activity was attributed to the increased cell surface area on chip and corroborated with a glucose uptake assay that measured 1.5-fold greater glucose uptake on chip as compared to static Transwell cultures. It is unclear whether these differences in cell height, morphology, barrier integrity, and differentiation between perfusion and static Caco-2 cultures are due to the higher nutrient supply and/or shear stress induced by perfusion. Finally, Kim et al. demonstrated that Caco-2 cells under perfusion could be co-cultured the intestinal microbe Lactobacillus rhamnosus GG (LGG) without impacting cell viability or barrier integrity as was found on static Transwell cultures. In fact, TEER increased when Caco-2
were co-cultured with LGG. This may be one of the greatest advantages of microfluidic IEC culture as compared to static Transwell culture; bacteria under static conditions rapidly overgrow and contaminate human cell cultures. In fact, transcriptome profiling across 22,097 genes demonstrated that Caco-2 co-cultured with commensal bacteria on chip were more similar to in-vivo ileum than Caco-2 Transwell cultures or Caco-2 on chip\textsuperscript{75}. Collectively, these functional studies indicate that organ chip technology may impart immortalized IECs with novel biological features and/or functions that can be leveraged to study drug metabolism, inflammation\textsuperscript{75}, and viral infection\textsuperscript{76}.

Ramadan et al.\textsuperscript{78} used PDMS soft lithography to create a microfluidic intestine model for studying the interaction between Caco-2 IECs and immune cells. The chip featured two, vertically stacked, fluidic compartments interfaced with a 0.4 µm pore size PET membrane that was manually cut from Transwell inserts. However, the chip design had some distinct features. The authors cited an incompatibility between IECs and immune cells. Therefore, IECs were cultured in the apical compartment and immune cells were cultured in a downstream basal compartment that was isolated via a valve. The valve was opened for monocyte stimulation and closed otherwise. Furthermore, another chamber located downstream of the monocytes contained magnetic beads for performing on-chip immunoassays.

In 2018, the Kasendra et al.\textsuperscript{79} extended the previously developed PDMS “gut-on-a-chip” technology to primary human organoid derived epithelium, whereas the previous works utilized Caco-2\textsuperscript{3,74-76}. This in itself is an important milestone because organ chips have been projected to enable personalized medicine. Similar to the previously published Caco-2 models\textsuperscript{3,74-76}, IEC developed 3D “crypt-villus” morphology under media
perfusion whereas static cultures did not. Importantly, both immunostaining and real-time quantitative polymerase chain reaction (RT-qPCR) detected the IEC lineages found in-vivo: enterocytes, goblet cells, enteroendocrine cells, Paneth cells, and stem cells. As discussed, this is a particularly important advantage of primary IECs as compared to immortalized IECs (Section 2.2). Furthermore, transcriptomic analysis of genes related to proliferation and host defense demonstrate that organ chip cultured primary human IECs better emulate the human duodenum from which organoids were derived than do 3D organoid cultures. However, organ chips were not compared to Transwells, so it is unclear whether organ chip culture of primary IECs improves biological function as compared to Transwells.

An increasing trend within the organ on a chip community is integrating multiple microfluidic chips in order to recapitulate a “human on a chip” for interrogating organ interaction and drug absorption, distribution, metabolism, and excretion (ADME). For example, Maschmeyer et al. integrated four human organ equivalents: the small intestine, a skin biopsy, a liver equivalent, and a kidney equivalent. The four compartment platform was fabricated using PDMS soft lithography. A bottom PDMS sheet was bonded to a glass slide via plasma. Then a commercial, polyester, track etched membrane with 1 µm pore diameter was glued to the PDMS layer; the glue was not specified. Finally, the apical PDMS sheet was bonded to the lower PDMS layer via plasma. A notable feature was two on-chip peristaltic pumps which were formed using the Quake valve architecture. This feature is attractive for commercialization since operators would not require an external pump. However, the on-chip pumps were pneumatically actuated so the operator required a compressed gas source, pressure
regulators, and an automated valve manifold. Therefore, the on-chip pumping system actually increased the operation complexity. The intestinal epithelium was modeled using commercially available primary human small IECs that grow on tissue culture inserts, supplied by MatTEK Corporation. Metabolic and gene analysis revealed that homeostasis was maintained across all four tissues for 28 days in-vitro.

Similarly, Kimura et al. fabricated a multi organ chip using PDMS soft lithography. Their chip recapitulated the small intestine, the liver, the lung, and major arteries connecting the aforementioned organs. Caco-2 cells were seeded on chip to model the intestinal epithelium. The device featured an upper and bottom PDMS layer interfaced by a PET microporous membrane. The authors bonded the membrane via an aminosilane coating followed by oxygen plasma and contact with the PDMS layers. One notable feature was a built in pumping mechanism. This group enclosed a magnetic stir bar into a dedicated chamber on both upper and lower PDMS layers. The device was then placed on a magnetic stir plate which spun the magnetic stir bar and recirculated media throughout the entire system. The stir bar based pump was much simpler compared to the pneumatic system developed by Maschmeyer et al. Another potential advantage is the ability to operate without a cell culture incubator if a combination stir and hot plate was used. Of course, without CO₂ control, the media pH would have to be maintained without bicarbonate based buffers.

As the predominant manufacturing technique for organ chips, PDMS soft lithography has both advantages and disadvantages. PDMS soft lithography is relatively affordable once a master mold is fabricated. Furthermore, special equipment is not required once the master mold is manufactured. A research lab only requires a scale to
weigh PDMS and curing agent, a vacuum chamber for removing air bubbles prior to PDMS curing, and an oven or hot plate for PDMS curing. When manufacturing single-layer devices, PDMS is easily bonded to a glass substrate via plasma oxidation. When manufacturing multi-layer devices, PDMS can bond to PDMS via plasma oxidation. Benchtop plasma generators are relatively common in most biological labs as they are used for pretreating coverslips for cell culture. PDMS has been extensively used to culture various cells, thereby demonstrating its high biocompatibility. Furthermore, O₂ and CO₂ gas are highly PDMS soluble and rapidly diffuse through PDMS, enabling the use of PDMS chips alongside gas controlled cell culture incubators.

PDMS has several important drawbacks. The manufacture of a photoresist or silicon master mold requires specialized equipment and can be costly. Most lithography is done in clean room facilities which are relatively uncommon. The equipment available in those facilities is typically too expensive for independent research labs to purchase. For example, a mask aligner can cost upwards of $20,000. The transparency masks used to manufacture master molds are expensive; high resolution glass masks are approximately $100-$900 per mask (pricing from Front Range Photomask) while lower resolution polymeric masks are approximately $30-$135 per mask (pricing from FineLineImaging). This cost can quickly accumulate due to frequent redesigns in the early stages of device development. PDMS is very hydrophobic and has been shown to adsorb small molecules and biologics. This is undesirable when assessing the effect of biochemical exposure on cultured cells. The high O₂ permeability of PDMS precludes establishing different oxygen concentrations in distinct fluidic compartments. This is particularly important for modeling the intestine in-vitro. In vivo, a steep oxygen gradient exists along the length of
the intestine and from the lumen to the serosa. Finally, PDMS soft lithography is recognized as a bench level rapid prototyping process that precludes high volume manufacturing. If organs on chips are going to augment preclinical drug discovery, the chips will have to be manufactured at high volume in order to decrease costs and incentivize pharmaceutical companies to test and transition to microfluidic technologies.

Today’s 3D printers, compute numerical control (CNC) mills, cutter plotters, and laser cutters are competitive alternative technologies for microfluidic manufacturing with resolutions that are greater than or equal to 100 micrometers. In fact, much ongoing microfluidics research is focused on faster, easier, and cheaper fabrication utilizing alternative methods. Though microfluidic small intestine models are predominately manufactured via PDMS soft lithography, Shat et al. created a microfluidic intestine model with several notable features using milling. Two polycarbonate (PC) cover plates sandwiched silicon rubber gaskets which in turn sandwiched PC membranes. The PC cover plates were fabricated using numerically controlled milling. Interior pockets were machined into both the upper and bottom PC cover plate to accommodate oxygen sensors. Laser cut silicone gaskets served as fluidic compartments. Medical grade double-sided adhesives bonded the gaskets to the microporous PC membranes. The device was quite distinct in that it featured three distinct cell culture chambers interfaced with porous membranes, integrated oxygen sensors, and integrated TEER sensors. Both sensors enabled live process monitoring. Furthermore, the PC construction enabled oxygen control and co-culture of Caco-2 IECs with LGG and/or Bacteroides caccae bacteria under aerobic or anaerobic conditions. The group demonstrated transcriptional, metabolic, and immunological responses in IECs co-cultured with LGG under aerobic
conditions that replicate in-vivo observations. Furthermore, co-culture of IECs with the obligate anaerobe Bacteroides caccae and LGG resulted in a transcriptional profile that was distinct from co-cultures of solely IECs and Caco-2. In the future, CNC milling, laser cutting, and 3D printing may enable scalable manufacturing organs on chips.

### 2.2.3 Cell Sources for Microfluidic Small Intestine Models

Organs on chips have been marketed as a potential tool to augment preclinical drug discovery and development\(^9\). One particular way to do so is to seed human cells in organ chips to minimize the discrepancies between preclinical animal models and human clinical trials. In this section, we discuss the current cell sources for microfluidic small intestine models.

Caco-2 cells are the most frequently used cell line in microfluidic small intestine models\(^3\)\(^,\)\(^7\)\(^1\)\(^,\)\(^7\)\(^3\)\(^-\)\(^7\)\(^7\)\(^,\)\(^9\)\(^6\). Caco-2 cells were originally obtained from human colon adenocarcinoma and spontaneously differentiate after extended culture on permeable membranes\(^9\)\(^8\)\(^,\)\(^9\)\(^9\). Differentiated Caco-2 express some morphological and biological characteristics of small intestinal enterocytes such as monolayer formation, polarization, TJ expression, apical microvilli, and small intestinal enzyme activities on the apical cell surface\(^9\)\(^8\)\(^,\)\(^9\)\(^9\). Several studies demonstrated functional differences between Caco-2 cultured on static Transwell inserts versus on continuously perfused microfluidic platforms\(^3\)\(^,\)\(^7\)\(^4\)\(^-\)\(^7\)\(^6\). On chip, Caco-2 cells: (1) exhibit an approximately 4-fold increase in cell height (2) form undulations and/or folds which exhibited the crypt-villus morphology found in-vivo, (3) form proliferative cell compartments in the basal “crypt” domain which migrate up the villi, (4) exhibit increased surface area, (5) exhibit higher TEER, (6) differentiate faster (7) exhibit drug metabolizing CYP3A4 enzyme, and (8) can be co-cultured with LGG
bacteria without impacting Caco-2 viability or barrier integrity. Transcriptome profiling across 22,097 genes demonstrated that Caco-2 co-cultured with commensal bacteria on chip were more similar to in-vivo ileum than Caco-2 Transwell cultures or Caco-2 on chip\textsuperscript{75}. Thus, organ chip technology imparts immortalized IECs with novel biological features and/or functions that can be leveraged to study drug metabolism, inflammation\textsuperscript{75}, and viral infection\textsuperscript{76}. Nevertheless, Caco-2 are a malignant cell type and it is unclear whether these cells faithfully represent native intestinal epithelium.

An increasing trend is the co-culture of the Caco-2 cell line and a second cell line to model adjacent tissue or downstream organs. This is most frequently done by culturing Caco-2 cells on top of the membrane in the upper fluidic compartment and culturing the second cell type in the adjacent fluidic compartment. For example, Ramadan et al.\textsuperscript{78} cultured the monocytic U937 cell line in the basal compartment in order to recapitulate the gut-immune axis. This cell line is derived from human lymphoma and can differentiate into a macrophage-like cell after chemical induction. Macrophages are a form of defensive cell capable of engulfing and digesting microbes, cancer cells, and cell debris. Similarly, Shah et al.\textsuperscript{96} created a three layer microfluidic chip to investigate the gut-microbiome. The bottom most layer served solely as a media perfusion layer. Caco-2 epithelial cells were cultured in the middle layer. Finally, LGG and/or Bacteroides caccae bacterial cells were cultured in the upper most layer. A key element to this co-culture was the maintenance of both aerobic and anaerobic microenvironments for Caco-2 cells and bacteria, respectively. In some experiments, primary human CD4\textsuperscript{+} T cells were added to the bottom most cell culture compartment in order to study gut-microbiome immune system interactions. Similarly, Kim et al.\textsuperscript{75} used their previously developed, two layer,
gut-on-a-chip to study bacterial-gut-immune cell interactions. They did so by introducing bacteria into the upper fluidic chamber on top of Caco-2 cells and primary human peripheral blood mononuclear cells (PBMCs) into the basal compartment.

Co-cultures have been expanded to include more than two cell types and interfacing cell types via fluidic channels connected in series. For example, Kimura et al.\textsuperscript{82} co-cultured epithelial Caco-2, liver HepG2, and lung A549 cell lines in order to simulate an organ-to-organ network for in vitro pharmacokinetic modeling. HepG2 is a human liver cancer cell line and A549 is a human lung cancer cell line. The small intestine was modeled via two fluidic compartments interfaced with a porous membrane. Both the lung and the liver were modeled by culturing A549 and HepG2 cells in their own dedicated compartments. To mimic in vivo blood flow, the media was first recirculated to the lung chamber, followed by a bifurcation that circulated the media to the liver and the small intestine chamber.

Similarly, Maschmeyer et al.\textsuperscript{80} created a chip featuring four discrete yet interfacing organs: the small intestine, the skin, the liver, and the kidney. The small intestine was modeled using a commercially available primary cell source; a Transwell insert containing a mature, differentiated monolayer of primary human IECs. Skin was modeled using a human juvenile biopsy which was placed in a commercial Transwell insert to shield the biopsy from convective fluid flow. The liver was modeled using a combination of the human HepaRG cell line, a cell line derived from human liver cancer, and commercially available human primary stellate cells. Finally, the kidney was modeled using the RPTEC/TERT1 cell line, an immortalized cell line derived from human kidney. Each of the modeled organs was compartmentalized in a distinct fluidic
chamber. At the time, this work was the only organ chip intestine model that used a primary human IEC instead of the immortalized Caco-2 cell line. This was an important stepping stone towards patient derived organ chips for personalized drug screening. However, the primary human IEC was supplied by MatTEK Corporation which specializes in commercial tissue engineered in-vitro models. Three years later, Kasendra et al. applied the Ingber lab’s previous gut-on-a-chip technology to primary human IECs which were expanded in house. Primary human organoid technology is still evolving, but adoption is growing (Figure 5), and it is anticipated that future intestine chip models will utilize primary human IECs rather than Caco-2 cells.

In summary, the immortalized cancer cell line Caco-2 is the currently predominant model of intestinal epithelium. Only one publication modeled the intestine via primary human intestinal cells. As discussed, organs on chips’ leading applications are modeling disease pathology and personalized drug screening. Primary cells, which are more representative of in vivo phenotypes compared to immortalized cell types, would better serve these applications. Due to recent advancements in intestinal biology, the next generation of intestine on a chip models may utilize primary, human, intestinal cells. Intestinal biology has been rejuvenated by the development of 3-dimensional intestinal cultures that support both murine and human primary cells. Furthermore, these cultures feature all of the in vivo intestinal cell types:
enterocytes, enter endocrine cells, Paneth cells, stem cells, progenitor cells, goblet cells, and tuft cells \[101\]. The expansion of primary, human, intestinal cells should facilitate the development of a patient derived intestine on a chip. It is notable that researchers investigating gut-immune interactions do tend to use human, primary, immune cells. This is most likely due to the availability of human, primary, immune cells via facile blood draw compared to intestinal biopsy. Furthermore, the protocols for immune cell isolation from whole blood are mature and well documented\[102\].

This dissertation seeks to leverage primary human organoids (Section 2.1.3) to develop a more physiologically relevant model for the first ever studies of cholinergic regulation of primary epithelial barrier integrity. More specifically, this dissertation seeks to corroborate and further probe the literature regarding muscarinic amelioration of epithelial barrier integrity (Section 2.1.2). Previous studies utilizing immortalized cell derived epithelium suggested that mAChR activation ameliorates epithelial barrier integrity following a variety of inflammatory stimuli. However, contradictory findings across the literature suggest that the results are still inconclusive. Finally, this dissertation describes a low cost, rapid, and scalable microfluidic organ chip fabrication technique that circumvents the facility, economic, throughput, and scalability disadvantages of the predominantly used PDMS soft lithography technique (Section 2.2.1). Given the growing interest in organ chips by both academia and industry, this technique enables adoption and technological development by researchers lacking microfabrication training and/or facilities. The new fabrication technique supports primary organoid derived epithelium, thereby enabling researchers to integrate emerging human intestinal organoid technology.
3. AIM 1: DEVELOP A PRIMARY ORGANOID DERIVED MODEL OF SMALL INTESTINAL EPITHELIUM IN HEALTHY AND DISEASED STATE

This aim served to leverage primary human intestinal organoids (Section 2.1.3) to develop a physiologically relevant model of the small intestinal epithelium. Intestinal organoids were dissociated and seeded on collagen I coated Transwell inserts. The primary human epithelium exhibited functional differences as compared to immortalized Caco-2 and HT-29 epithelium: increased barrier integrity, IL-8 secretion, and the presence of both absorptive enterocytes and secretory goblet cells. Next, pro-inflammatory cytokine TNF-α was used to model an inflamed state. Treatment with TNF-α led to impaired barrier integrity, apoptosis, and polarized increase of IL-8. Single cell segmentation of fluorescent microscopy images revealed an intact IEC monolayer with altered cell morphology and TJ disorder, demonstrating TNF-α reduces epithelial integrity through both apoptosis and TJ rearrangement. The primary human small intestine epithelium presented here is a robust and phenotypically more relevant model of in-vivo intestine, as compared to immortalized IECs. Treatment with pro-inflammatory factors may be used to model diseased intestine and ultimately, to test therapeutic approaches to GI disease.

*This work will be submitted for publication in 2019.*
3.1 Introduction

A single layer of epithelial cells form the main component of the mucosal barrier in the small intestine\textsuperscript{103}. The single layer epithelium consists of a variety of specialized cells including enterocytes, goblet cells, Paneth cells, enteroendocrine cells, transit amplifying cells, and stem cells\textsuperscript{103}. Some cells perform specialized functions to fortify the mucosal barrier, such as mucin or antimicrobial peptide secretion by goblet and Paneth cells, respectively\textsuperscript{103}. But, epithelial permeability is largely regulated by the expression of cohesive junctional complexes, such as tight junctions (TJs), which seal the paracellular space between adjacent epithelial cells\textsuperscript{104}. Intestinal bowel disease (IBD) patients exhibit increased epithelial permeability and TJ abnormalities\textsuperscript{105}. Increased epithelial permeability may initiate pathogen translocation from the intestinal lumen to activate the mucosal immune response by underlying immune cells. Thus, IBD patients exhibit increased expression of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-\(\alpha\))\textsuperscript{106}. TNF-\(\alpha\) is produced by cells occupying the lamina propria such as macrophages, monocytes, and differentiated Th1 cells\textsuperscript{107,108}. In return, TNF-\(\alpha\) induces epithelial apoptosis\textsuperscript{108,109}, disrupting reorganization of TJs, thereby further propagating the inflammatory response\textsuperscript{109}. Though it is unclear whether increased epithelial permeability precludes pro-inflammatory cytokine production or is a consequence of mucosal immune activation\textsuperscript{104}, ameliorating epithelial permeability is critical for IBD treatment.

Previous in-vitro studies demonstrated that cholinergic activation of intestinal epithelial cells (IECs) decreased epithelial permeability following pro-inflammatory challenge\textsuperscript{21,39-41,43}. However, these studies utilized murine models\textsuperscript{21} or immortalized...
Caco-2\textsuperscript{43} and HT-29\textsuperscript{10,41,43} cell lines. Caco-2 and HT-29 cells were originally obtained from human colon adenocarcinoma and spontaneously differentiate after extended culture on permeable membranes\textsuperscript{98,99}. Differentiated Caco-2 express some morphological and biological characteristics of small intestinal enterocytes such as monolayer formation, polarization, TJ expression, apical microvilli, and small intestinal enzyme activities on the apical cell surface\textsuperscript{98,99}. The differentiated HT-29 phenotype is similar to small intestinal enterocytes, except HT-29 cells also produce mucin like intestinal goblet cells\textsuperscript{99}. Nevertheless, both Caco-2 and HT-29 are malignant cell types and it is unclear whether these cells faithfully represent native intestinal epithelium.

Previous in-vivo studies using murine models also demonstrated cholinergic amelioration of epithelial permeability\textsuperscript{21}, but differences between human and murine species limit the study relevance\textsuperscript{110}. Utilizing human small intestinal tissue explants is hindered by tissue availability, particularly for experimental replicates. Furthermore, both in-vivo studies and whole tissue explants complicate mechanistic studies due to multicellular tissue complexity. For example, it is widely accepted that nAChR stimulation of intestinal macrophages reduces TNF-\(\alpha\) production\textsuperscript{111-113}. Thus, studying cholinergic modulation of epithelial permeability using a primary cell derived intestinal model is a desirable advancement to uncover epithelium specific processes.

Recently developed intestinal organoids have largely addressed scalable culture of primary intestinal tissue\textsuperscript{54,55}. Three dimensional (3D) intestinal organoids are established by embedding biopsy or resection derived LGR5\textsuperscript{+} intestinal stem cells in a complex extracellular matrix, Matrigel, and supplementing the medium with essential growth factors: Wnt-3A, epidermal growth factor (EGF), noggin, and R-spondin\textsuperscript{55}. Organoids
form cystic structures, contain all of the intestinal epithelial cell lineages found in the native intestine, and can be indefinitely passaged to generate enough tissue for experimental replication\textsuperscript{56}. Though organoids are both physiologically similar to the native intestinal epithelium and scalable, the lumen is relatively inaccessible thereby complicating permeability measurements. Two dimensional (2D) monolayers derived from dissociated organoids enable access to both apical and basal media compartments, greatly facilitating epithelial perturbation and analysis\textsuperscript{64-69}.

In this study, we describe the development and characterization of small intestinal epithelium derived from primary human organoids to model impaired epithelial barrier integrity induced by pro-inflammatory cytokine TNF-\(\alpha\). This primary cell derived intestinal model recapitulated the hallmarks of differentiated small intestinal epithelium such as polarization, apical microvilli, TJ formation, small intestinal enzyme expression, but also contained both absorptive enterocytes and mucus secreting goblet cells which account for the majority of the intestinal epithelium in-vivo. Comparison of the primary cell derived model to Caco-2 and HT-29 monolayers demonstrated increased throughput, barrier integrity, and interleukin-8 production (IL-8), a critical cytokine of the intestinal epithelium in-vivo\textsuperscript{114,115}. Treatment of primary cell monolayers with TNF-\(\alpha\) led to decreased barrier integrity, apoptosis, and polarized increase of IL-8. Single cell segmentation of fluorescent microscopy images revealed an intact IEC monolayer with altered cell morphology and TJ disorder, demonstrating TNF-\(\alpha\) reduces epithelial integrity through both apoptosis and TJ rearrangement. Collectively, we demonstrate a simple and robust primary human organoid derived model of the small intestinal epithelium which can be used to study GI disease.
3.2 Methods

Primary intestinal organoid culture and expansion

De-identified endoscopic tissue biopsies were collected from grossly unaffected (macroscopically normal) areas of the duodenum patients undergoing endoscopy for gastrointestinal complaints. Informed consent and developmentally appropriate assent were obtained at Boston Children’s Hospital from the donors’ guardian and the donor, respectively. All methods were approved and carried out in accordance with the Institutional Review Board of Boston Children’s Hospital (Protocol number IRB-P00000529). Cells were cultured as 3D organoids embedded in 50 μL of Matrigel (cat no. 354230, Corning) on a 24-well plate as previously described\textsuperscript{116}. Wnt-3A, epidermal growth factor (EGF), noggin, and R-spondin 3 containing organoid expansion medium, termed WENR medium, was prepared from a mixture of Advanced DMEM/F12 medium (cat no. 12634028, Gibco) and 50% LWRN conditioned medium, which was prepared from L-WRN cells, as previously described\textsuperscript{117}. This cell line produces Wnt-3A, R-spondin 3, and noggin. WENR medium was supplemented with GlutaMAX (1x, cat no. 35050061, Gibco), HEPES (10 mM, cat no. 15630080, Gibco), Primocin (0.1 mg/mL, Invivogen), B-27 supplement (0.5x, cat no. 12587010, Gibco), N-2 supplement (0.5x, cat no. 17502048, Gibco), nicotinamide (10 mM, cat no. N0636, Sigma-Aldrich), N-acetyl cysteine (0.5 mM, cat no. A7250, Sigma-Aldrich), epidermal growth factor (50 ng/mL, cat no. 315-09, Peprotech), gastrin (50 nM, cat no. A7250, Sigma-Aldrich), A-83-01 (500 nM, cat no. SML0788, Sigma), prostaglandin E2 (10 nM, cat no. 14010, Cayman Chemical), and SB202190 (10 μM, cat no. S7067, Sigma-Aldrich). Y-27632 ROCK inhibitor (10 μM, cat no. Y0503, Sigma-Aldrich) was added to the organoid medium for
the first 48 hours following crypt isolation or passage. The culture medium was refreshed every 48 hours using 500 μL per well. Cell culture was performed in a humidified, 37°C, 5% CO₂ incubator.

Every 7-10 days, the organoids were passaged to new 24-well plates at a ratio of 1:4-1:8 depending on culture density. Matrigel droplets were scratched off the 24-well plate using a 1000 μL pipette tip and collected into a 15 mL conical tube. The organoids were centrifuged at 500g for 5 minutes at room temperature. After aspirating the cell culture medium, the organoids were suspended in 0.5 mM ethylenediaminetetraacetic acid (EDTA, cat no. AM9260G, Gibco) in phosphate buffered saline (PBS) without Ca²⁺ or Mg²⁺ and re-centrifuged at 300g for 5 minutes at room temperature. After aspirating the EDTA, the organoids were re-suspended in Trypsin-EDTA and incubated in a 37°C bath for 2 minutes. The Trypsin-EDTA was then quenched via a 2:1 dilution with Caco-2 culture medium containing 10% FBS and the organoid suspension was tritured ~10x using a 1000 μL pipette tip to produce single cells and small organoid fragments. The cells were pelleted at 300g for 5 minutes at room temperature. The cells were suspended in 4°C Matrigel and plated on new 24-well plates. The plated Matrigel was incubated at 37°C for 15 minutes before adding 500 μL of culture medium containing 10 μM Y-27632 ROCK inhibitor.

**Primary intestinal monolayer culture**

Polyester Transwell inserts (cat no. 353095, Corning, Corning, NY) in a 24-well plate were coated with 200 μL of a 400 μg/mL rat tail type I collagen in Dulbecco’s Modified Eagle Medium (DMEM, cat no. 11995-065, Thermo Fisher Scientific) for at least 1 hour at 37°C inside a humidified cell culture incubator with 5% CO₂. Organoids
were harvested for dissociation and monolayer seeding after 7-10 days of culture. Matrigel droplets were harvested and processed in Trypsin-EDTA as described above. The Trypsin-EDTA was then quenched via a 2:1 dilution with Caco-2 culture medium containing 10% FBS and the organoid suspension was triturated ~20x using a 1000 μL pipette tip to produce single cells and small organoid fragments. The cell suspension was filtered through a 40 μm cell strainer (cat no. 22-363-547, Fisher) into a 50 mL conical tube and pelleted at 300g for 5 minutes at room temperature. The cells were resuspended in WENR medium with 10 μM ROCK inhibitor. Transwell inserts were seeded using 200 μL of cell suspension (seeding density of 9.09 x 10^5 cells/cm²) and then 600 μL of media + 10 μM ROCK inhibitor was added to the basolateral compartment. ROCK inhibitor was used for the first 48 hours of cell culture and the apical and basal cell culture medium was refreshed every other day. Following 2 days of culture in EM medium, the apical and basal medium was replaced with differentiation medium (DM) containing Advanced DMEM/F12 + 20% FBS + 4mM GlutaMAX supplement + 100 U/mL Penicillin-Streptomycin. Apical and basal DM medium was replenished every 48 hours.

**Caco-2 cell culture**

Caco-2 epithelial cells were obtained from the American Type Culture Collection (ATCC, HTB-37) and cultured in Dulbecco’s Modified Eagle Medium (DMEM, cat no. 11995-065, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, cat no. 35-011-CV, Corning), and 100 U/mL Penicillin-Streptomycin (cat no. 15140122, Thermo Fisher Scientific). Caco-2 cells were cultured on 0.4 μm polyester (cat no. 353095, Corning, Corning, NY) Transwell inserts in a 24 well plate. Prior to cell seeding, the inserts were coated with 200 μL of a 400 μg/mL rat tail type I collagen in DMEM for
at least 1 hour at 37°C inside a humidified cell culture incubator with 5% CO₂. Post at least 1 hour, the collagen solution was removed and Caco-2 cells were seeded on the inserts by adding 200 uL of Caco-2 cell suspension (seeding density of 2.6 x 10⁵ cells/cm²) and then adding 600 uL of media to the basolateral compartment. The apical and basal cell culture medium was refreshed every other day.

**HT-29 cell culture**

HT-29 epithelial cells were obtained from the American Type Culture Collection (ATCC, HTB-38) and cultured in RPMI 1640 Medium with GlutaMAX and HEPES (cat no. 72400047, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, cat no. 35-011-CV, Corning), and 100 U/mL Penicillin-Streptomycin (cat no. 15140122, Thermo Fisher Scientific). HT-29 cells were cultured on 0.4 μm polyester (cat no. 353095, Corning, Corning, NY) Transwell inserts in a 24 well plate. Prior to cell seeding, the inserts were coated with 200 μL of a 400 µg/mL rat tail type I collagen in DMEM for at least 1 hour at 37°C inside a humidified cell culture incubator with 5% CO₂. Post at least 1 hour, the collagen solution was removed and HT-29 cells were seeded on the inserts by adding 200 uL of HT-29 cell suspension (seeding density of 2.6 x 10⁵ cells/cm²) and then adding 600 uL of media to the basolateral compartment. The apical and basal cell culture medium was refreshed every other day. HT-29 cells were used at passage 69-70.

**Tumor necrosis factor-α stimulation of monolayers**

TNF-α (cat no. 300-01A, PeproTech) was dissolved in deionized water with 0.1% bovine serum albumin at 10,000 µg/mL and stored at -80°C. Stocks were thawed only once prior to use. TNF-α was spiked in the basal media compartment at the concentration
and duration specified in the figure caption following nicotinic or muscarinic stimulation.

**Alkaline phosphatase measurement**

Alkaline phosphatase (AP) expression was measured using a commercial kit (AS-71109, AnaSpec, Fremont, CA). All kit components were prepared as specified by the manufacturer. Cell lysate from Transwell inserts was prepared as follows: medium was removed, and the inserts were washed 2x with PBS in both apical and basal compartments. Next, 200 µL of sterile 10x TrypLE Select (cat no. A1217701, Thermo Fisher Scientific) was added to the apical side of each insert prior to incubation at 37°C for ~15 minutes. Cells were collected into a centrifuge tube and inserts were washed with 800 µL of PBS and pelleted at 300g for 5 min at room temperature. The pellet was re-suspended in 150 µL of AP kit supplied buffer, washed and re-suspended in 150 µL of buffer. A 10 µL aliquot of cell suspension was removed to quantify the cell number via hemocytometer. The cells were centrifuged and suspended in 0.2% Triton X-100 (AC327371000, Fisher Scientific). The cells were incubated for 10 minutes at 4°C with agitation and then centrifuged at 2500g for 10 minutes at 4°C. The supernatant was used for the AP assay as specified in the kit instructions. The fluorescence was measured via plate reader (EnSightTM, PerkinElmer) using 485nm and 528nm emission and excitation wavelengths, respectively. The actual amount of AP was interpolated using a calibration curve generated with the kit supplied AP standard.

**Transepithelial electrical resistance (TEER) measurement**

To minimize temperature variation, 24 well plates were allowed to cool to room temperature for 20 minutes prior to TEER measurement. TEER was measured using an epithelial Volt/Ohm meter (EVOM2, World Precision Instruments, Sarasota, FL) coupled
to a chopstick-like electrode (STX2, World Precision Instruments, Sarasota, FL). TEER values (Ω x cm²) were determined by subtracting the baseline resistance value measured in the absence of cells and then multiplying the remaining ‘specific’ resistance value (Ω) times the cell culture surface area (cm²).

**IL-8 measurement**

IL-8 secretion was measured using an enzyme-linked immunosorbent assay (ELISA) kit (cat no. 88-8086-22, Thermo Fisher) according to the manufacturer’s instructions. Note that cell culture supernatant samples were incubated overnight at 4°C to maximize ELISA sensitivity. Prior to the ELISA, cell culture supernatants were collected, frozen at -80 °C, and thawed only 1-2 times prior to use to minimize sample degradation.

**Immunostaining and imaging**

For fixation, Transwell monolayers were washed with PBS, fixed for 15-20 min at room temperature with 4% formaldehyde, washed with PBS, permeabilized in 0.1% Triton X-100 for 15-20 minutes at room temperature, and washed with PBS. Monolayers were blocked with 2.5% goat serum for 1-2 hours at room temperature or overnight at 4°C. Then, primary antibodies were diluted in 2.5% goat serum and applied for 1-2 hours at room temperature or overnight at 4°C. The following primary antibodies were used: anti-ZO-1 (1:200, cat no. 33-9100, Thermo Fisher Scientific), anti-MUC2 (1:200, cat no. PA1-23786, Thermo Fisher Scientific), anti-occludin (1:200, 71-1500, Thermo Fisher Scientific), anti-Ezrin (1:200, cat no. PA5-17518, ThermoFisher), and phalloidin (1:500, cat no. A22287, Thermo Fisher Scientific). Transwell membranes were isolated and mounted on a standard glass slide and coverslip with Gold Antifade Mountant (cat no.
Secondary antibodies were host species goat Alexa Fluor 488, 546, and 647 conjugates from ThermoFisher.

**Cellular morphology quantification**

Cellular morphology was quantified by fixing and immunostaining monolayers for cell nuclei (DAPI) and tight junctions (ZO-1) as described above. Fluorescence microscopy was performed on a Zeiss Axio Observer.Z1 microscope equipped with an ORCA-Flash4.0 camera (cat no. C11440-22CU, Hamamatsu). Image processing was performed with ImageJ\(^{118}\) (https://imagej.nih.gov/ij/) . Prior to cell segmentation, Z-stacks were processed with the Extended Depth of Field plugin\(^{119}\) to obtain an image of tight junctions without interruptions. Cell segmentation was performed with an open source watershed algorithm (https://biii.eu/tissue-cell-segmentation). The segmentation of each image was manually corrected and improperly segmented cells were removed. Cell elongation was quantified via the parameter ‘Aspect Ratio’, which was calculated by first fitting an ellipse to the segmented cell and calculating the ratio of the major:minor ellipse axes. Tight junction rippling was quantified via the difference between the ‘Convex Area’ and ‘Area’; kinked or rippled tight junctions increase the convex hull selection among a set of points, thereby increasing this difference.

**Statistical analysis**

Student’s t-tests, ANOVA followed by a Tukey’s post-hoc correction, or two-way ANOVA with Sidak’s multiple comparisons test was used to determine statistical significance as indicated in figure legends. Data are presented as mean ± standard error of the mean (SEM). p values < 0.05 were considered to be significant. Exact p values are indicated in the Results section.
## 3.3 Results

**Human intestinal organoids recapitulate differentiated intestinal epithelium in-vitro**

Despite in-vitro studies demonstrating cholinergic modulation of intestinal epithelial inflammation and barrier injury\(^{21,39-41,43}\), these results have not been demonstrated on a primary human intestinal model. In order to develop a primary human small intestinal model for the purpose of studying TNF-\(\alpha\) induced inflammation and cholinergic modulation, we first established in-vitro organoid cultures via biopsy derived intestinal stem cells (Figure 6a) based on previously published reports\(^{54,116}\).

**Figure 6**: Dissociated human intestinal organoids form differentiated intestinal epithelial monolayers. (a) Schematic representation of the protocol to generate biopsy derived human small intestinal organoids. (b) Schematic representation of a monolayer formed on a permeable insert by seeding organoid derived intestinal cells for 2 days in WENR medium and differentiation media for 5 days. (c) Biopsy derived human small intestinal organoids were expanded in WENR medium and dissociated to establish monolayers (d) on permeable inserts. Scale bars denote 500 and 100 \(\mu\)m, respectively. (e) Representative images of monolayers grown in WENR media or differentiation media (f) for 7 days showed increased proliferation marker Edu+ cells (red) or goblet cell marker MUC2+ cells (green), respectively. Scale bars denote 50 \(\mu\)m. (g) Edu+ cells significantly decreased in 20% FBS cultured monolayers from 39.6% to 16.6% while MUC+ cells significantly increased in 20% FBS from to 0.4% to 12% (* \(p <0.05\) by student’s t-test). Data are presented as mean + SEM from 3 independent experiments using monolayers generated from one donor.
Then, we characterized the formation of heterogeneous, polarized, and differentiated epithelial monolayers on commercially available permeable membrane supports (Figure 6b). Organoids cultured and expanded in 3-dimensional (3D) Matrigel under a Wnt-3A, epidermal growth factor (EGF), noggin, and R-spondin 3 rich media (Figure 6c), termed WENR media, were dissociated and seeded onto collagen type I coated permeable membrane supports Figure 6d), enabling both apical and basal media sampling, perturbation, and analysis. Several previous studies have demonstrated that culture of organoid or crypt derived monolayers under a WENR media maintained proliferative cells, most likely LGR5+ stem cells and transit amplifying cells, which populate the native small intestinal crypt. In contrast, removing WENR and/or adding one of several small molecules resulted in a differentiated monolayer of primarily mucus secreting goblet cells and absorptive enterocytes, which populate the native small intestinal villi. As previous studies of cholinergic modulation of intestinal epithelial inflammation utilized cell lines differentiated on permeable supports, primary organoid derived monolayers in this study were cultured in both WENR media (Figure 6e) or a 20% FBS media (Figure 6f) and characterized for differentiation. Fluorescence microscopy of monolayers stained for proliferative cell marker EdU and mucin-2 (MUC2) goblet cell marker showed that the culturing monolayers under 20% FBS media rather than WENR Media reduced proliferative cells from 39.6% to 16.6% (p = 0.0063) and increased goblet cells from 0.4% to 12% (p < 0.0001) (Figure 6g). Immunostaining of cell monolayers revealed that phospho-ezrin expressing enterocytes (Figure 7c) account for a large proportion of cells when cultured under 20% FBS media. Confocal microscopy of monolayers stained for F-actin (Figure 7a), and ZO-1 (Figure
b) demonstrated polarized brush border formation, and robust tight junction formation. Taken together, these data confirmed a differentiated primary human intestinal model with structural and cellular features of the native small intestinal villi.

**Figure 7:** Morphological and lineage analysis of primary organoid derived small intestinal epithelium. (a) Orthogonal x-y projection and cross sectional views of confocal microscopic images showing intestinal epithelium immunostained for F-actin (magenta) and DAPI (blue). (b) Orthogonal x-y projection and cross sectional views of confocal microscopic images showing intestinal epithelium immunostained for ZO-1 (red) and DAPI (blue). (c) Orthogonal x-y projection and cross sectional views of confocal microscopic images showing intestinal epithelium immunostained for phospho-ezrin (yellow) and DAPI (blue). (d) Orthogonal x-y projection and cross sectional views of confocal microscopic images showing intestinal epithelium immunostained for MUC2 (green) and DAPI (blue).

Given that previous research regarding cholinergic modulation of intestinal inflammation and barrier injury utilized immortalized cell lines\textsuperscript{39-41,43}, the primary intestinal epithelial model was functionally compared to Caco-2 and HT-29 monolayers. To enable in-vitro study of the intestinal barrier, Caco-2 cells are cultured on permeable supports for 3 weeks, during which the cells differentiate toward an enterocyte phenotype expressing brush border enzymes\textsuperscript{99}. Brush border enzyme alkaline phosphatase (AP) is used as a differentiation marker\textsuperscript{122} while transepithelial electrical resistance (TEER) measurements across intestinal cell monolayers indicate barrier integrity. The expression of AP (129.3 pg/10\textsuperscript{5} cells) by a 7 day primary intestinal model was equivalent to the AP expression (156 pg/10\textsuperscript{5} cells) by a 21 day Caco-2 intestinal model (p = 0.34) (**Figure 8a**).
Figure 8: Functional comparison of organoid derived primary human intestinal monolayers and immortalized Caco-2 and HT-29 cell derived human intestinal monolayers (a) Primary human monolayers cultured for 5 days in 20% FBS comparably expressed alkaline phosphatase as Caco-2 monolayers cultured for 21 days (p > 0.05 by student’s t-test). (b) Primary human monolayers cultured for 5 days in 20% FBS showed comparable or greater TEER as Caco-2 monolayers cultured for 5 days (* and # p < 0.05 by ANOVA followed by Tukey’s HSD test, # denotes comparison to Caco-2 monolayers). (c) HT-29 monolayers and primary human monolayers cultured for 7 days both showed significantly increased apical IL-8 secretion compared to basal secretion. Primary human monolayers showed significantly increased apical and basal IL-8 secretion compared to HT-29 monolayers (* p < 0.05 by two-way ANOVA with Sidak’s multiple comparisons test). All data are presented as mean + SEM from at least 3 independent experiments from one donor unless specified as in panel b.

Primary intestinal monolayers exhibited equal to (Donor 1: 818 Ω x cm², p = 0.1270) or greater than TEER (Donor 2: 1008 Ω x cm², p = 0.0001; Donor 3: 974 Ω x cm², p = <0.0001; Donor 4: 1097 Ω x cm², p = <0.0001) as compared to Caco-2 monolayers (Caco-2: 604 Ω x cm²) (Figure 8b). Furthermore, there was significant variation in the average TEER observed among primary monolayers originating from different donors (Figure 8b). Interleukin-8 (IL-8) is a major intestinal proinflammatory molecule which is released upon intestinal infection or injury, resulting in neutrophil recruitment and a further proinflammatory cascade\textsuperscript{123,124}. Thus, IL-8 may serve as an immunological marker for in-vitro intestinal epithelial inflammation or barrier injury\textsuperscript{69}. IL-8 was secreted in a polarized manner for both HT-29 and primary intestinal epithelium: primary and HT-29 monolayers secreted 2.1x (p < 0.0001) and 4.7x (p = 0.0494) more IL-8, respectively, in the apical compartment as compared to the basal
compartment (Figure 8c). However, primary intestinal epithelium secreted more IL-8 than HT-29 monolayers in both the apical (Mean diff = 3873 pg, p < 0.0001) and basal compartment (Mean diff = 1928 pg, p < 0.0001) (Figure 8c). These results indicated that while Caco-2 and HT-29 monolayers bear some similarity to primary monolayers (AP expression and polarized IL-8 secretion, respectively), primary monolayers exhibited increased IL-8 secretion, increased barrier integrity, reduced differentiation time as measured by AP expression, and both absorptive and secretory cell types.

**Tumor necrosis factor-α decreases alters cell morphology and decreases barrier integrity through apoptosis and tight junction disorder**

To study cholinergic modulation of inflammation and barrier injury using the primary intestinal epithelial model, we characterized barrier disruption induced by tumor necrosis factor-α (TNF-α). TNF-α is a pro-inflammatory cytokine upregulated in inflammatory bowel disease (IBD)\(^{108}\) and was previously used to investigate cholinergic modulation of inflammation endothelial\(^{125}\) and epithelial HT-29\(^{40,41}\) monolayers. Primary monolayers were cultured for 7 days as described, and then varying doses (specified in figures and figure captions) of TNF-α were applied to the basal media compartment. Phase contrast microscopy of control (Figure 9a) and TNF-α treated monolayers (Fig. 4b) revealed a higher number of dead cells floating above the monolayer, particularly at doses of 25 and 50 ng/mL. Furthermore, multicellular structures (Figure 9c), likely remnant from seeding undissociated organoid fragments, were particularly susceptible to TNF-α induced cell death (Figure 9d). Apoptotic cells, identified by nuclear fragmentation and blebbing\(^{126-128}\), were quantified for control and TNF-α treated monolayers. The percentage of apoptotic cells increased (Figure 9e) from 1.6% (control monolayers) to 3.2% (p = 0.6180), 5.6% (p = 0.0292), and 5.1% (p = 0.0656) for 4, 25, and
The monolayer density was quantified and while the cell density decreased from 173,208 cells/cm² to 151,557, 143,718, and 148,197 cells/cm² for 4, 25, and 50 ng/mL TNF-α, respectively, the results were non-significant (Figure 9f). To quantify barrier injury induced by TNF-α, TEER was measured before and after TNF-α exposure and the final TEER value was normalized by the initial TEER value. Normalized TEER values significantly decreased from 1.62 to 1.29 (p = 0.0021), 0.74 (p < 0.0001), and 0.64 (p < 0.0001) for 4, 25, and 50 ng/mL TNF-α, respectively (Figure 9g). Furthermore, the differences in TEER between a low dose of 4 ng/mL TNF-α and higher doses of 25 and 50 ng/mL
TNF-α were statistically different (p < 0.0001). These data demonstrate that even a low dose of 4 ng/mL TNF-α decreases primary intestinal epithelial barrier integrity, at least partly due to cell apoptosis. However, given that apoptotic cells and cell density were non-significantly altered following a 4 ng/mL TNF-α dose exposure, the primary intestinal epithelial barrier may also be compromised by TJ alterations.

Immunofluorescent labeling of control and TNF-α treated monolayers for TJ protein ZO-1 revealed alterations in both cell morphology and TJ structure (Figure 11a-d). Qualitatively, TNF-α treated cells appeared both more elongated and larger as compared to non-treated cells. To quantitatively analyze TNF-α induced cell morphology alterations, immunofluorescent images of tight junction protein ZO-1 were processed using a segmentation algorithm implemented in ImageJ (Figure 11e). Following cell segmentation, all of the segmented cells were manually verified for accuracy. Only an exposure to 50 ng/mL TNF-α resulted in significant (p < 0.0001) cell elongation in comparison to control (Figure 11f). Cell size was quantified via the parameter ‘Area’ which was simply the area of the segmented cell. Both 4 and 50 ng/mL TNF-α exposures elicited significant cell size increase (p < 0.0001 and p = 0.0013, respectively) in comparison to control while 25 ng/mL TNF-α did not (p = 0.1018) (Figure 11g). Interestingly, cell size increased significantly more following a 4 ng/mL TNF-α exposure in comparison to a 50 ng/mL TNF-α exposure. Qualitatively, tight junction appeared rippled and/or kinked following TNF-α exposure (Figure 11a-d). Tight junction rippling was quantified via the difference between the ‘Convex Area’ and ‘Area’; kinked or rippled tight junctions increase the convex hull selection among a set of points, thereby
increasing this difference (Figure 10).

Tight junction rippling significantly increased following TNF-α exposure to 4 (p < 0.0001), 25 (p = 0.0388), and 50 ng/mL (p < 0.0001) (Figure 11h). Taken together, these data demonstrate that TNF-α induces a leaky primary intestinal epithelium through both apoptosis, and cellular or tight junction reorganization.

**Figure 10**: The ‘convex area’ is defined by a selection wrapped around the ‘area’.
Figure 11: Quantitative morphological analysis of primary human epithelium exposed to TNF-α. (a) The monolayer morphology post 5 day culture in 20% FBS was visualized by immunofluorescent microscopy of TJs (ZO-1, green) and nuclei (blue). (b-d) Representative images of monolayers exposed to varying doses of basal TNF-α for 48 hours demonstrated increased area, elongation and rippled tight junctions. Scale bars denote 50 µm. (e) Schematic representation of monolayer segmentation of the image in panel a, using TJ protein ZO-1 for cell border detection. (f) Cell aspect ratio significantly increased following exposure to 50 ng/mL TNF-α for 48 hours. (g) Cell size significantly increased following exposure to 4 and 50 ng/mL TNF-α for 48 hours. (h) The difference between the cell convex area and area significantly increased following exposure to 4, 25, and 50 ng/mL TNF-α for 48 hours. All panels: * and # p < 0.05 by ANOVA followed by Tukey’s HSD test, # denotes comparison to control. All data are presented as mean + SEM from at least 3 independent experiments from one donor.

3.4 Discussion

In this study, we described an in-vitro human small intestinal model featuring a primary intestinal epithelium on a permeable membrane support (Figure 6b and Figure 6d). Derived from primary 3D intestinal organoids (Figure 6a and Figure 6c), this system recapitulates small intestinal functionality more accurately compared to highly prevalent immortalized cell line models. Using the in-vitro human small intestinal model, we simulated an inflamed intestine by treating the primary epithelium with cytokine TNF-α.
The in-vitro human small intestinal model was established using solely primary human cells by obtaining and expanding LGR5+ stem cells in 3D Matrigel culture as previously described (Figure 6a). Following differentiation, monolayers exhibited cellular heterogeneity, containing both mucus secreting goblet cells (Figure 7d) and absorptive enterocytes (Figure 7c), thereby mimicking the cellular phenotype of the native small intestinal villi. Furthermore, differentiated monolayers exhibited an F-actin rich brush border in the apical plasma membrane (Figure 7a), indicating a highly polarized epithelium as found on native small intestinal villi. As previously demonstrated, maintenance of the primary epithelium in WENR media (Figure 6e) mimicked the cellular phenotype of the native small intestinal crypts. Namely, monolayers remained highly proliferative with practically zero goblet cells present (Figure 6g). Therefore, the protocol described herein may be adapted to model distinct compartments of the native intestinal crypt-villus axis, depending on the biological phenomena under investigation. In the present study, we formed monolayers representative of the villus compartment by differentiating cells under a 20% FBS medium.

Differentiated monolayers exhibited robust tight junction formation (Figure 7b), which led to TEER values higher than similarly formed Caco-2 monolayers (Figure 8b). The epithelial barrier integrity was comparable across monolayers derived via biopsy samples from multiple human donors, indicating high reproducibility (Figure 8b). Compared to Caco-2 monolayers, the in-vitro human small intestinal model differentiated approximately 3x faster to equally express alkaline phosphatase (Figure 8a), thereby significantly increasing experimental throughput while simultaneously recapitulating the
native small intestine with higher fidelity. Furthermore, we demonstrated significantly higher IL-8 (Figure 8c) by primary intestinal epithelium compared to similarly formed HT-29 monolayers. Collectively, the data highlights the functional differences between primary and immortalized cell derived intestinal models. While all of the monolayers described herein were derived from small intestinal duodenal segments, it was demonstrated that LGR5⁺ stem cells recapitulate the cellular phenotype of the intestinal segments from their origin. Thus, the protocol described herein could be adapted to model the diversity of the human intestine.

Intestinal cell derived monolayers cultured on permeable membrane supports are frequently used to study the epithelial barrier in response to pro-inflammatory cytokine, viral, or bacterial challenge. In the present study, we exposed the basal side of the primary epithelium to cytokine TNF-α to simulate an inflamed intestine. The barrier integrity of the epithelial monolayer significantly decreased following exposure to TNF-α (Figure 9g). Analysis of TNF-α exposed monolayers with reduced barrier integrity revealed significantly increased apoptosis (Figure 9e) and decreased epithelial monolayer density (Figure 9f), indicative of cell shedding. Indeed, TNF-α is a potent stimulus of IEC shedding. Interestingly, basal exposure to TNF-α strongly targeted cells growing as multicellular clumps on the apical cell surface (Figure 9c and Figure 9d). This may suggest that “villus-like” cells are more susceptible to TNF-α induced apoptosis. In accordance, previous in-vivo studies demonstrated that acute TNF-α exposure significantly blunts villi. Though the mechanisms of IEC apoptosis are still under debate, IECs show differential susceptibility to apoptosis depending on IEC differentiation or location.
While apoptosis was clearly present in response to TNF-α, immunostaining of monolayers for tight junction protein ZO-1 and nuclei revealed that confluent monolayers with intact tight junctions spanned the permeable support. Using image segmentation (Figure 11e), we quantitatively analyzed the cell morphology of >10^3 cells in response to basal TNF-α exposure. Cells exposed to even low doses of TNF-α exhibited significant enlargement (Figure 11g). Regardless of TNF-α dose, cells exhibited rippled tight junctions (Figure 11b-d). This TJ alteration was quantitatively analyzed using a new parameter, the convex hull area – cell area (Figure 10), which demonstrated significant tight junction rippling in response to TNF-α at all doses (Figure 11h). A high dose of TNF-α also resulted in a significant aspect ratio increase (Figure 11f). In vivo, IECs undergoing apoptosis alter TJ and integrins in order to detach from the basal membrane and the adjacent IECs extend their cytoplasm underneath the shedding IEC to create new junctions that maintain an intact epithelial barrier\textsuperscript{136}. The observed TJ rippling may indicate TJ remodeling while the increased cell size and aspect ratio may show cells adopting a larger and migratory phenotype to close TJ gaps induced by cell apoptosis. Together, these data suggest that TNF-α impairs epithelial barrier integrity through apoptosis and TJ remodeling which synergistically works with morphological changes to maintain an intact epithelial barrier.

3.5 Conclusion

This study demonstrated a primary cell derived in-vitro model of the human small intestinal epithelium, which exhibited hallmarks of in-vivo intestinal epithelium such as polarized F-actin, robust tight junctions, and contained both absorptive enterocytes and mucus secreting goblet cells. The in-vitro model is expected to be phenotypically more
similar to in-vivo intestine than immortalized IECs. Alkaline phosphatase expression by primary intestinal epithelium was equivalent to a Caco-2 model. Primary intestinal epithelial TEER was equal to or greater than a Caco-2 model. Polarized IL-8 secretion by primary intestinal epithelium was greater than a HT-29 model. The results provided insight into TNF-α induced disruption of epithelial barrier integrity: TNF-α reduced epithelial barrier integrity via both TJ alterations, and apoptosis which preferentially targeted “villus-like” multicellular tissue. The intestinal model described here was reproducible and practical in regard to setup, throughput and ease of perturbing and analyzing the intestinal epithelial barrier. Therefore, the model and protocol presented here is a powerful tool to study cholinergic regulation of epithelial barrier integrity.
4. AIM 2: INVESTIGATE CHOLINERGIC AMELIORATION OF EPITHELIAL BARRIER INTEGRITY IN A DISEASED STATE

This aim served to leverage the primary human intestinal epithelium model and the TNF-α induced inflammation model (Section 3) to investigate cholinergic regulation of the epithelial barrier. To systematically investigate the effects of cholinergic signaling in the gut, nicotine and bethanechol were used as nAChR and mAChR specific agonists, respectively. Nicotinic stimulation of primary intestinal epithelium did not ameliorate TNF-α induced paracellular permeability. Similarly, muscarinic stimulation of primary intestinal epithelium did not ameliorate TNF-α induced paracellular permeability. Furthermore, mAChR activation did not inhibit TNF-α induced IL-8 production. However, muscarinic stimulation of primary intestinal epithelium decreased transcellular transport of a 70 kDa dextran. The results presented here are contradictory to several immortalized IEC studies demonstrating muscarinic amelioration of epithelial barrier integrity or increased endocytosis. The discrepancy may be due to intrinsic biological differences between immortalized IECs and primary human IECs. Alternatively, previous results indicate the discrepancy may be due to differences in cellular kinetics and/or receptor recycling. The results warrant further investigation into the differences between immortalized primary human IECs in regard to muscarinic regulation.

*This work will be submitted for publication in 2019.*
4.1 Introduction

Previous in-vitro studies demonstrated that cholinergic activation of intestinal epithelial cells (IECs) decreased epithelial permeability following pro-inflammatory challenge\textsuperscript{21,39-41,43}. Acetylcholine (ACh) is a critical IEC regulator, controlling ion transport\textsuperscript{137}, mucus secretion\textsuperscript{138}, and cell proliferation\textsuperscript{58}. In the small intestine, acetylcholine is secreted by enteric neurons of the lamina propria\textsuperscript{139} and possibly by IECs themselves\textsuperscript{58,59}. Intestinal epithelial cells express both nicotinic (nAChR) and muscarinic acetylcholine receptors (mAChRs) that interact with ACh\textsuperscript{137}. However, these studies utilized murine models\textsuperscript{21} or immortalized Caco-2\textsuperscript{43} and HT-29\textsuperscript{40,41,43} cell lines. Caco-2 and HT-29 cells were originally obtained from human colon adenocarcinoma and spontaneously differentiate after extended culture on permeable membranes\textsuperscript{98,99}. Differentiated Caco-2 express some morphological and biological characteristics of small intestinal enterocytes such as monolayer formation, polarization, TJ expression, apical microvilli, and small intestinal enzyme activities on the apical cell surface\textsuperscript{98,99}. The differentiated HT-29 phenotype is similar to small intestinal enterocytes, except HT-29 cells also produce mucin like intestinal goblet cells\textsuperscript{99}. Nevertheless, both Caco-2 and HT-29 are malignant cell types and it is unclear whether these cells faithfully represent native intestinal epithelium. Previous in-vivo studies using murine models also demonstrated cholinergic amelioration of epithelial permeability\textsuperscript{21}, but differences between human and murine species limit the study relevance\textsuperscript{110}. Utilizing human small intestinal tissue explants is hindered by tissue availability, particularly for experimental replicates. Furthermore, both in-vivo studies and whole tissue explants complicate mechanistic studies due to multicellular tissue complexity. For example, it is widely accepted that
nAChR stimulation of intestinal macrophages reduces TNF-α production\textsuperscript{111-113}. Thus, studying cholinergic modulation of epithelial permeability using a primary cell derived intestinal model is a desirable advancement to uncover epithelium specific processes.

In this study, we describe the application of a model small intestinal epithelium derived from primary human organoids (Section 3) to investigate cholinergic amelioration of epithelial barrier integrity. Muscarinic stimulation reduced epithelial transcytosis but neither nAChRs or mAChRs stimulation ameliorated TNF-α induced epithelial permeability, potentially highlighting a functional difference between previously published immortalized cell line models.

4.2 Methods

Primary intestinal organoid culture and expansion

De-identified endoscopic tissue biopsies were collected from grossly unaffected (macroscopically normal) areas of the duodenum patients undergoing endoscopy for gastrointestinal complaints. Informed consent and developmentally appropriate assent were obtained at Boston Children’s Hospital from the donors’ guardian and the donor, respectively. All methods were approved and carried out in accordance with the Institutional Review Board of Boston Children’s Hospital (Protocol number IRB-P00000529). Cells were cultured as 3D organoids embedded in 50 μL of Matrigel (cat no. 354230, Corning) on a 24-well plate as previously described\textsuperscript{116}. Wnt-3A, epidermal growth factor (EGF), noggin, and R-spondin 3 containing organoid expansion medium, termed WENR medium, was prepared from a mixture of Advanced DMEM/F12 medium (cat no. 12634028, Gibco) and 50% LWRN conditioned medium, which was prepared from L-WRN cells, as previously described\textsuperscript{117}. This cell line produces Wnt-3A, R-
spondin 3, and noggin. WENR medium was supplemented with GlutaMAX (1x, cat no. 35050061, Gibco), HEPES (10 mM, cat no. 15630080, Gibco), Primocin (0.1 mg/mL, Invivogen), B-27 supplement (0.5x, cat no. 12587010, Gibco), N-2 supplement (0.5x, cat no. 17502048, Gibco), nicotinamide (10 mM, cat no. N0636, Sigma-Aldrich), N-acetyl cysteine (0.5 mM, cat no. A7250, Sigma-Aldrich), epidermal growth factor (50 ng/mL, cat no. 315-09, Peprotech), gastrin (50 nM, cat no. A7250, Sigma-Aldrich), A-83-01 (500 nM, cat no. SML0788, Sigma), prostaglandin E2 (10 nM, cat no. 14010, Cayman Chemical), and SB202190 (10 μM, cat no. S7067, Sigma-Aldrich). Y-27632 ROCK inhibitor (10 μM, cat no. Y0503, Sigma-Aldrich) was added to the organoid medium for the first 48 hours following crypt isolation or passage. The culture medium was refreshed every 48 hours using 500 μL per well. Cell culture was performed in a humidified, 37°C, 5% CO2 incubator.

Every 7-10 days, the organoids were passaged to new 24-well plates at a ratio of 1:4-1:8 depending on culture density. Matrigel droplets were scratched off the 24-well plate using a 1000 μL pipette tip and collected into a 15 mL conical tube. The organoids were centrifuged at 500g for 5 minutes at room temperature. After aspirating the cell culture medium, the organoids were suspended in 0.5 mM ethylenediaminetetraacetic acid (EDTA, cat no. AM9260G, Gibco) in phosphate buffered saline (PBS) without Ca$^{2+}$ or Mg$^{2+}$ and re-centrifuged at 300g for 5 minutes at room temperature. After aspirating the EDTA, the organoids were re-suspended in Trypsin-EDTA and incubated in a 37°C bath for 2 minutes. TheTrypsin-EDTA was then quenched via a 2:1 dilution with Caco-2 culture medium containing 10% FBS and the organoid suspension was triturated ~10x using a 1000 μL pipette tip to produce single cells and small organoid fragments. The
cells were pelleted at 300g for 5 minutes at room temperature. The cells were suspended in 4°C Matrigel and plated on new 24-well plates. The plated Matrigel was incubated at 37°C for 15 minutes before adding 500 μL of culture medium containing 10 μM Y-27632 ROCK inhibitor.

**Primary intestinal monolayer culture**

Polyester Transwell inserts (cat no. 353095, Corning, Corning, NY) in a 24-well plate were coated with 200 μL of a 400 μg/mL rat tail type I collagen in Dulbecco’s Modified Eagle Medium (DMEM, cat no. 11995-065, Thermo Fisher Scientific) for at least 1 hour at 37°C inside a humidified cell culture incubator with 5% CO2. Organoids were harvested for dissociation and monolayer seeding after 7-10 days of culture. Matrigel droplets were harvested and processed in Trypsin-EDTA as described above. The Trypsin-EDTA was then quenched via a 2:1 dilution with Caco-2 culture medium containing 10% FBS and the organoid suspension was triturated ~20x using a 1000 μL pipette tip to produce single cells and small organoid fragments. The cell suspension was filtered through a 40 μm cell strainer (cat no. 22-363-547, Fisher) into a 50 mL conical tube and pelleted at 300g for 5 minutes at room temperature. The cells were resuspended in WENR medium with 10 μM ROCK inhibitor. Transwell inserts were seeded using 200 μL of cell suspension (seeding density of 9.09 x 10⁵ cells/cm²) and then 600 μL of media + 10 μM ROCK inhibitor was added to the basolateral compartment. ROCK inhibitor was used for the first 48 hours of cell culture and the apical and basal cell culture medium was refreshed every other day. Following 2 days of culture in EM medium, the apical and basal medium was replaced with differentiation medium (DM) containing Advanced DMEM/F12 + 20% FBS + 4mM GlutaMAX supplement + 100 U/mL Penicillin-
Streptomycin. Apical and basal DM medium was replenished every 48 hours.

**Tumor necrosis factor-α, nicotinic, and muscarinic stimulation of monolayers**

Nicotine (cat no. N3876, Sigma-Aldrich) and bethanechol (cat no. 5057730001, Sigma-Aldrich) stocks were prepared at 50 mM and stored at -20°C. Stocks were thawed only once prior to use. Nicotine or bethanechol was added to the basal media compartment and incubated at 37°C and 5% CO₂ for 30 minutes prior to TNF-α exposure. Nicotine or bethanechol exposure was done using 575 µL of cell culture media in the basal compartment, followed by a 25 µL spike of TNF-α to achieve the desired TNF-α concentration. TNF-α (cat no. 300-01A, PeproTech) was dissolved in deionized water with 0.1% bovine serum albumin at 10,000 µg/mL and stored at -80°C. Stocks were thawed only once prior to use. TNF-α was spiked in the basal media compartment at the concentration and duration specified in the figure caption following nicotinic or muscarinic stimulation.

**Transepithelial electrical resistance (TEER) measurement**

To minimize temperature variation, 24 well plates were allowed to cool to room temperature for 20 minutes prior to TEER measurement. TEER was measured using an epithelial Volt/Ohm meter (EVOM2, World Precision Instruments, Sarasota, FL) coupled to a chopstick-like electrode (STX2, World Precision Instruments, Sarasota, FL). TEER values (Ω x cm²) were determined by subtracting the baseline resistance value measured in the absence of cells and then multiplying the remaining ‘specific’ resistance value (Ω) times the cell culture surface area (cm²).
**IL-8 measurement**

IL-8 secretion was measured using an ELISA kit (cat no. 88-8086-22, Thermo Fisher Scientific) according to the manufacturer’s instructions. Note that cell culture supernatant samples were incubated overnight at 4°C to maximize ELISA sensitivity. Prior to the ELISA, cell culture supernatants were collected, frozen at -80 °C, and thawed 1-2 times.

**Paracellular permeability measurement**

Paracellular permeability was measured by measuring the flux of small molecular weight (457 Da) Lucifer Yellow dye (cat no. L0259, Sigma-Aldrich) across primary human intestinal monolayers. Lucifer Yellow salt was dissolved in water at 2.25 mM and stored at -20°C. Stocks were thawed and diluted to a working concentration of 100 µM in sterile cell culture medium for the assay. 100 µL of Lucifer Yellow was added to the apical side of monolayers while 600 µL of fresh cell culture medium was added to the basal side of monolayers. After 60-90 minutes, 50 µL the basal cell culture medium was sampled and the fluorescence intensity of the samples was measured at 360 nm and 528 nm emission and excitation wavelengths, respectively.

**Transcellular permeability measurement**

Transcellular permeability was measured by measuring the flux of large molecular weight (70 kDa) tetramethylrhodamine (TRM) conjugated dextran (cat no. 50-152-6775, Fisher) across primary human intestinal monolayers. 25 mg/mL dextran stock was stored at 4°C. The stock was diluted to a working concentration of 3 mg/mL in sterile cell culture medium for the assay. 100 µL of Lucifer Yellow was added to the apical side of monolayers while 600 µL of fresh cell culture medium was added to the
basal side of monolayers. After 60-90 minutes, 50 µL the basal cell culture medium was sampled and the fluorescence intensity of the samples was measured at 530 nm and 590 nm emission and excitation wavelengths, respectively.

Endocytosis by primary human intestinal monolayers was measured by quantifying the internalization of a 70 kDa TRM conjugated lysine-fixable dextran (cat no. D1818, Thermo Fisher Scientific). Dextran was dissolved in sterile PBS with Ca^{2+} and Mg^{2+} at 20 mg/mL and stored at -20°C. Stocks were thawed and diluted to a working concentration of 3 mg/mL in sterile cell culture medium for the assay. Monolayers were incubated in 600 µL of fresh, basal, cell culture medium with or without 50 µM bethanechol for 4 hours. The apical medium was not changed. After 4 hours, the apical medium was removed and the 3 mg/mL dextran solution was added. After 60-90 minutes, the apical and basal medium was removed and the monolayers were washed 5x with PBS with Ca^{2+} and Mg^{2+}. Then, the monolayers were fixed in 4% formaldehyde (cat no. 28906, Thermo Fisher Scientific) for 20 minutes in the dark. Post fixation, the monolayers were washed 2x with PBS and permeabilized in 0.1% Triton X-100 (AC327371000, Fisher Scientific) for 15 minutes in the dark. Next, the monolayers were washed 2x with PBS and blocked overnight in 2.5% goat serum at 4°C. The next day, the monolayers were stained with anti-ZO-1 antibody (1:200, 2 hours, cat no. 339188, Thermo Fisher Scientific) at room temperature in the dark. The monolayers were washed 2x with PBS and stained with secondary antibody (1:1000, 1 hour, cat no. A-21235, Thermo Fisher Scientific) in 2.5% goat serum at room temperature in the dark. The monolayers were washed 2x with PBS and mounted on a standard glass slide and coverslip with Gold Antifade Mountant (cat no.
Confocal microscopy was performed on an LSM 710 confocal microscope (Zeiss) equipped with Zen software (Zeiss). Fluorescence microscopy was performed on a Zeiss Axio Observer.Z1 microscope equipped with an ORCA-Flash4.0 camera (cat no. C11440-22CU, Hamamatsu). Images from the Axio Observer were used for quantification of TRM conjugated dextran internalization, rather than confocal microscopy images. The integrated raw fluorescence intensity of the area occupied by the TRM emission was summed for all images of a sample. The summed integrated fluorescence intensity of the TRM emission was then normalized by the total cell area occupied by the DAPI fluorescence. Image processing was performed with CellProfiler (www.cellprofiler.org).

**Immunostaining and imaging**

For fixation, Transwell monolayers were washed with PBS, fixed for 15-20 min at room temperature with 4% formaldehyde, washed with PBS, permeabilized in 0.1% Triton X-100 for 15-20 minutes at room temperature, and washed with PBS. Monolayers were blocked with 2.5% goat serum for 1-2 hours at room temperature or overnight at 4°C. Then, primary antibodies were diluted in 2.5% goat serum and applied for 1-2 hours at room temperature or overnight at 4°C. The following primary antibodies were used: anti-ZO-1 (1:200, cat no. 33-9100, Thermo Fisher Scientific), anti-MUC2 (1:200, cat no. PA1-23786, Thermo Fisher Scientific), anti-occludin (1:200, 71-1500, Thermo Fisher Scientific), anti-Ezrin (1:200, cat no. PA5-17518, Thermo Fisher Scientific), and phalloidin (1:500, cat no. A22287, Thermo Fisher Scientific). Transwell membranes were isolated and mounted on a standard glass slide and coverslip with Gold Antifade Mountant (cat no. P36931, Thermo Fisher Scientific). Secondary antibodies were host
species goat Alexa Fluor 488, 546, and 647 conjugates from Thermo Fisher Scientific.

**Statistical analysis**

Student’s t-tests, ANOVA followed by a Tukey’s post-hoc correction, or two-way ANOVA with Sidak’s multiple comparisons test was used to determine statistical significance as indicated in figure legends. Data are presented as mean ± standard error of the mean (SEM). p values < 0.05 were considered to be significant. Exact p values are indicated in the Results section.

**4.3 Results**

**Muscarinic receptor activation decreases primary intestinal epithelial transcytosis but does not ameliorate TNF-α induced barrier leakiness or IL-8 production**

We used the primary human intestinal epithelial model to investigate cholinergic amelioration of TNF-α induced leaky gut. The enteric nervous system resides adjacent to the intestinal epithelium, and releases the neurotransmitter acetylcholine. Acetylcholine interacts with intestinal epithelial cells via nicotinic (nAChR) or muscarinic cholinoreceptors (mAChR) to regulate epithelial fluid and electrolyte transport across the intestinal lumen. Previously published data demonstrated protective effects on intestinal barrier integrity via cholinergic activation. However, all previous evidence was acquired using murine models immortalized cell line models of the intestinal epithelium.

Primary monolayers were cultured for 7 days as previously described, and then 25 ng/mL TNF-α was applied to the basal media compartment following a 30 min exposure to 50 µM nicotine in the basal media compartment. After TNF-α exposure, TEER significantly decreased compared to control, regardless if cell monolayers were pretreated
with nicotine (p < 0.0001) or not (p = 0.0006) (Figure 12a). Nicotine treatment alone did not affect TEER (p = 1.000). To complement paracellular permeability measurement via TEER, the flux of Lucifer Yellow (457 Da) added to the apical compartment was measured by sampling the basal compartment. Corroborating TEER measurements, Lucifer Yellow flux significantly increased compared to control, regardless of if cell monolayers were pretreated with nicotine (p < 0.0001) or not (p < 0.0001) (Figure 12b). Nicotine treatment alone did not affect Lucifer Yellow flux (p = 0.9669). To investigate transcellular transport, the flux of a 70 kDa tritcethylrhodamine (TRM) conjugated dextran added to the apical compartment was measured by sampling the basal compartment. Transcellular transport did not significantly change following under any treatment (Figure 12c). Together, these results indicated that nAChR activation did not alter primary intestinal epithelial barrier integrity under healthy or TNF-α induced inflammatory conditions.
Figure 12: Primary human intestinal epithelial barrier integrity after exposure to nicotine and/or TNF-α. (a) TEER significantly decreased following exposure to 25 ng/mL TNF-α in the basal compartment for 4 hours. TEER did not change following exposure to 50 µM nicotine in the basal compartment for 4 hours. Pre-incubation with 50 µM nicotine in the basal compartment for 30 minutes prior to TNF-α did not eliminate TNF-α induced TEER decrease. (b) Lucifer yellow flux significantly increased following exposure to 25 ng/mL TNF-α in the basal compartment for 4 hours. Lucifer yellow flux did not change following exposure to 50 µM nicotine in the basal compartment for 4 hours. Pre-incubation with 50 µM nicotine in the basal compartment for 30 minutes prior to TNF-α did not eliminate TNF-α increased lucifer yellow permeability. (c) 70 kDa dextran flux did not change following exposure to 25 ng/mL TNF-α or 50 µM nicotine in the basal compartment for 4 hours. All panels: * and # p < 0.05 by ANOVA followed by Tukey’s HSD test, # denotes comparison to control. All data are presented as mean + SEM from at least 3 independent experiments from one donor.

To investigate mAChR activation, 25 ng/mL TNF-α was applied to the basal media compartment following a 30 min exposure to 50 µM muscarinic receptor agonist bethanechol in the basal media compartment. Similar to nAChR experiments, TEER significantly decreased after TNF-α as compared to control, regardless if cell monolayers were pretreated with bethanechol (p = 0.0002) or not (p = 0.0002) (Figure 13a).

Bethanechol treatment alone did not affect TEER (p = 0.7256). Similarly, Lucifer Yellow flux corroborated TEER measurements; Lucifer Yellow flux significantly increased compared to control, regardless if cell monolayers were pretreated with bethanechol (p = 0.0002) or not (p < 0.0001) (Figure 13b). Bethanechol treatment alone did not affect Lucifer Yellow flux (p = 0.0904). Though neither nAChR or mAChR activation with nicotine or bethanechol, respectively, did not alter paracellular barrier integrity, bethanechol treatment of monolayers significantly decreased transcellular transport under
healthy \((p = 0.0002)\) and TNF-\(\alpha\) induced inflammatory conditions \((p < 0.0001)\) (Figure 13c). To further investigate mAChR dependent macromolecule transport, we designed a second assay allowing visualization and quantification of macromolecule internalization. Primary epithelium was exposed to a 70 kDa TRM-conjugated ‘lysine fixable’ dextran in the presence or absence of 50 \(\mu\)M bethanechol. Monolayers were extensively washed, fixed, stained for tight junction protein ZO-1 and cell nuclei, and imaged via fluorescence microscopy, allowing for direct visualization of internalized 70 kDa dextran. Fluorescence microscopy revealed bethanechol treated monolayers (Figure 13e) with qualitatively more dextran internalization as compared to non-treated monolayers (Figure 13d). The normalized fluorescent intensity revealed 2.7x higher internalization for un-treated monolayers as compared to bethanechol treated monolayers (Figure 13f).
Figure 13: Primary human intestinal epithelial barrier integrity after exposure to bethanechol and/or TNF-α. (a) TEER significantly decreased following exposure to 25 ng/mL TNF-α in the basal compartment for 4 hours. TEER did not change following exposure to 50 µM bethanechol in the basal compartment for 4 hours. Pre-incubation with 50 µM bethanechol in the basal compartment for 30 minutes prior to TNF-α did not eliminate TNF-α induced TEER decrease. (b) Lucifer yellow flux significantly increased following exposure to 25 ng/mL TNF-α in the basal compartment for 4 hours. Lucifer yellow flux did not change following exposure to 50 µM bethanechol in the basal compartment for 4 hours. Pre-incubation with 50 µM bethanechol in the basal compartment for 30 minutes prior to TNF-α did not eliminate TNF-α increased lucifer yellow permeability. (c) 70 kDa dextran flux did not change following exposure to 25 ng/mL TNF-α in the basal compartment for 4 hours. 70 kDa dextran flux significantly decreased following exposure to 50 µM bethanechol with or without TNF-α co-incubation in the basal compartment for 4 hours. Panels a-c: * and # p < 0.05 by ANOVA followed by Tukey’s HSD test, # denotes comparison to control. Panels a-c: data is presented as mean ± SEM from at least 3 independent experiments from one donor. (d) A representative images of a monolayer showing internalization of an apically introduced lysine-fixable TMR conjugated 70 kDa dextran. (e) A representative images of a monolayer incubated with 50 µM bethanechol for 4 showing a lack of internalization of an apically introduced lysine-fixable TMR conjugated 70 kDa dextran. (f) The integrated raw fluorescence intensity due to internalized TRM conjugated dextran normalized by the nuclei area occupied by DAPI fluorescence was lower for monolayers exposed to 50 µM bethanechol in the basal compartment for 4 hours. Data are presented as mean ± SEM from 2 independent experiments from one donor.
TNF-α results in pro-inflammatory IL-8 production by intestinal epithelial cells. Correspondingly, IBD patients typically exhibit elevated TNF-α and IL-8 levels. Previous results demonstrated significant IL-8 suppression by pre-treatment of HT-29/B6 monolayers, with carbachol, a cholinergic agonist stimulating both nAChRs and mAChRs, prior to TNF-α. Furthermore, pre-treatment of HT-29/B6 monolayers with atropine, a cholinergic antagonist, negated the effects of carbachol. Given that bethanechol reduced macromolecular transcytosis in primary human epithelium, we analyzed IL-8 secretion by TNF-α and bethanechol treated monolayers. The apical production of IL-8 remained unchanged despite treatment with TNF-α, bethanechol, or both. Exposure to 25 ng/mL TNF-α in the basal compartment for 4 hours significantly increased basal IL-8 secretion but apical IL-8 remained constant. Exposure to 50 µM bethanechol in the basal compartment for 4 hours did not alter IL-8 secretion. Pre-incubation with 50 µM bethanechol in the basal compartment for 30 minutes prior to 25 ng/mL TNF-α did not eliminate TNF-α increased basal IL-8 secretion (* and # p < 0.05 by ANOVA followed by Tukey’s HSD test, # denotes comparison to control). Data are presented as mean ± SEM from at least 3 independent experiments from one donor.
4.4 Discussion

Intestinal cell monolayers derived from immortalized cell lines were used to study nAChR and mAChR stimulation for amelioration of intestinal epithelial permeability\(^\text{39-41,43}\). The present study is the first utility of a primary human intestinal epithelium towards said application. Nicotinic stimulation of primary epithelium did not alter barrier integrity or transcytosis nor did it rescue TNF-\(\alpha\) induced barrier impairment (**Figure 12a-c**). Similarly, a previous study demonstrated that nicotine did not impact interleukin 1 beta (IL-1\(\beta\)) induced epithelial permeability of Caco-2 monolayers\(^\text{43}\). However, a plethora of in-vivo studies demonstrated amelioration of epithelial injury via nAChR activation by nicotine\(^\text{17}\), alternative nicotinic agonists\(^\text{17}\), or vagal nerve stimulation\(^\text{16,22,24,33,112,113}\). Thus, nAChR activation most likely does not target epithelial cells directly, but rather exerts anti-inflammatory effects via another cell type found in the intestinal mucosa. Indeed, these studies demonstrated reduced TNF-\(\alpha\) production by macrophages\(^\text{112}\) and enteric glial cell (EGC) activation\(^\text{24}\). Further supporting this hypothesis, in-vitro studies utilizing co-cultures of intestinal epithelium and EGCs demonstrated that nAChR activation targeted EGCs to exert protective effects on the intestinal epithelium\(^\text{33,34,142}\).

Similarly, mAChR stimulation with bethanechol did not rescue ameliorate TNF-\(\alpha\) barrier impairment (**Figure 13a-b**). However, mAChR stimulation significantly decreased transcytosis in both a basal healthy state and post TNF-\(\alpha\) treatment (**Figure 13c**). To further verify the effect of mAChR activation on transcytosis, we visualized a ‘lysine-fixable’ 70 kDa dextran (**Figure 13d-e**) and quantified the results via image processing (**Figure 13f**). Though mAChR stimulation did not ameliorate TNF-\(\alpha\) barrier impairment, we tested whether the decreased transcytosis extended to a decrease in IL-8
release following TNF-α exposure, which would minimize the immune response and further barrier impairment. Results demonstrated that mAChR did not reduce TNF-α induced secretion (Figure 14a), suggesting that intestinal epithelial transcytosis and IL-8 secretion are dependent on different cellular pathways.

These results are interesting as they potentially highlight functional differences between in-vitro experiments utilizing immortalized cell lines, primary human cells, and ex-vivo experiments using whole human mucosa. In contrast to our results, one study demonstrated that bethanechol stimulation of mAChR-3 increases macromolecular permeability of horseradish peroxidase (HRP) in ex-vivo human mucosa and T84 cell monolayers, another human colorectal derived epithelial cell line. However, a contradicting study demonstrated that carbachol, a nonspecific cholinergic agonist, did not alter HRP permeability of T84 cell monolayers. Contrary to our results, mAChR activation of Caco-2 monolayers via bethanechol ameliorated IL-1β induced barrier injury. However, mAChR activation did not decrease IL-8 production, in agreement with our results. The discrepancies in the literature may be due to differential mAChR expression between immortalized cells, primary cells, and ex-vivo tissue, which is frequently unreported or assessed using functional tests with antagonists rather than direct observation at the mRNA or protein level. Furthermore, it is plausible that differing tissue culture protocols for one cell line may produce different cell phenotypes, resulting in differential mAChR expression. Indeed, it was reported that mAChR subtypes are differentially expressed across the crypt-villus axis in mouse small intestine. Thus, it may be plausible that even a single immortalized cell line may exhibit differential mAChR expression dependent on differentiation. Given these findings
and the rapid advancement of primary human intestinal organoids, the 2D intestinal epithelium described herein is a powerful tool to study mAChRs with higher phenotype fidelity and cellular heterogeneity.

4.5 Conclusion

Using a primary cell derived in-vitro model of the human small intestinal epithelium (Section 3), this study suggested that cholinergic activation has significantly different effects on intestinal epithelium derived from immortalized IECs as compared to primary IECs. Neither nicotinic nor muscarinic activation of primary intestinal epithelium ameliorated TNF-α induced epithelial barrier injury. Muscarinic activation of primary intestinal epithelium decreased the transcellular transport of a 70 kDa dextran. Given the contradiction with previous immortalized IEC studies, the results warrant further investigation, including a characterization of primary IEC mAChR expression as a function of differentiation.
5. AIM 3: DEVELOP A LOW COST, RAPID, AND SCALABLE MICROFLUIDIC ORGAN CHIP FABRICATION TECHNIQUE

This aim served to develop an alternative organ chip fabrication technique that circumvents some disadvantages of PDMS soft lithography (Section 2.3.2), namely manufacturing cost, throughput, scalability and microfabrication facility requirements. The newly developed organ chip was manufactured via laser micromachining of commercially available adhesives, plastics, and track etched membranes. The fabrication technique was first validated by culturing both Caco-2 and primary human organoid derived IECs on a traditional bilayer organ chip. Next, the fabrication technique was used to develop a tri-layer organ chip integrating both 2D monolayer and 3D culture of primary human intestinal organoids. The technique presented here rapidly (hours) produced inexpensive (~$2 per chip) organ chips using an inexpensive, ($12,450) benchtop laser. CAD-based manufacturing enabled iterative design with zero tooling or mold costs. Collectively, the technique presented here had significant advantages in cost, throughput, scalability, and equipment requirements as compared to PDMS soft lithography.

*This work was submitted to Biofabrication in 2018, in review.*
5.1 Introduction

Two-dimensional (2D) tissue culture originated one century ago and remains invaluable for studying biology and developing therapeutics. Nevertheless, 2D cultures inaccurately represent native tissues. In vivo, continuous nutrient supply and waste product removal occurs via luminal flow via the circulatory and lymphatic systems, which maintain a homeostatic steady state. Native cells experience physical cues such as fluid shear forces or multilateral mechanical stretching and chemical cues from heterogeneous tissue-tissue interfaces. Microfluidic organs-on-chips are cell culture models that recapitulate heterogeneous tissue-tissue interfaces and integrate continuous media perfusion to maintain biochemical homeostasis and flow-induced shear stress.

The predominant embodiment of organs-on-chip is a bi-layer design featuring two channels interfaced by a porous membrane or hydrogel. Culturing different cell types on opposing membrane surfaces or in adjacent channels mimics heterogeneous tissue-tissue interfaces. The bi-layer chip has been used to model the blood-brain barrier, the hematopoietic stem cell niche, the gut microbiome-epithelial-immune interface, the lung alveolar-capillary interface, and the placental barrier. Future organs-on-chips integrating patient-derived cells may enable personalized medicine. Interconnecting multiple organs-on-chip via an artificial circulatory system, termed body-on-a-chip, may permit in-vitro pharmacokinetics. Despite these advances, organs-on-chips have been concentrated among bioengineering research groups and have yet to transition to mass clinical or diagnostic applications. Chip automation and parallelization remains challenging, and complex, multi-layered (>

...
2 layers) chips are limited.

Facile, rapid, economic, and reliable organ-on-chip fabrication would promote interdisciplinary adoption and technological development. Organs-on-chips are most frequently fabricated via poly(dimethylsiloxane) (PDMS) soft lithography.\textsuperscript{3,71,75,152,161} The advantages of PDMS organs-on-chips include high feature resolution, biocompatibility, optical transparency, and gas permeability enabling culture oxygenation and pH control in standard CO\textsubscript{2} incubators.\textsuperscript{162} But, PDMS organs-on-chips have several drawbacks. PDMS’ gas permeability\textsuperscript{162} prohibits O\textsubscript{2} tension control, which is necessary for recapitulation of hypoxic tissue conditions, as seen in the small intestinal lumen\textsuperscript{87}. PDMS’ water vapor permeability\textsuperscript{162} results in evaporation induced bubble formation or high osmolarity, which can block flow and impact cell fate and viability\textsuperscript{163}. PDMS also absorbs hydrophobic molecules\textsuperscript{162}, complicating drug pharmacokinetic studies. While PDMS easily bonds to both itself and glass via plasma activation, bonding to polymers requires additional processing such as silanization.\textsuperscript{72} PDMS soft lithography requires significant microfabrication training and capital infrastructure.\textsuperscript{151} Moreover, initial prototyping may require multiple iterations and lithographic mold fabrication can be prohibitively expensive ($150-$500 per design from 3rd party manufacturers). Other investigators have 3D printed microfluidic cell culture models\textsuperscript{164,165}, but these single channel devices do not integrate membranes for recapitulating tissue-tissue interfaces. Rapid and economic microfluidic devices were fabricated with laser or razor cut thermoplastic or adhesive sheets, though these were typically analytical microdevices\textsuperscript{166-168}, and not cell culture platforms. A recent work comparing adhesive tape based cell culture platforms against traditional 96-well plates and analogous PDMS platforms found
that tape based platforms compared favorably in regards to cell viability and morphology across three different human breast cancer cell lines\textsuperscript{169}. This study highlighted additional benefits of tape based cell culture platforms such as economical fabrication, high throughput, and facile bonding of disparate materials such as polystyrene and glass. However, cell culture experiments were limited to 24 hours, immortalized human cell lines, and performed on 2D substrates, either glass or polystyrene\textsuperscript{169}. Therefore, in this study, we aimed to fabricate multi-layered, membrane integrated organs-on-chips for long term primary cell culture without PDMS soft lithography.

A single layer epithelium lines the intestinal wall and forms the rate-limiting barrier to drug absorption\textsuperscript{170}. Therefore, oral drug absorption in humans can be approximated using an in vitro differentiated, intestinal epithelium\textsuperscript{171}. The human colon carcinoma Caco-2 cell line cultured on permeable supports differentiates into a monolayer with some features of the native small intestine\textsuperscript{172}. Organ-on-chip technology was used to develop Caco-2 models with greater fidelity to human intestinal structure and function\textsuperscript{3,74,75}. Nevertheless, the immortalized Caco-2 cell line has limited genetic similarity to human intestinal epithelium. Recent advances in intestinal biology have enabled primary human cultures containing intestinal stem cells (self-renewal), Paneth cells (antimicrobial peptide secretion), goblet cells (mucus production), enteroendocrine cells (hormone production), and enterocytes (absorption)\textsuperscript{55,173}. Primary, three dimensional (3D) organoids are established from biopsy- or resection-derived intestinal stem cells embedded in Matrigel\textsuperscript{173}. While intestinal organoids are genetically and phenotypically more closely related to the native epithelium, organoids form closed lumens that complicate intestinal transport studies. To enable luminal access, researchers
cultured primary intestinal monolayers on permeable supports\textsuperscript{64,67,132}, but these models failed to emulate the native 3D tissue structure. More recently, primary intestinal monolayers exhibiting crypt-villus like tissue organization were formed on microengineered scaffolds\textsuperscript{174} and organs-on-chips.\textsuperscript{153} Thus, in this study, we too aimed to integrate primary intestinal monolayers and organoids on organ chips.

Here, we describe a “cut and assemble” process for manufacturing thermoplastic organs-on-chips. Most importantly, our technique produced multilayer devices with integrated polymeric membranes and Luer fluidic interfaces faster than soft lithography (hours versus days) at minimal cost (~$2 per device) without specialized bonding. The resulting biocompatible, thermoplastic chips are water vapor impermeable, thereby eliminating evaporation-induced bubble formation and osmolarity shifts while potentially enabling O\textsubscript{2} tension control. The cut and assemble manufacturing technique was validated by reengineering a recently described gut-on-a-chip\textsuperscript{3,75} using Caco-2 cells and primary human intestinal organoids. Caco-2 cells and primary organoids cultured in a bilayer chip formed confluent monolayers expressing tight junctions and low permeability comparable to static Transwell\textsuperscript{TM} controls. Furthermore, Caco-2 cultures on chip differentiated four times faster towards the enterocyte phenotype as compared to controls and produced mucus, corroborating previously published results.\textsuperscript{175} We integrated primary intestinal monolayers and 3D intact organoids in a dual membrane, tri-layer organ chip. Monolayers exhibited 3D tissue structure spanning $10^2 \, \mu m$ in height and organoids formed typical cystic structures in close proximity to monolayers, potentially enabling paracrine signaling. The rapid, benchtop, fabrication process presented here has great potential to enable microphysiological modeling of multicellular tissues, 3D cell
culture, and the study of paracrine signaling. Although the presented experiments integrate existing technologies such as primary organoid culture, membrane-integrated organs-on-chips, and microfluidic device fabrication via adhesive films, they represent a scientific work that enables wider adoption of organs-on-chips and drives technological development.

5.2 Methods

Chip design and fabrication

In contrast to practically all previous iterations of gut-on-a-chip, the bi-layer chip discussed herein was fabricated without lithography. The rapid cut and assemble manufacturing process required only a laser cutter/engraver, double-sided adhesive tape, and sheets of poly(methyl methacrylate) (PMMA) and polyester (PET). First, all chip layers were simultaneously designed using CAD software (SOLIDWORKS, Waltham, MA). Then, a laser cutter (Epilog Zing 16, Epilog Laser, Golden, CO) was used to transfer the CAD design to the various materials. The 3/16” PMMA upper layer (McMaster-Carr, Robbinsville, NJ) featured four circular through-holes, which served as fluidic inlets and outlets for both the upper and bottom fluidic layers. The second layer was comprised of a 0.003” polyester or a 1/16” PMMA sheet (McMaster-Carr, Robinsville, NJ), which was sandwiched between 50 µm thick double-sided adhesive tape (966 adhesive, 3M, Maplewood, Minnesota). This 1 mm wide channel served as the upper fluidic channel used for cell culture. The circular inlets and outlets matched the diameter of the through-holes in the uppermost 3/16” PMMA sheet. The third layer, a polycarbonate (PC), track etched membrane with 1.0 µm diameter pores (cat no. 7091-4710, GE Healthcare, Marlborough, MA), featured two circular through-holes with
identical diameters as the previous layers. The membrane through-holes enabled fluidic access to the bottom fluidic channel. The fourth layer was comprised of a 0.003” polyester sheet sandwiched between 50 µm double-sided adhesive tape. The features of this layer are identical to the upper fluidic layer but reflected 90°. The fifth and final layer was a No. 1 glass coverslip. During laser cutting, the protective paper lining on both the PMMA sheets and the double-sided adhesives minimized exposing the materials to burn products. Post laser cutting, 10-32 UNF threads were hand tapped in each of the circular through-holes on the top PMMA sheet. Next, the protective paper lining on both upper and lower PMMA sheets were removed and the plastics were serial cleaned by rinsing with deionized water, Contrad 70 detergent (Fisher), deionized water, isopropyl alcohol and then dried with compressed nitrogen. The laser cut layers were then assembled layer by layer using a custom jig to facilitate hole and channel alignment. The two layers of double sided adhesive tape served as the apical and basal fluidic channels and simultaneously bonded the entire chip. Post assembly, the devices were stored under vacuum at 37 °C overnight in order to eliminate outgassing induced bubble formation. Threaded, polypropylene, male Luer lock fittings (cat no. EW-45518-84, Cole-Parmer, Vernon Hills, IL) were connected to the chip via the threaded inlets and outlets. Barbed PC connectors with female luer lock connections (cat no. 11733, Qosina, Ronkonkoma, NY) were connected to the chip. Tubing (cat no. SC-95802-01, silicone, ID 1/32”, OD 3/32”, Cole-Parmer, Vernon Hills, IL) was fitted over the barbed ends to perfuse culture medium through the device.

The bi-layer chip was modified for use with organoid derived primary human intestinal epithelial cells to enable seeding at a lower cell concentration while enabling
confluent monolayer formation post-seeding. The top fluidic channel was replaced with a 1/16” PMMA sheet sandwiched between 50 µm thick double-sided adhesive tape (966 adhesive, 3M, Maplewood, Minnesota) for a final channel height of 1.6876 mm. Furthermore, the top 3/16” PMMA cover was not bonded until the day of use at which point the PC membrane was treated with oxygen plasma (50 Watts, 30s, March PX-250 Plasma System).

A dual membrane tri-layer chip was designed and fabricated for co-culture of 2D primary monolayers and 3D organoids (Figure 21). Two additional components were added to the bi-layer architecture: (1) a 1/16” PMMA sandwiched between two pieces of 50 µm thick double-sided adhesive tape as with the 3D gel channel, and (2) a PC, track etched membrane with 30.0 µm diameter pores (cat no. PCT30047100, Sterlitech, Kent, WA).

**Caco-2 cell culture**

Caco-2 epithelial cells were obtained from the American Type Culture Collection (ATCC) and cultured in Dulbecco’s Modified Eagle Medium (DMEM, cat no. 11995-065, Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS, cat no. 35-011-CV, Corning), and 100 U/mL Penicillin-Streptomycin (cat no. 15140122, Thermo Fisher Scientific). Cells were cultured in a 37°C, 5% CO₂ incubator. All experiments were done with Caco-2 cells between passage numbers 40-50.

After fabrication, the bi-layer chip was sterilized via UV irradiation (300 mJ/cm²) of the top and bottom chip surfaces (Spectrolinker XL-1000, Spectronics Corporation, Westbury, NY). All tubing and fittings were preassembled and sterilized via autoclave. Both apical and basal fluidic channels were coated with a 400 µg/mL solution of rat tail
type I collagen (cat no. 354249, Corning, Corning, NY) in DMEM for at least one hour at 37°C inside a humidified cell culture incubator with 5% CO₂. The device and tubing were then flushed with Caco-2 culture medium via a sterile, plastic syringe. Caco-2 cells were harvested from a sub confluent T75 flask via 0.25% Trypsin-EDTA (cat no. 25200056, Thermo Fisher Scientific) and incubation at 37 °C. After cell detachment, the Trypsin-EDTA was diluted with an equal volume of cell culture media and centrifuged at 300g for 5 minutes at room temperature. The cells were suspended in cell culture medium at 5 x 10⁶ cells/mL. The outlet to the bottom fluidic channel was clamped and the harvested cells were infused into the top fluidic channel via a sterile 1 mL syringe. The chips were placed in a 37 °C, 5% CO₂ cell culture incubator for 1-2 hours for cell attachment. Post attachment, culture medium was perfused through the apical channel via a syringe pump (PhD 2000, Harvard Apparatus, Holliston, MA) at a rate of 0.84 uL/min. The next day, culture medium was perfused through both the apical and basal channels at a rate of 0.84 uL/min.

As controls, Caco-2 cells were cultured on 0.4 µm polyester (cat no. 353095, Corning, Corning, NY) Transwell™ inserts in a 24 well plate. Prior to cell seeding, the inserts were coated with 200 µL of the collagen solution for at least 1 hour at 37 °C inside a humidified cell culture incubator with 5% CO₂. Caco-2 cells were seeded on the inserts by adding 200 µL of Caco-2 cell suspension (seeding density of 2.6 x 10⁵ cells/cm²) and then adding 600 µL of media to the basolateral compartment. The apical and basal cell culture medium was refreshed every other day.
Organoid culture of biopsy derived intestinal stem cells

De-identified endoscopic tissue biopsies were collected from grossly unaffected (macroscopically normal) areas of the duodenum in children undergoing endoscopy for gastrointestinal complaints. Informed consent and developmentally appropriate assent were obtained at Boston Children’s Hospital from the donors’ guardian and the donor, respectively. All methods were approved and carried out in accordance with the Institutional Review Board of Boston Children’s Hospital (Protocol number IRB-P00000529). Cells were cultured as 3D organoids embedded in 50 µL of Matrigel (cat no. 354230, Corning) on a 24-well plate as previously described. Expansion Medium (EM) for expanding intestinal organoids was prepared from a mixture of Advanced DMEM/F12 medium (cat no. 12634028, Gibco) and 50% L-WRN conditioned medium, which was prepared from L-WRN cells, as previously described. This cell line produces Wnt-3A, R-spondin 3, and noggin. EM was supplemented with GlutaMAX (1x, cat no. 35050061, Gibco), HEPES (10 mM, cat no. 15630080, Gibco), Primocin (0.1 mg/mL, Invivogen), B-27 supplement (0.5x, cat no. 12587010, Gibco), N-2 supplement (0.5x, cat no. 17502048, Gibco), nicotinamide (10 mM, cat no. N0636, Sigma-Aldrich), N-acetyl cysteine (0.5 mM, cat no. A7250, Sigma-Aldrich), epidermal growth factor (50 ng/mL, cat no. 315-09, Peprotech), gastrin (50 nM, cat no. A7250, Sigma-Aldrich), A-83-01 (500 nM, cat no. SML0788, Sigma), prostaglandin E2 (10 nM, cat no. 14010, Cayman Chemical), and SB202190 (10 µM, cat no. S7067, Sigma-Aldrich). Y-27632 ROCK inhibitor (10 µM, cat no. Y0503, Sigma-Aldrich) was added to the organoid medium for the first 48 hours following cell isolation or passage. The culture medium was refreshed every 48 hours using 500 µL per well. Cell culture was performed in a humidified, 37°C,
5% CO₂ incubator.

Every 7-10 days, the organoids were passaged to new 24-well plates at a ratio of 1:4-1:8 depending on culture density. Matrigel droplets were scratched off the 24-well plate using a 1000 µL pipette tip and collected into a 15 mL conical tube. The organoids were centrifuged at 500g for 5 minutes at room temperature. After aspirating the cell culture medium, the organoids were re-suspended in 0.5 mM ethylenediaminetetraacetic acid (EDTA, cat no. AM9260G, Gibco) in 1x PBS and re-centrifuged at 300g for 5 minutes at room temperature. After aspirating the EDTA, the organoids were re-suspended in Trypsin-EDTA and incubated in a 37°C bath for 2 minutes. The Trypsin-EDTA was then quenched via a 2:1 dilution with Caco-2 culture medium containing 10% FBS and the organoid suspension was triturated ~10x using a 1000 µL pipette tip to produce single cells and small organoid fragments. The cells were pelleted at 300g for 5 minutes at room temperature. The cells were suspended in 4°C Matrigel and re-plated on new 24-well plates. The plated Matrigel was incubated at 37°C for 15 minutes before adding 500 µL of culture medium containing 10 µM ROCK inhibitor.

**Monolayer culture of primary human intestinal epithelial cells**

Polyester Transwell™ inserts in a 24-well plate were coated with 200 µL of collagen solution for at least 1 hour at 37 °C inside a humidified cell culture incubator with 5% CO₂. Organoids were harvested for dissociation and monolayer seeding after 7-10 days of culture. Matrigel droplets were harvested and processed in Trypsin-EDTA as described above. The Trypsin-EDTA was then quenched via a 2:1 dilution with Caco-2 culture medium containing 10% FBS and the organoid suspension was triturated ~20x using a 1000 µL pipette tip to produce single cells and small organoid fragments. The cell
suspension was filtered through a 40 µm cell strainer (cat no. 22-363-547, Fisher) into a 50 mL conical tube and pelleted at 300g for 5 minutes at room temperature. The cells were resuspended in EM medium with 10 µM ROCK inhibitor. Transwell™ inserts were seeded using 200 µL of cell suspension (seeding density of 9.09 x 10^5 viable cells/cm²) and then 600 µL of media + 10 µM ROCK inhibitor was added to the basolateral compartment. ROCK inhibitor was used for the first 48 hours of cell culture and the apical and basal cell culture medium was refreshed every other day. Following 2 days of culture in EM medium, the apical and basal medium was replaced with differentiation medium (DM) containing Advanced DMEM/F12 + 20% FBS + 4mM GlutaMAX supplement + 100 U/mL Penicillin-Streptomycin. Apical and basal DM medium was replenished every 48 hours.

For seeding primary human intestinal epithelial cells on bi-layer chips, the cells were harvested as described above and suspended at a concentration of 10 x 10^6 cells/mL. Cell viability was assessed via trypan blue exclusion by incubating cells with an equal volume of 0.4% Trypan Blue Solution (15250061, Thermo Fisher Scientific). Prior to seeding, the bi-layer chip was treated with O₂ plasma (50 Watts, 30s, pure O₂, March PX-250 Plasma System) and the 3/16” acrylic cover was bonded. Note that primary cell adhesion required a plasma treated membrane whereas Caco-2 cells adhered without plasma treatment. The chip was sterilized via UV irradiation as previously described. The chip was coated with collagen solution for 2 hours after which the collagen was flushed with EM medium containing 10 µM ROCK inhibitor. The cell suspension was perfused through the apical channel and the chips were maintained under static conditions in a 37 °C humidified cell culture incubator with 5% CO₂ for 5-6 hours to enable cell attachment.
Then, apical medium was perfused at 1.48 µL/min with EM medium for 3 days before changing to DM medium for 2 additional days. The basal medium was manually refreshed every 24 hours, with EM and DM media, as above.

**Alkaline phosphatase measurement**

Alkaline phosphatase (AP) expression was measured using a commercial kit (AS-71109, AnaSpec, Fremont, CA). All kit components were prepared as specified by the manufacturer. Cell lysate from Transwell™ inserts was prepared as follows: medium was removed, and the inserts were washed two times with sterile PBS in both apical and basal compartments. Next, 200 µL of sterile 10x TrypLE™ Select (A1217701, Thermo Fisher Scientific) was added to the apical side of each insert prior to incubation at 37°C for ~15 minutes. Cells were collected into a sterile centrifuge tube and inserts were washed with 800 µL of sterile PBS and pelleted at 300g for 5 min at room temperature. The pellet was re-suspended in 150 µL of AP kit supplied buffer, washed and re-suspended in 150 µL of buffer. A 10 µL aliquot of cell suspension was removed to quantify the cell number via hemocytometer. The cells were centrifuged and suspended in 0.2% Triton X-100 (AC327371000, Fisher Scientific). The cells were incubated for 10 minutes at 4°C with agitation and then centrifuged at 2500g for 10 minutes at 4°C. The supernatant was used for the AP assay. 50 µL of the supernatant was moved to a well of a black, polystyrene, 96 well plate (12-566-620, Fisher Scientific). Then, 50 µL of the reaction mixture was added to each well and the plate was manually mixed for 30 seconds. Following a 30 minutes incubation at 37°C, 50 µL of stop solution was added to each well. The plate was manually mixed for 30 seconds. The fluorescence was measured via plate reader (EnSight™, PerkinElmer) using 485nm and 528nm emission and excitation wavelengths,
respectively. For Caco-2 on chip, the same protocol was used except that the 10x TrypLE™ and the subsequent PBS wash was infused via a sterile, plastic syringe to detach cells. The actual amount of AP was interpolated using a calibration curve generated with the kit supplied AP standard.

**Paracellular permeability measurement**

The apparent permeability coefficient for tetramethylrhodamine (TRITC) labeled dextran (4.4 kDa) (cat no. T1037, Sigma-Aldrich) was determined by measuring transport across the Caco-2 cell monolayer. TEER values of cell monolayers were measured prior to the permeability assay and monolayers with TEER values below 165 Ω x cm² were not used. For control Transwell cultures, 300 µL of 500 µM dextran in cell culture medium was applied to the apical compartment. 100 µL was immediately sampled from the apical compartment, transferred to a black, polystyrene, 96 well plate and stored at 4°C. Transwells were maintained in a humidified, 37°C + 5% CO₂ incubator. 100 µL aliquots were sampled from the basolateral compartment every 30 minutes over 3 hours and 100 µL of fresh medium preheated to 37°C was added to replace the aliquoted volume. The fluorescence intensity of the collected basolateral samples was measured at 557nm and 576nm emission and excitation wavelengths, respectively. The interpolated dextran concentration was determined using a standard curve. The apparent permeability coefficient was calculated as specified. For Caco-2 on chip, the dextran solution was perfused through the upper channel and cell culture media was perfused through the lower channel at a rate of 0.84 µL/min. Aliquots were sampled from the lower channel every hour and stored at 4°C. The apparent permeability coefficient for Lucifer Yellow (450 Da) (cat no. L0259, Sigma-Aldrich) across primary organoid derived monolayers on
static inserts and bi-layer chips was determined as described above.

**Monolayer morphology and mucus production measurement**

Cell morphology was assessed by staining F-actin, nuclei, and ZO-1 tight junction protein. Monolayers were washed and stained at room temperature. All monolayers were washed three times with PBS and fixed in 4% formaldehyde (cat no. 28906, Thermo Fisher Scientific) for 20 minutes. Post fixation, the monolayers were permeabilized in 0.1% Triton X-100 for 20 minutes and blocked overnight in 2% bovine serum albumin solution (BSA, cat no. 97061-416 VWR). The next day, the monolayers were stained with anti-ZO-1 antibody (1:200, 1 hour, cat no. 339188, Thermo Fisher Scientific), phalloidin (1:500, 1 hour, cat no. A22287, Thermo Fisher Scientific), and DAPI (1:1000, 10 minutes, cat no. D1306, Thermo Fisher Scientific) diluted in 1% BSA solution. Transwell membranes were isolated and mounted on a standard glass slide and coverslip with Gold Antifade Mountant (cat no. P36931, Thermo Fisher Scientific).

Mucus production was assessed by alcian blue and immunostaining for MUC2 protein. Monolayers were washed and stained at room temperature. For alcian blue staining, monolayers were washed three times with PBS and fixed with 4% formaldehyde for 20 minutes. Next, the cell monolayers were washed three times with PBS and stained for 20 minutes with a 1% alcian blue solution in 3% acetic acid (pH=2.5, cat no. 50-319-30, Fisher Scientific) that was filtered via a 0.1µm syringe filter. Post staining, monolayers were washed five times with PBS. For MUC2 immunostaining, the monolayers were washed, fixed, permeabilized, and blocked as previously described. Mucin was detected via an anti-mucin 2 primary antibody (1:200, 1 hour, cat no. PA1-23786, Thermo Fisher Scientific) and an Alexa Fluor 647 secondary antibody (1:1000, 1
hour, A-21244, Thermo Fisher Scientific). For monolayers in the bi-layer chip, the protocol remained the same, but washes and stains were applied via syringe pumps to minimize damage to the monolayer.

The tri-layer chip was stained as follows. All steps were performed at room temperature and all washes and stains were applied to both apical and basal channels via syringe pump to minimize damage to the monolayer. The chip was washed with 500 µL of PBS followed by 4% formaldehyde for 30 minutes. The chip was washed with 500 µL of PBS prior to application of 0.1% Triton X-100 for 30 minutes. The chip was then washed with 2.5% goat serum applied via syringe pump overnight. The chip was washed with 500 µL of PBS prior to the addition of a cocktail of DAPI and anti-Ki67 antibody (1:200, MA514520, Thermo Fisher Scientific) incubated for 2 hours. The chip was washed with 500 µL of PBS via syringe pump and secondary antibody (1:500, A-11029, Thermo Fisher Scientific) was applied overnight. The chip was washed with 500 µL of PBS and imaged immediately.

Fluorescence microscopy was performed on a Zeiss Axio Observer.Z1 microscope equipped with an ORCA-Flash4.0 camera (cat no. C11440-22CU, Hamamatsu). Color images of alcian blue stained monolayers were obtained on an Olympus IX51 microscope equipped with an Olympus DP70 camera.

Confocal microscopy was performed on an LSM 710 confocal microscope (Zeiss) equipped with Zen software (Zeiss) using the Plan-Apochromat 10x/.45 M27 objective. The 405-nm laser was used to excite DAPI. A 512x512 pixel scan format was used. Z-slices were acquired at 2.87-µm intervals with each slice representing the average of 8 scans.
Statistical analysis

Student’s t-test or ANOVA followed by a Tukey’s post-hoc correction was used to determine statistical significance as indicated in figure legends (error bars indicate standard error of the mean (SEM); p values < 0.05 were considered to be significant).

5.3 Results and discussion

Rapid and facile cut and assemble of bi-layer organs-on-chips

The cut and assemble manufacturing technique eliminated PDMS elastomer and microfabrication to overcome the aforementioned limitations of soft lithography. The bi-layer chip presented here (Figure 15a-c) featured apical and basal channels interfaced via a PC track etched membrane across a 10 mm length and 1 mm width. The channel height was application dependent. In one embodiment, Caco-2 cells were cultured in a 196 µm tall channel. In a second embodiment to enable monolayer seeding with fewer cells, primary human intestinal cells were cultured in a 1.6875 mm tall channel. Both apical and basal channels had independent inlet and outlets for cell seeding and medium perfusion. Each bi-layer chip consisted of 9 discrete layers (Figure 15a) irreversibly bonded to membrane interfaced channels (Figure 15b). While the bi-layer chip featured 9 layers, each device was constructed in 4 steps (Figure 15d) to align and bond 5 components: (1) a top 3/16” acrylic layer that sealed the chip and provided fluidic connections, (2) a thermoplastic sheet flanked by two adhesive tape layers for the apical channel, (3) a track etched PC membrane to interface the basal and apical channels, (4) a thermoplastic sheet flanked by two adhesive tape layers for the basal channel, and (5) a bottom glass coverslip that sealed the chip. Device geometries were designed in a CAD program and transferred to the respective layers using a benchtop laser cutter. Post laser
cutting, 10-32 UNF threads were tapped on the top acrylic layer, enabling commercial Luer lock connections. Then, the device was assembled layer by layer (Figure 15d).

**Figure 15**: Laser cut and assembled membrane integrated bi-layered organ chip. a) A schematic showing the integration of 9 discrete layers to form a bi-layered organ chip. b) A schematic of an assembly cutaway view of the bi-layered organ chip showing the inner apical and basal microchannels. c) A photograph of the bi-layered organ chip composed of clear PMMA, acrylic adhesive, polycarbonate track etched membrane, and glass coverslip. d) 5 layers are aligned and irreversibly bonded in 4 steps to form the apical and basal channels separated by a polycarbonate track etched membrane with a pore diameter of 1.0 µm.

Cut and assemble fabrication boasts several advantages compared to soft lithography. By eliminating lithographic mold fabrication and the subsequent PDMS degassing, curing, and bonding, chips were cut and assembled in approximately one hour while soft lithography fabrication spans several days. The total material cost per cut and assembled chip was approximately $2 (Table 2). Economical and rapid prototyping is particularly useful in early project phases for iterative design whereas photomasks may be cost prohibitive ($150-$500 per mask depending on feature size) and photo plotting may require several days for turnaround. Furthermore, eliminating lithography enables researchers without microfabrication experience or facilities to build organs-on-chips with minimal capital investment. Our laser cutting system (along with all peripheral equipment including an air compressor and a fume extraction system) cost a total of $12,450 whereas a mask aligner may cost between $30,000-$100,000 and does not include peripheral equipment such as a spin coater and vacuum systems. Makerspaces
equipped with laser cutters, plotter cutters, and 3D printers further reduce capital costs and increase accessibility.\textsuperscript{88} Furthermore, cut and assemble is more amenable to high volume manufacturing techniques such as die cutting, injection molding, and industrial laser cutting as compared to soft lithography.\textsuperscript{177}

<table>
<thead>
<tr>
<th>Component</th>
<th>Bulk Cost ($)</th>
<th>Per Chip Cost ($)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3/16” Acrylic (12”x12” sheet)</td>
<td>8.06</td>
<td>0.08</td>
</tr>
<tr>
<td>3M 966 Adhesive (12”x12” sheet)</td>
<td>6.72</td>
<td>0.24</td>
</tr>
<tr>
<td>Whatman 7060-4710 Cyclopore Membrane (100 pc)</td>
<td>152.2</td>
<td>1.52</td>
</tr>
<tr>
<td>0.003” Polyester Film (40” x 10’ sheet)</td>
<td>15.53</td>
<td>0.01</td>
</tr>
<tr>
<td>Fisherbrand 24x60 mm coverglass (66 pc)</td>
<td>12.54</td>
<td>0.19</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>179.52</strong></td>
<td><strong>2.04</strong></td>
</tr>
</tbody>
</table>

Table 2: Cost of materials to fabricate a cut and assembled bi-layer organ chip. Per chip cost of sheet based materials was calculated by dividing the sheet area by the chip area.

Though often neglected, facile but reliable fluidic connections simplify chip use, automation, and high-throughput application. Luer Lock and Luer Cone are fluidic interfacing standards but most fabrication techniques, including soft lithography, are incompatible with threaded fluidic connectors. Therefore, we engineered cut and assembled organ chips with threaded ports to accept standard Luer fittings. Cut and assembled organ chips potentially enable O\textsubscript{2} tension control to recapitulate hypoxic tissues because O\textsubscript{2} permeability in acrylic is an order of magnitude lower than PDMS. Similarly, PDMS’ high vapor permeability results in significant evaporation relative to the microliter-sized compartments, thereby increasing media osmolarity and compromising cell viability.\textsuperscript{163} However, evaporation induced osmolarity shifts are a
minor concern, however, because most organ chips are operated under perfusion.\textsuperscript{3,71,75,96,161,178} The channel geometry can be easily modified, and additional channels and membranes can be added. This may be useful when emulating multiple biological barriers such as an epithelium alongside an endothelium.\textsuperscript{152}

The advantages discussed above are a trade off with reduced feature resolution compared to traditional microfabrication. In the x-y plane, soft lithography reliably produces micron-sized features while laser cutting is limited to $10^2$-10$^3$ μm. Figure 16 shows the average deviation of channels cut from the 196 μm tall bilayer chip channel element (0.003” PET sheet flanked by 966 adhesive tape on both sides). Note that measurements showed overcutting (positive error) regardless of the nominal channel width. Overcutting may be an effect of the laser spot size during material ablation; the CAD drawing specifies a nominal channel width but a large laser spot size leads to overcutting past the nominal lines. Furthermore, the results (Figure 16) showed a prohibitively large error (~33 % and ~70 %) at nominal channel widths of 250 and 125 μm, respectively. However, error was limited to 9.6 % and 12.6 % for nominal channel widths of 1000 μm and 500 μm, respectively. These errors may be reduced by compensating for laser overcutting by specifying nominal channel width of 900 μm channels to achieve an observed channel width of 1000 μm, for example. While a few previously described tissue chips utilized channel widths...
less than 500 µm\textsuperscript{151,152,154}, channel widths of 1 mm or greater are more frequently used.\textsuperscript{74,96,151,153,155,156} Therefore, we deemed laser cut and assemble suitable for the fabrication of organ chips with channel widths of 500 µm or greater. Further, high performance laser cutters claim a focused spot size of 25 µm, thereby reducing overcutting and approaching lithographic resolutions. Note, however, that laser cutting microfluidic devices introduces error via manual operations such as laser focusing. Additionally, the error may vary depending on the processed material.

In the z plane, traditional microfabrication enables tunable feature height by controlled photoresist deposition. Feature heights of 10^2-10^3 µm conventionally require multiple spin coatings and long subsequent baking steps. Theoretically, the minimum cut and assemble feature height is limited to one adhesive tape layer as a channel element, 60 µm in our case, though alternative tapes may be available. Intermediate feature heights are partially constrained; feature height is increased by layering inert thermoplastic materials such as polyester (PET) film between two adhesive tapes. For example, the Caco-2 bi-layer chip featured 196 µm tall channels composed of a 0.003” PET film sandwiched by two 60 µm adhesive tapes, while the primary cell bi-layer chip featured a 1.6875 mm tall channel composed of a 1/16” acrylic sheet sandwiched by two 60 µm adhesive tapes. The maximum cut and assemble feature height is theoretically without limits.

**Recapitulating the human intestine on cut and assemble chips**

To assess biocompatibility, human intestinal Caco-2 cells were cultured in the cut and assemble bi-layer chip under apical and basal medium perfusion. Prior to cell seeding, both channels were coated with rat tail type I collagen to promote cell adhesion.
The medium flow rate of 0.84 µL/min delivered a shear stress of 0.015 dyne/cm² across the epithelial monolayer as previous work suggested an intestinal lumen shear stress of 0.002-0.08 dyne/cm².³ Caco-2 cells were cultured for 5 days on chip and compared to cells grown on static Transwell™ inserts for 5 and 21 days. After these time points, the cells were fixed and stained for F-actin, tight junction protein ZO-1, and cell nuclei. Cells formed confluent monolayers with comparable morphology, F-actin expression and tight junctions under all three culture conditions (Figure 17). Importantly, Caco-2 cells formed confluent monolayers across the integrated membrane demonstrating suitability for studying epithelial barrier function. While laser cut, or cutter plotter processed adhesives were previously used to fabricate microfluidic devices, these were analytical microfluidic devices.¹⁷⁹-¹⁸² This study demonstrates that acrylic based adhesives are biocompatible channel elements that support human intestinal epithelial cell culture.
Figure 17: Evaluation of biocompatibility of laser cut and assemble chips. Cell morphology is visualized by immunostaining against ZO-1 tight junctions (green), DAPI nuclei (blue), and F-actin cytoskeleton (red) of Caco-2 cells grown on static Transwell inserts for 5 days (top), 21 days (middle), or on laser cut and assemble chips for 5 days (bottom). Scale bar denotes 20 µm.

Microfluidic perfusion culture impacts human intestinal function in vitro

Following validation of biocompatibility of cut and assemble bi-layer chips we next assessed cellular function. As Caco-2 cells cultured on porous membranes are used to model intestinal transport\cite{183}, we quantified barrier integrity by measuring the apical to basal paracellular transport of a fluorescent dextran (4.4 kDa). Paracellular transport is governed by molecular diffusion through tight junctions rather than active transport via cell membrane bound transporters. The apparent permeability of dextran was calculated as previously described.\cite{184} We did not observe a significant difference ($p = 0.9748$) between the 5 and 21-day static Transwell\textsuperscript{TM} models (Figure 18a). Furthermore, the results were consistent with previous permeability measurements of a similarly sized dextran across Caco-2 monolayers.\cite{185-187} In contrast, the apparent permeability across the
cut and assemble chip Caco-2 monolayers was 40 and 100 times higher than the static 5-day and 21-day Transwell™ models, respectively (p < 0.0001) (**Figure 18a**). This finding may be partly explained by ongoing perfusion increasing flux at the monolayer surface.188

During extended culture, typically 3 weeks, Caco-2 cells on static Transwell™ inserts differentiate toward an intestinal enterocyte phenotype expressing transport proteins and brush border enzymes.170 Among brush border enzymes, alkaline phosphatase (AP) is a frequently used differentiation marker.122,189,190 As expected, analysis of AP expression revealed a significant 1.7-fold increase (p = 0.0317) between Caco-2 on static Transwell™ inserts at 5 versus 21 days (**Figure 18b**). However, between Caco-2 cells cultured for = 5 days on chip versus static, AP expression significantly increased 2.2-fold (p = 0.0035) (**Figure 18b**). The increased AP expression was consistent with a previous study that reported 4-fold increased AP activity by human proximal tubular epithelial cells in response to perfusion.191 Perfusion of media may expedite cell differentiation via flow-induced shear stress, increased exposure to nutrients and/or decreased exposure to cellular metabolites and waste products compared to static conditions where media is replenishment every 48 hours.
Figure 18: Characterization of epithelial barrier function and Caco-2 monolayer differentiation. 
a) The apparent paracellular permeability quantified by tracking a 4.4 kDa fluorescent dextran through Caco-2 monolayers cultured on Transwell inserts for 5 or 21 days and Caco-2 monolayers cultured on chip for 5 days. Data are presented as mean + SEM from 3 independent experiments each utilizing 3 static inserts and 3 chips (* p < 0.05 by ANOVA followed by Tukey’s HSD test). b) Alkaline phosphatase expression of Caco-2 cells cultured on Transwell inserts for 5 or 21 days compared to Caco-2 cells cultured on chip for 5 days. Data are presented as mean + SEM from 3 independent experiments each utilizing 3 static inserts and 3 chips (* p < 0.05 by ANOVA followed by Tukey’s HSD test). c) Analysis of mucus production by Caco-2 monolayers on Transwell™ and on chip. Left-most panels depict representative images of Caco-2 monolayers stained with Alcian Blue. In right-most panels, cells are visualized by DAPI nuclei (blue) staining and mucus protein is visualized by anti-MUC2 staining (red) of Caco-2 cells grown on static Transwell inserts for 5 days (top) and on laser cut and assembled chips for 5 days (bottom). Scale bars denote 20 µm.

We next compared mucus production by Caco-2 cells on static Transwell™ inserts versus chip. Alcian blue, a polyvalent dye, was used to identify gastrointestinal
mucins\textsuperscript{192} and immunostaining was used to specifically identify Mucin 2, the most abundant structural protein of the gastrointestinal mucus layer.\textsuperscript{193} Analysis of alcian blue staining suggested that Caco-2 cells on chip produce more mucus compared cells grown on static Transwells (Figure 18c). These results are consistent with two previous studies combining Caco-2 and other gastrointestinal cell lines with media perfusion that reported increased mucus production in response to mechanical stimulation via fluid flow\textsuperscript{74, 45}.

**Cut and assemble organs-on-chips support primary human intestinal epithelium**

While Caco-2 cells on permeable supports are frequently used to model enterocytes for transport studies across the small intestinal epithelium, as a model, Caco-2 are limited by their colorectal adenocarcinoma origin. Caco-2 cells contain unknown genetic mutations, fail to fully recapitulate the gut’s heterogeneous cell population (stem cells, transit-amplifying cells, Paneth cells, goblet cells, enteroendocrine cells and enterocytes), and may not accurately represent any one cell type. Therefore, we sought to establish a more physiologically relevant intestine model by utilizing human primary intestinal epithelial cells expanded as organoids derived from intestinal biopsies (Figure 19a). Organoids were dissociated primarily to single cells (82%) (Figure 19b), with 71% viability. Then, cells were plated on either Transwell\textsuperscript{TM} inserts (Figure 19c) or on cut and assemble chips (Figure 19d) for 5 or 7 days. Static monolayers were maintained in organoid expansion medium (EM) for 2 days followed by differentiation medium (DM) for 5 days while chip monolayers were maintained in EM for 3 days followed by DM for 2 days. Monolayers were then fixed and stained for F-actin, tight junction protein ZO-1, and nuclei. Primary cells formed confluent monolayers with comparable morphology and tight junctions under both culture conditions (Figure 20a-b), demonstrating suitability
for future epithelial barrier studies. We next quantified barrier integrity (comparing primary monolayers on chip versus on static inserts) by measuring the apical to basal paracellular transport of fluorescent Lucifer Yellow (450 Da). We observed a significant increase ($p=0.0029$) in the apparent permeability between the 5-day static and chip models (Figure 20c), consistent with observations for Caco-2 cells (Figure 18a). These results indicate that cut and assemble chips support primary intestinal cells to form confluent monolayers expressing tight junctions and low permeability in response to continuous perfusion.

![Figure 19](image)

**Figure 19**: Formation of primary human intestinal monolayers from biopsy-derived organoid cultures. a) Organoid expansion in 3D Matrigel. Scale bar denotes 500 µm. b) The percentage of cells/clumps occurring as single cells, doublets, triplets, or clumps of 4 or more cells after organoid dissociation from 3 independent experiments. c) Transwell™ based monolayers were maintained for 7 days; the phase contrast microscopy image shows Transwell™ primary human epithelial monolayers at 7 days (note that EM was switched to DM at Day 2). d) Phase contrast images of primary human epithelial monolayers in the bi-layer chip at 1, 3, and 5 days post seeding (note that EM was switched to DM at Day 3). Scale bar denotes 100 µm.
Figure 20: Primary human intestinal epithelium on chip. a) Representative images of cells immunostained for ZO-1 tight junctions (green), DAPI nuclei (cyan), and F-actin cytoskeleton (red); primary intestinal cells grown on laser cut and assembled chips for 5 days (bottom) and static inserts for 7 days (top). Scale bar denotes 20 µm. b) Representative phase contrast images showing primary human epithelium at 5 days across the entire length of the chip and a higher magnification of the area denoted by the white rectangle (bottom, scale bar denotes 500 µm). c) The apparent paracellular permeability quantified by tracking Lucifer Yellow through primary epithelial monolayers cultured on Transwell inserts or on bi-layer chips for 5 days. Data are presented as mean ± SEM from 3 independent experiments each utilizing 3 static inserts and 3 chips generated from organoids isolated from one donor (*p = 0.0029 by Student’s t-test).

Integration of primary human intestinal monolayers and intact organoids in tri-layered organ chips

In the native intestine, epithelial cells are maintained by their surrounding physical, biochemical, and cellular niche. The cellular niche includes myofibroblasts, fibroblasts, endothelial cells, immune cells, glial cells, neural cells, and smooth muscle cells which are embedded in the ECM underlying the epithelium. The cellular niche
regulates epithelial cells via both paracrine and contact dependent signaling. While the bi-layer organ chip is the most predominant design, modeling multi-cellular tissues would benefit from more complex organ chip architectures that enable the integration of more than 1-2 cell types or 3D tissue culture within a matrix. To demonstrate the versatility of the cut and assemble method toward fabricating multi-layered architectures enabling 3D tissue culture, we fabricated a dual membrane, tri-layer organ chip (Figure 21a-c) and integrated 2D and 3D tissue culture of primary intestinal monolayers and intact organoids in adjacent compartments. Organoids cultured in standard plasticware thrive throughout approximately 1.5 mm thick Matrigel (Figure 19a). Therefore, we engineered the tri-layer organ chip with a 1.6875 mm tall, central, organoid culture channel (Figure 21c). To promote physical interaction between the differentiated monolayer and the central, organoid laden compartment, the monolayer was cast on a 30 µm pore diameter PC membrane. First, intestinal stem cell laden Matrigel was polymerized in the central channel for 30 min at 37 °C. Then, the apical channel was seeded with dissociated organoids to generate a confluent epithelial monolayer. Organoid expansion medium (EM) was perfused through the apical and basal channels for 6 days. Then, the apical medium was altered to differentiate the epithelial monolayer and perfusion continued for 4 days. Monolayers achieved confluency in 1-2 days and remained confluent over the 10-day culture duration (Figure 22). Similarly, stem cells formed closed organoids in 1-2 days, which expanded over 10 days and maintained their characteristic cystic morphology (Figure 22). 3D confocal microscopy of the central organoid channel confirmed the presence of single organoids with their characteristic cystic morphology (Figure 23c) as well as larger, morphologically complex organoids (Figure 23d).
While sparse 3D tissue structures spanning a few cell layers were observed in the bi-layer organ chip (Figure 20a-b), 3D tissue structures in the tri-layer organ chip were present across the entire chip length at an even shorter duration of culture (Figure 22). To visualize the tissue height, we acquired monochrome 3D confocal stacks and differentially colored each slice to represents the z-depth. Color coded maximum intensity projections of primary monolayers in the tri-layer chip revealed 3D tissue growth spanning approximately $10^2 \mu$m (Figure 23a). 3D, multicellular, tissue structures on chip were recently reported and attributed to the presence of media flow and human intestinal microvascular endothelial cells (HIMECs). Here, we demonstrate 3D tissue growth in the absence of HIMECs. However, the previously reported villus-like structures formed on a collagen I and Matrigel coated membrane. Therefore, it is plausible that biochemical cues originating from laminin and collagen IV rich Matrigel promote 3D tissue growth. In the tri-layer chip presented here tissue growth may be promoted by direct contact between the monolayer and Matrigel through the 30 µm pores as the bi-layer chip was only collagen I coated. Confocal fluorescence microscopy revealed organoids in close proximity to the basal regions of the monolayer and 3D tissue
structures (Figure 23b). It is possible, though unproven, that intact organoids adjacent to the epithelium communicate with the differentiated epithelial monolayer via paracrine signaling to drive morphological changes. For example, intestinal hedgehog signaling in the intervillus pockets of the developing epithelium is involved in crypt-villus axis formation during development and the adult small intestine retains Indian Hedgehog (Ihh) ligands in the differentiated villi.\textsuperscript{194,195} Thus, integration of the 2D and 3D microenvironments in the tri-layer gut chip may exhibit more native functionality of intestinal epithelium and stem cells than independent cultures, while allowing evaluation of monolayer and organoid behavior simultaneously.

Figure 22: Phase contrast images of primary human epithelial monolayers and organoids in the tri-layer chip at 2, 4, 6, and 10 days post seeding (note that apical Expansion Media was switched to Differentiation Media at Day 6). Scale bar denotes 500 µm.
Figure 23: Structural analysis of a dual membrane tri-layered organ chip integrating primary human intestinal monolayers and intact organoids. a) Z-depth color coded maximum intensity projections of the monolayer cultured on chip for 10 days and stained with DAPI when viewed from above by confocal microscopy. The color bars above the image specify the range of z-depths in µm. Both images in panel a were observed on the same chip. b) A representative maximum intensity z-projection and the corresponding orthogonal view of the monolayer cultured for 10 days and stained with DAPI when viewed from above by confocal microscopy. The dashed white lines indicate the upper and lower surfaces of the primary monolayer while the dashed white circles indicate underlying intact organoids in close proximity to the monolayer. c) Representative orthogonal views of intact organoids cultured on chip for 10 days, stained with DAPI, and imaged by confocal microscopy. d) Representative 3D reconstruction of confocal immunofluorescent micrographs of intact organoids cultured on chip for 10 days and stained for DAPI. Scale bars denote 100 µm.

Primary monolayers enable intestinal transport studies across an epithelium mainly comprising enterocytes and goblet cells but typically lacking proliferative cell compartments. Conversely, 3D organoids contain proliferative cell compartments but limit intestinal transport study due to poor luminal access. Confocal fluorescence microscopy of organoids on chip revealed Ki67+ proliferative stem cells (Figure 24).
thereby demonstrating that the tri-layer chip could enable studying intestinal transport across a differentiated epithelium and the subsequent effects on intestinal stem cells. As paracrine Hedgehog signaling between epithelial and mesenchymal cells promotes stromal niche formation which affects epithelial proliferation and differentiation, the tri-layer organ chip presented here is a particularly powerful tool for integrating the small intestine’s mesenchymal components (fibroblasts, endothelial cells, enteric neurons, and glia) and studying paracrine or cell-to-cell contact-dependent (e.g., enteroendocrine cell-enteric glia) signaling.

**Figure 24**: Representative 3D reconstruction of confocal immunofluorescent micrographs of intact organoids cultured on chip for 10 days and stained for DAPI and Ki-67. Scale bar denotes 100 µm.

### 5.4 Conclusion

Microfabricated organs-on-chips may potentially improve preclinical models, while providing platforms for controlled biological evaluation. In order to commercially succeed, organs-on-chips should be simple to use, automated, and high throughput. Currently, automation and throughput is limited by chip cost and fabrication complexity. The microfabrication free, cut and assemble manufacturing technique presented herein provides rapid (hours), facile, and inexpensive (~$2 per chip) access to multilayer organs-on-chips with standard fluidic connectors. Caco-2 cells cultured on cut an assemble chips
formed confluent monolayers expressing tight junctions, which enabled molecular permeability assays. Caco-2 cells cultured on chips also differentiated ~4 times faster than on Transwell™ inserts, increasing experimental throughput. The cells on chip also produced mucus and alkaline phosphatase, emulating native intestinal functions. Moreover, cut and assemble chips supported primary human intestinal monolayers with tight junction functional barriers.

The versatility of cut an assemble chips was further demonstrated by generating a dual membrane tri-layer gut chip. This tri-layer organ chip may be particularly useful for integrating intestinal monolayers with 3D culture of mesenchymal cells (fibroblasts, endothelial cells, enteric neurons and glial). These cells occupy the ECM rich lamina propria and communicate with the epithelium via paracrine and cell-to-cell contact dependent signaling. In a proof-of-principle experiment we co-cultured primary 2D monolayers with 3D organoids. Remarkably, the monolayer formed multicellular 3D structures spanning 10^2 µm, possibly aided by paracrine signaling between the differentiated epithelial monolayer and the proliferative proximate organoids. This platform may enable characterization of intestinal transport and organoid biology towards improved screening and disease modeling, and further design improvements could include enhanced imaging capabilities and increased cell-monolayer interactions. This could be accomplished be decreasing the central channel’s height and/or using an apical membrane with pores > 30µm.

The many features of cut and assemble chips, including the low gas and water vapor permeability of thermoplastics, compared to PDMS, the rapid, easy, and economical fabrication method as well as the ability to make custom multilayered chips,
make cut and assemble fabrication well suited for wider adoption and development of organs-on-chips.

CONCLUSION

Cholinergic regulation of intestinal epithelial barrier integrity has been documented using both in-vivo\textsuperscript{16,17,24,111-113,197} and in-vitro\textsuperscript{25-27,33-36,38-40,43} systems. In 2000, Borovikova et al.\textsuperscript{14} hypothesized that the cholinergic parasympathetic nervous system can modulate a systemic inflammatory response. Using both in-vitro and in-vivo models, the researchers demonstrated that vagal nerve stimulation (VNS) ameliorated lipopolysaccharide (LPS) induced epithelial barrier injury via $\alpha_7$nAChR activation of intestinal macrophages. This seminal work catalyzed a series of in-vivo investigations regarding VNS for modulating intestinal inflammation and barrier integrity\textsuperscript{16,17,24,111-113,197}. Chapter 2, Section 2.1.1 discusses these studies in detail. Based on these studies, it can be concluded that VNS locally modulates the gastrointestinal (GI) inflammatory process with limited systemic effect through a $\alpha_7$nAChR dependent mechanism. It is widely believed that macrophage $\alpha_7$nAChR is activated via the release of acetylcholine (ACh) from the efferent vagus nerve though one study observed enteric glial cell (EGC) activation following VNS. In-vitro studies utilizing immortalized intestinal epithelial cells (IECs) confirmed that EGCs contribute to nicotinic and VNS induced amelioration of epithelial inflammation and/or barrier integrity\textsuperscript{25,27,33,34}. Chapter 2, Section 2.1.2 discusses these studies in detail. Zero studies indicated that nicotinic activation regulates barrier integrity via IECs.

Though VNS induced ACh released may regulate epithelial barrier integrity via IEC independent nicotinic mechanisms, ACh also binds to muscarinic G-protein coupled
receptors (GPCRs). Several in-vitro studies utilizing immortalized IECs demonstrated muscarinic amelioration of epithelial barrier integrity\textsuperscript{35,36,38-40,43}. Chapter 2, Section 2.1.2 discusses these studies in detail. Based on these studies, it can be concluded that mAChR stimulation modulates IECs in-vitro, oftentimes with a protective effect against epithelial barrier injury. However, the type and extent of effect varied from study to study. Furthermore, the implicated mAChR subtypes and downstream mechanisms varied.

Table 1 summarizes the effects and mechanisms. One plausible explanation for the discrepancies is that the various IEC cell lines (T84, Caco-2, HT-29/B6) differentially express mAChR and/or respond to mAChR stimulation. Several studies highlight functional differences between common GI cell lines\textsuperscript{45-47}. Furthermore, it is plausible that differing tissue culture protocols for one cell line may produce different cell phenotypes\textsuperscript{48-51}, resulting in differential mAChR expression. This hypothesis is supported by a recent in-vivo study demonstrating that all five M\textsubscript{1}-M\textsubscript{5} mAChR subtypes were detected in mature enterocytes while while M\textsubscript{2} co-localized with crypt Paneth cells\textsuperscript{52,53}. This finding indicates that cell differentiation may impact mAChR expression, thus complicating experimental design and data interpretation. Moreover, it indicates that some mAChR subtypes, such as M\textsubscript{2}, may not be recapitulated via immortalized IECs; the previously discussed immortalized IEC studies only specified M\textsubscript{1} and M\textsubscript{3} subtype detection\textsuperscript{35,36,38-40,43}. Together, these factors motivate the need for a primary human in-vitro model of the GI epithelium that faithfully recapitulates in-vivo IEC phenotypes.

Recently developed human intestinal organoids have largely addressed scalable culture of primary intestinal tissue\textsuperscript{54,55}. Organoids form cystic structures, contain all of the intestinal epithelial cell lineages found in the native intestine, and can be indefinitely
passaged to generate enough tissue for experimental replication\textsuperscript{56}. It was demonstrated that primary intestinal organoids exhibit broad similarity to human GI tissue, suggesting similar global transcriptional activity\textsuperscript{57}. Thus, human intestinal organoids are an excellent platform for studying mAChR and nAChR signaling with higher fidelity to human biology in-vivo.

Several studies utilized whole organoids to study mAChR and nAChR signaling; \textbf{Chapter 2}, Section 2.1.3 discusses these studies in detail. Using whole organoids, Takashi et al.\textsuperscript{58,59} found that mAChR activation stunted organoid growth and proliferation via control of LGR5\textsuperscript{+} stem cells. Various subtypes were involved, M\textsubscript{1}-M\textsubscript{4}, which were expressed across the entire organoid. In contrast, nAChR activation enhanced organoid growth and proliferation via Paneth cell associated $\alpha_2$ and $\beta_4$ nAChRs that regulate Wnt5a production and thus LGR5\textsuperscript{+} stem cell proliferation. Separately, Liu et al.\textsuperscript{60} noted carbachol (CCh) induced Paneth cell degranulation in mouse organoids which may explain the stunted organoid growth observed by Takashi\textsuperscript{58}. Pastula et al.\textsuperscript{61} co-cultured murine intestinal organoids with murine myenteric neurons showing that co-cultured neurons were able to replace exogenous Wnt necessary for organoid growth. The growth factors responsible were undefined but it may very well be that neuron derived ACh stimulated Paneth cell associated nAChRs, increasing Wnt5a production, as postulated by Takashi two years later\textsuperscript{59}.

\textbf{Intestinal epithelial permeability and cholinergic regulation}

Though organoids are both scalable genetically similar to the human intestinal epithelium in-vivo, organoids are closed cystic structures which complicates studying epithelial barrier integrity. Two dimensional (2D) monolayers derived from dissociated
organoids enable access to both apical and basal media compartments, greatly facilitating epithelial perturbation and analysis\textsuperscript{64-69}. However, mAChR or nAChR regulation of epithelial barrier integrity has not been studied using 2D organoid derived monolayers.

In Chapter 3, we use primary human intestinal organoids to develop a 2D, physiologically relevant model of the small intestinal epithelium. Intestinal organoids were dissociated and seeded on collagen I coated Transwell inserts. The primary human epithelium exhibited functional differences as compared to immortalized Caco-2 and HT-29 epithelium: increased barrier integrity, increased polarized IL-8 secretion, and the presence of both absorptive enterocytes and secretory goblet cells. We found that approximately 12\% of cells were mucus secreting goblet cells while the remainder were enterocytes; in-vivo, goblet cells comprise 8\% to 10\% and enterocytes comprise approximately 90\% of the epithelium\textsuperscript{198}. Next, pro-inflammatory cytokine tumor necrosis factor alpha (TNF-\alpha) was used to model an inflamed state. IBD patients exhibit increased expression of TNF-\alpha\textsuperscript{106} which is produced by macrophages, monocytes, and differentiated Th1 cells\textsuperscript{107,108}. Treatment with TNF-\alpha led to impaired barrier integrity, apoptosis, and polarized increase of interleukin 8 (IL-8). Indeed, TNF-\alpha is a potent stimulus of IEC shedding\textsuperscript{133}. Primary IECs growing as multicellular aggregates were particularly sensitive to TNF-\alpha induced apoptosis. This may suggest that “villus-like” cells are more susceptible to TNF-\alpha induced apoptosis. In accordance, previous in-vivo studies demonstrated that acute TNF-\alpha exposure significantly blunts villi\textsuperscript{134}. Though the mechanisms of IEC apoptosis are still under debate, IECs show differential susceptibility to apoptosis depending on IEC differentiation\textsuperscript{135} or location\textsuperscript{136}. Despite increased apoptosis and decreased monolayer density, fluorescent microscopy images revealed a
confluent IEC monolayer with intact TJ. Using single cell segmentation, we quantitatively analyzed the cell morphology of >10^3 cells and demonstrated an altered cell morphology and TJ disorder in response to basal TNF-α exposure. Post TNF-α exposure, IECs were larger, elongated, and exhibited TJ kinking or rippling. In-vivo, IECs adjacent to apoptotic cells extend their cytoplasm underneath the shedding IEC to create new junctions that maintain an intact epithelial barrier\textsuperscript{136}. The observed TJ rippling may indicate TJ remodeling while the increased cell size and aspect ratio may show cells adopting a larger and migratory phenotype to close TJ gaps induced by cell apoptosis. Together, these data suggest that TNF-α impairs epithelial barrier integrity through apoptosis and TJ remodeling which synergistically works with morphological changes to maintain an intact epithelial barrier.

Based on previous studies utilizing immortalized IECs\textsuperscript{25-27,33-36,38-40,43}, we hypothesized (Chapter 4) that muscarinic and/or nicotinic receptor activation may antagonize TNF-α impaired epithelial barrier integrity. By pre-incubating differentiated primary human organoid derived 2D monolayers with nicotinic or muscarinic specific agonists, nicotine or bethanechol, respectively, we demonstrated that neither nAChR nor mAChR activation ameliorated TNF-α induced paracellular permeability. Previous reports demonstrated that mAChR activation ameliorated paracellular permeability across HT-29/B6\textsuperscript{40,41} and Caco-2\textsuperscript{43} monolayers induced by pro-inflammatory cytokines TNF-α and IL-1β, respectively. Based on previous reports, we performed an IL-8 enzyme-linked immunosorbent assay (ELISA) to confirm that mAChR activation did not inhibit TNF-α induced immune activation of IECs. In contrast, it was demonstrated that mAChR stimulation of HT-29/B6 IECs with carbachol (CCh) inhibited IL-8 production induced
by TNF-α stimulation via an M₃ dependent mechanism⁴⁰,⁴¹. The discrepancy between these and our results may be due to intrinsic differences between primary and HT-29/B6 cells in regards to mAChR expression and/or biological function. Alternatively, the discrepancy may be due to kinetic factors; we investigated mAChR regulation of TNF-α induced IL-8 production during a 24 hour span rather than an 8 hour span in the previous works⁴⁰,⁴¹. It was demonstrated that CCh activates M₃ mAChR which results in shedding of TNF receptor TNFR1 within 5 minutes⁴⁰. Therefore, it is plausible that TNFR1 shedding via mAChR activation is a one-time event and given enough time, the cell surface will present new TNFR1. This hypothesis is supported by results that showed mAChR inhibition of TNF-α induced paracellular permeability across HT-29/B6 monolayers at 8 and 12 hour whereas mAChR activation was ineffective at 24 hours⁴¹. Even though we did not demonstrate mAChR reduced paracellular permeability at 4 hours, the kinetics of TNFR1 shedding and/or recycling may be different than HT-29/B6 cells. Using a 70 kDa dextran, we performed a permeability assay demonstrating that mAChR activation reduced transcellular transport across primary epithelium. The result was confirmed using a 70 kDa lysine fixable dextran: dextran internalization was visualized via fluorescent confocal microscopy and quantified using image analysis software. In contrast, previous works demonstrated that mAChR activation via bethanechol and CCh increased endocytosis of horseradish peroxidase (HRP) across T84⁴⁶ monolayers and ex-vivo rat ileum⁵⁷, respectively. The discrepancy between these two studies and our results may be due to intrinsic differences between T84 IECs, multicellular (EGCs, neurons, immune cells, etc) ex-vivo rat ileum, and our primary IEC model. Interestingly, it is well established that TNF induced apoptotic signaling requires
endocytosis and endosomal machinery. Thus, we might expect reduced endocytosis to correlate with amelioration of TNF-α induced epithelial injury in our study. Most likely, TNFR1 uses many internalization mechanisms.

To summarize, in Chapter 3, we develop a facile, robust, and phenotypically more relevant model of small intestinal epithelium, as compared to immortalized IECs. We modeled an inflamed state using pro-inflammatory cytokine TNF-α, revealing that epithelial barrier integrity is compromised via IEC apoptosis and TJ disruption and that IECs adopt a distinct morphology to maintain a cellular monolayer sealed by TJs. In Chapter 4, we provided evidence that mAChR activation reduces IEC endocytosis but does not inhibit TNF-α induced paracellular permeability. The results are contradictory with previous research demonstrating mAChR increased endocytosis and decreased paracellular permeability. We postulate that the discrepancy is due to intrinsic differences between primary and immortalized IEC function, cellular kinetics and/or receptor cycling.

**Low cost, rapid, and scalable microfluidic organ chips**

Despite the advantages of primary human organoid derived 2D monolayers over immortalized IECs, microfluidic cell culture devices, termed organ chips, lend additional advantages to traditional Transwell monolayers. For example, Kim et al. demonstrated that Caco-2 on chip exhibited an approximately 4-fold increase in cell height as compared to Transwell cultured Caco-2 and formed undulations which mimicked the crypt-villus morphology found in-vivo. The basal adjacent “crypt” contained EdU+ proliferative cells which migrated up the “villi”. Caco-2 on chip for 5 days exhibited aminopeptidase activity higher than 5 day static Caco-2 cultures and equal activity to 21
day static Caco-2 cultures, demonstrating accelerated differentiation under perfusion\(^3\). Similarly, Caco-2 on chip exhibited drug metalizing cytochrome P450 3A4 (CYP3A4) enzyme activity whereas static cultures did not\(^74\). Finally, Kim et al. demonstrated that Caco-2 cells on chip could be co-cultured with the intestinal microbe Lactobacillus rhamnosus GG (LGG) without impacting cell viability or barrier integrity as was found on static Transwell cultures. This may be one of the greatest advantages of microfluidic IEC culture as compared to static Transwell culture; bacteria under static conditions rapidly overgrow and contaminate human cell cultures. Transcriptome profiling across 22,097 genes demonstrated that Caco-2 co-cultured with commensal bacteria on chip were more similar to in-vivo ileum than Caco-2 Transwell cultures or Caco-2 on chip\(^75\). Collectively, these functional studies indicate that organ chip technology may impart immortalized IECs with novel biological features and/or functions. Chapter 2, Section 2.2 discusses manufacturing of organ chips and current organ chip intestinal models in detail. Currently, microfluidic organ chips are predominantly manufactured via polydimethylsiloxane (PDMS) soft lithography, which has some functional and significant manufacturability disadvantages.

In Chapter 5, we developed an alternative organ chip fabrication technique that circumvents some disadvantages of PDMS soft lithography, namely manufacturing cost, throughput, scalability and microfabrication facility requirements. The newly developed organ chip was manufactured via laser micromachining of commercially available adhesives, plastics, and track etched membranes. The fabrication technique was validated by culturing both Caco-2 and primary human organoid derived IECs on a traditional bilayer organ chip. Caco-2 cultured on laser machined chips formed confluent
monolayers expressing tight junctions (TJs), which enabled molecular permeability assays. Caco-2 on chips also differentiated ~4 times faster than on Transwell™ inserts, produced mucus, and expressed alkaline phosphatase, emulating native intestinal functions. Laser machined chips supported primary human intestinal monolayers expressing TJs. The versatility of laser machined chips was further demonstrated by generating a dual membrane tri-layer gut chip. This tri-layer organ chip may be particularly useful for integrating intestinal monolayers with 3D culture of mesenchymal cells (fibroblasts, endothelial cells, enteric neurons and glial). These cells occupy the extracellular matrix (ECM) rich lamina propria and communicate with the epithelium via paracrine and cell-to-cell contact dependent signaling. In a proof-of-principle experiment we co-cultured primary 2D monolayers with 3D organoids. The monolayer formed multicellular 3D structures spanning 10^2 μm, possibly aided by paracrine signaling between the differentiated epithelial monolayer and the proliferative Ki-67^+ proximate organoids.

In summary, in **Chapter 5**, we developed an organ chip manufacturing technique that rapidly (hours) produced economic (~$2 per chip) organ chips using an inexpensive, ($12,450) benchtop laser. CAD-based manufacturing enabled iterative design with zero tooling or mold costs. Collectively, the manufacturing technique had significant advantages in cost, throughput, scalability, and equipment requirements as compared to PDMS soft lithography. The many features of laser machined chips, including the low gas and water vapor permeability of thermoplastics, the rapid, easy, and economical fabrication method, and the ability to fabricate multilayered chips, make laser machine fabrication well suited for wider adoption and development of organ chips.
FUTURE DIRECTIONS

This thesis represents a small step toward a more physiologically relevant model of small intestinal epithelium as compared to the predominantly used immortalized cell derived models. The small intestinal epithelium is a physical barrier between the body and the luminal environment which may contain a variety of pathogens. The intestine metabolizes drugs and is thus of upmost importance to the design and synthesis of orally administered therapeutics. Finally, the intestine is home to the gut microbiome which has attracted unprecedented attention in the past decade. Therefore, a robust and simple in-vitro intestinal model that mimics human biology with high fidelity is significant. Our observations regarding muscarinic regulation of epithelial barrier integrity are in contradiction with previously published research with immortalized Caco-2, T84, and HT-29/B6 cells. A first step toward resolving these discrepancies and advancing our understanding of cholinergic regulation of epithelial function may be a thorough study of muscarinic receptor and nicotinic receptor expression by primary intestinal epithelial cells as a function of differentiation. Based on previously published research, we hypothesized that cellular kinetics and/or TNF receptor recycling may explain the observed discrepancies which could be investigated via temporally resolved studies of muscarinic activation and TNF receptor presentation. Advancing our understanding of cholinergic regulation of intestinal epithelial function has a clinical implication – bioelectric medicine.

Bioelectric medicine is not a novel concept. In fact, vagal nerve stimulation has been clinically applied to improve active Crohn’s disease\textsuperscript{200}. Furthermore, vagal nerve stimulation was shown to improve rheumatoid arthritis, another TNF-\(\alpha\) mediated
The rationale for targeting the vagus nerve in inflammatory bowel disease is a reduction in TNF-α production by both splenic and gut resident macrophages due to increased acetylcholine produced by splenic lymphocytes and vagus nerve interfacing neurons. However, based on our findings and the previously published research, it is evident that our molecular and cellular understanding of cholinergic regulation of the intestinal epithelium is in its infancy. Nevertheless, bioelectric medicine has the potential to augment inflammatory bowel disease treatment and this warrants further investigation of cholinergic signaling in the intestinal epithelium.
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APPENDIX A: OTHER WORK

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Microfluidic Sample Preparation for Single Cell Analysis
Sanjin Hosic,† Shashi K. Murthy,†‡ and Abigail N. Koppes*†
†Department of Chemical Engineering and ‡Barnett Institute of Chemical and Biological Analysis, Northeastern University, 360 Huntington Avenue, Boston, Massachusetts 02115, United States

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Single cell analysis is not only driven by stochasticity of homogeneous cell populations as in cell cultures but also by the need to analyze tissues composed of multiple distinct cell types and the need to identify discrete subpopulations among seemingly identical cells. For example, the intestinal stem cell niche is a tissue composed of several different cell types such as stem cells, Paneth cells, Goblet cells, enterocytes, and enteroendocrine cells. Currently, researchers are investigating the existence of distinct intestinal stem cell populations. Much of the current literature supports the existence of a proliferative stem cell population responsible for epithelial homeostasis and a quiescent stem cell population responsible for regeneration in response to injury. However, conflicting reports preclude definitive stem cell biomarkers for each population. Nonbiased single cell molecular analysis may settle the debate over intestinal stem cell markers once and for all.

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Such findings have driven the development of new analytical systems to probe biology at the resolution of a single cell. In order to study single cells accurately and efficiently, systems with high sensitivity and throughput are needed. The small dimensions of microfluidic systems enable single cell and reagent manipulation with minimal dilution, resulting in high sensitivity assays. Furthermore, microfluidic systems offer several key advantages toward the study of single cells including facile automation, parallelization, and reagent reduction. Early researchers found that sample preparation such as cell manipulation, compartmentalization, and lysis was significantly more difficult to implement at the single cell scale compared to in bulk. However, sample preparation preceding molecular analysis has also been miniaturized, allowing facile sample processing. As such, microfluidic systems have been developed and applied toward the study of single cells extensively. Given microfluidics’ instrumental role in single cell analysis up to this point, we can expect continued innovations in microfluidics to better enable single cell biology.

In this review, novel microfluidic techniques currently used toward sample preparation and subsequent single cell analysis are highlighted. Techniques are discussed in terms of discrete sample preparation steps that may be necessary for characterizing single cells; tissue dissociation into cell suspensions, sorting heterogeneous cell populations into homogeneous populations, isolating, and lysing single cells (Figure 1). With each discrete step, conventional approaches are discussed first and then microfluidic based strategies are reviewed. Finally, the future direction for developing microfluidic single cell analysis technology is discussed.

**SAMPLE PREPARATION**

**Tissue Dissociation. Conventional Approaches.** The first step toward single cell analysis is obtaining cells from a source. To enable researchers regarding the function of an organ or even a whole organism via single cell data, it is vital that the cells are representative of that specific organ or organism. Intact tissues obtained via biopsy are an excellent source of cells and are representative of their native microenvironment. To obtain suspended cells from the harvested intact tissue, the extracellular matrix and cell–cell junctions holding the cells together in a 3D structure must be disrupted. Conventional methods consist of incubating the intact tissues with enzymes such as collagenase in order to digest proteins in the extracellular matrix. Exposure to chelating agents such as ethylenediaminetetraacetic acid (EDTA) binds to Ca$^{2+}$ and disrupts the cell-to-cell adherens junctions regulated by transmembrane cadherin proteins. After chemical exposure, intact tissue is often dissociated into a cell suspension via gentle mechanical agitation such as pipetting or inversion. For example, Robin et al. described a procedure to isolate human myogenic cells following a patient muscle tissue biopsy. The procedure called for the addition of Dispase II and collagenase D to minced tissue followed by pipetting of the mixture. The same study described a similar protocol to isolate fibroblasts from a patient skin biopsy by exposing the tissue to collagenase followed by mincing. Many more dissociation enzymes are in use, for example, trypsin, elastase, and hyaluronidase to name a few. For liquid biopsies, such as a blood draw, cell dissociation is not required. In these cases, cell sorting may be the first sample preparation step. This is common for circulating tumor cell enumeration, which is discussed in later sections.

A critical goal of any tissue dissociation protocol is to yield as many viable cells as possible for downstream workup. Several protocol parameters may affect the outcome of the tissue dissociation such as animal species, tissue type, which enzyme and or chelator is used, chemical concentration, incubation time, temperature, agitation method, etc. Parameters that can be controlled are determined empirically through trial and error to maximize the cell yield and viability. Though developing a dissociation protocol appears an arduous task, a plethora of scientific literature can serve as a starting point for isolating cells from various mammalian tissue including but not limited to murine brain tissue, murine, rat, and human heart tissue, and murine intestine. Much effort has been dedicated to optimizing tissue dissociation because errors
upstream can propagate to the downstream cell assay, negatively impacting the data.

The enzymatic and mechanical tissue dissociation protocols described above have inherent drawbacks making these conventional protocols less than ideal. For example, isolating human adipose derived adult stem cells from tissue obtained via liposuction, rather than bone marrow, required 8–10 h of continuous labor. In addition to being highly labor intensive, long protocols significantly increase contamination risk, operator error, and variability. Furthermore, enzymatic digestion may result in a loss of cell surface protein expression. A loss of cell surface proteins can negatively impact the ability to sort cells via fluorescent activated cell sorting (FACS), a current workhorse in cell isolation and applications, emerging microfluidic devices are addressing downstream applications, emerging microfluidic devices are addressing issues in the upstream tissue dissociation step.

Microscale Approaches. Traditional batch tissue culture operations rely on supplying cells with nutrients and refreshing culture medium at discrete time points, thereby creating variability in nutrient and waste product concentrations throughout the cells’ life cycle. To create an environment that better mimics the flux of in vivo tissue, Hattersley et al. developed a microfluidic device for on-chip perfusion culture, analysis, and dissociation of intact rat liver tissue (Figure 2A). On-chip tissue dissociation was performed following a viability assay. In brief, the immobilized tissue was perfused with ethylene glycol tetraacetic acid (EGTA) to scavenge $Ca^{2+}$ ions followed by Earl’s balanced salt solution (EBSS) to remove EGTA, which may prevent collagenase inhibition. Next, the tissue was digested by perfusion with collagenase solution. Finally, the tissue was perfused with ice-cold dispersal buffer at a high flow rate ($500 \mu$L/min). The cells were collected at the device output directly into a centrifuge tube to be used for cell pelleting. Following on-chip enzymatic dissociation, a Trypan blue assay indicated that 78 ± 2.4% cells were viable, comparable with traditional dissociation methods. While still utilizing an enzymatic mechanism, this microfluidic approach reduces the duration of cell or tissue exposure to nonsterile conditions, thereby minimizing contamination risk. However, only approximately 30 000 cells were isolated from 4 mm$^3$ of tissue via a 2 h collagenase perfusion. These results indicate a low yield of total cells, potentially due to an overly gentle procedure. Additionally, a 2 h dissociation procedure may be unsuitable for applications where gene expression analysis is the ultimate goal, since gene expression occurs on the same time scale. With a similar goal of reducing foreign contamination, Wallman et al. built a microfluidic device, termed “biogrid”. The device addressed contamination risk in addition to nonenzymatic requirements and scalability in the clinical or commercial production of neural stem cell therapies. Neural stem cells can be cultured in suspension as stem and progenitor cell aggregates called neurospheres, requiring periodic passaging and subsequent expansion. To conform to good manufacturing practice (GMP) guidelines in a clinical or commercial setting, contamination risk should be minimized by passaging neurospheres in a closed, sterile environment without the use of proteolytic enzymes. Proteolytic enzymes are biologically active molecules and therefore have the potential...
to introduce contamination or toxicity. Nonenzymatic methods such as mechanical means involving scalpels are not scalable and pose a significant contamination risk due to environmental exposure or contaminated tools. The described device consisted of a 3 mm × 3 mm, 170 μm thick silicon chip, with a sharp edged grid in the center. The grid’s edges were 20 μm thick with 200 μm edge to edge spacing (Figure 2C). By passing a neurosphere suspension through the biogrid, neurospheres greater than the grid-to-grid spacing were mechanically dissociated while aggregates and cells smaller than the spacing were unaffected. Comparing the biogrid with traditional enzymatic protocols, the growth rate of passaged neurospheres was more reproducible. However, enzymatically passaged cultures exhibited a greater growth rate compared to biogrid passaged cultures. Cultures passaged with both methods indicated equivalent growth active fractions in passaged cultures. Grid spacing and edge width can be tailored to dissociate various cell aggregates, such as embryonic stem cell spheres, as demonstrated by Wallman et al. Despite offering a closed, scalable, nonenzymatic method for neurosphere dissociation, strictly single cell dissociation was not achieved with aggregates remaining post processing. Single cell dissociation would be critical for optimal passaging and cell culture operation by increasing yield of the culture system. Furthermore, single cell dissociation is crucial for downstream workups such as FACS or single cell molecular analysis.

To achieve strictly single cell dissociation from neurospheres, Lin et al. designed a microfluidic cell dissociation chip, termed "μ-CDC". The single channel device consists of micropillars 50 μm wide and 167 μm tall with 20 μm spacing between adjacent pillars (Figure 2B). Neurospheres of DC115 and KT98 brain tumor mouse models were dissociated into single cells by passage through the pillar array at 3–15 mL/min via a syringe pump. The yield of single cells following μ-CDC dissociation was 91–95% with single cell viabilities of 80–85%, compared to 50% achieved via trituration. However, the rate of neurosphere reformation by microfluidic dissociated cells was lower compared to enzymatically dissociated neurospheres, indicating that the induced shear stress may negatively affect cells. Furthermore, recovery of KT98 cells and DC115 cells was approximately 75% and 93%, respectively, indicating that the cell type affects device performance. The authors stated that optimizing the geometry and flow rate may potentially address recovery and neurosphere reformation for improved cell expansion.

Low cell recovery during tissue dissociation is also a concern in cell based analyses of tumor tissue, particularly for smaller and harder to obtain clinical samples, such as those obtained by fine needle aspirate biopsy. Qui et al. designed a microfluidic device for dissociating tumor tissue equal to or less than 1 mm into single cells (Figure 2D). The device consisted of branching channels. The dissociation is performed by repeated constriction and expansion along each channel, generating shear forces during fluid flow. The device process was evaluated with cell suspensions featuring small HCT 116 colon cancer cell clusters, intact cell monolayers grown from HCT 116 cells, and tumor spheroid derived from HCT 116, LS 174T, and NCI-H1650 cancer cell lines via the hanging drop method. With respect to intact cell monolayers, nonenzymatic device dissociation achieved the same cell yields but showed a significant increase in the single cell population, from 61% to 95% when compared to a control dissociation method utilizing trypsin-EDTA, vortexing, and pipetting. With respect to tumor spheroids, a combination of device dissociation with 5 min EDTA pretreatment resulted in an increase in single cell population from ~60% to 90% compared to dissociating tumors via vortexing and pipetting following 5 min EDTA pretreatment. In addition to increasing the single cell yield, the device dissociation protocols were completed in 10 min or less across all experiments and enabled nonenzymatic spheroid dissociation. However, it was noted that the model cell lines used in the study may not be representative of actual human clinical specimens. Patient samples are more complex in that they contain a heterogeneous cell population, blood vessels, and lower stromal content. As such, patient derived tumor tissue may respond differently compared to the immortalized cell lines and remains to be tested. Nevertheless, the device provides high single cell yields, crucial for limited samples. Furthermore, the device offers rapid and nonenzymatic tissue dissociation, which is critical for measuring endogenous molecular expression.
Microfluidic tissue dissociation is still nascent when compared to other microfluidic applications, but the advantages are already clear. Performing tissue dissociation in flow offers easier scale-up and higher productivity, both necessary for commercialization. Cell and/or tissue handling is minimized by utilizing closed systems, thereby reducing contamination risk. Flow environments within laminar microfluidics can be tightly controlled, offering higher reproducibility and the dissociation mechanisms are on the size scale of cellular samples, resulting in higher single cell yields. The smaller process volumes inherent to microfluidics also allow facile and reliable dissociation of smaller and limited clinical samples. Finally, nonenzymatic methods are particularly attractive from a regulatory standpoint and allow scientists to isolate single cells that maintain endogenous surface marker expression. As such, we can expect this research field to gain significant traction in the future.

**Cell Sorting. Conventional Approaches.** Bulk tissue dissociation typically results in a heterogeneous cell suspension. However, scientists are often interested in studying one target cell type within the greater tissue population; hence, the next step in single cell workflow is to sort the heterogeneous cell suspension into homogeneous fractions. The most commonly used method for sorting populations, FACS, relies on conjugating cell surface proteins specific to one cell type with an antibody coupled to a fluorescent dye. Suspended cells then flow past a laser beam, which excites the dye and a detector records several parameters including the emitted color (Figure 3A). Single cell droplets are given a specific charge based upon the fluorescent dye color and then deflected into the correct container by an electric field. Since its invention in 1969,29 FACS has evolved and maintains its position as a cell enrichment workhorse capable of detecting as many as 14 fluorescent markers and sorting 50 000 cells per second.30 For example, Sanchez-Freier et al.31 performed cell sorting of cultured human embryonic stem cells and induced pluripotent cells to exclude partially differentiated and dead cells. Using a five laser FACS system, double-positive cells were directly sorted to each well of a 96 well plate where quantitative real-time polymerase chain reaction (qPCR) was carried out. Direct integration with well plates for single cell compartmentalization is another advantage of FACS. Nevertheless, FACS has several limitations. FACS instruments are costly, typically several hundred thousand dollars. As such, they are usually only available at core facilities, which limits widespread use to smaller research programs. Furthermore, decreased cell viability is frequently observed due to long processing times.

An alternative to FACS is the use of magnetic fields to separate cells with marker specific magnetic labels, termed magnetic activated cell sorting or MACS (Figure 3B). Since the development of magnetic-activated cell sorting (MACS) over 20 years ago, this technique has evolved considerably.32 Treutlein and co-workers33 used MACS to enrich distal lung epithelial cells from a heterogeneous population containing leukocytes and alveolar macrophages for single cell RNA sequencing. MACS offers cost-effective cell enrichment compared to FACS and several commercial MACS systems are in existence. The FDA approved CELLSEARCH system allows for automated staining, imaging, and enumeration of circulating tumor cells (CTCs) from a 7.5 mL blood sample. This system is useful for bulk CTC enrichment but cannot be integrated directly with well plates for single cell molecular analysis. To overcome this, Neves et al.34 developed a protocol for CELLSEARCH based CTC enrichment, FACS sorting, and isolation into PCR tubes followed by single cell molecular analysis via qPCR. This study illustrated the combination of CELLSEARCH and FACS for molecular analysis of single CTCs.

Despite wide adoption, both FACS and MACS have drawbacks that limit their widespread use or the fidelity of subsequent single cell analyses. For example, FACS machines require high technical expertise to operate and can be costly, ranging anywhere from $100 000 to $500 000. MACS introduces an inherent risk of sample contamination due to batch-wise processing. Both methods rely on labeling of cell surface markers to separate cells, which adds to the overall processing time and viability reduction. It has been shown that immunomagnetic labeling can alter gene expression,35 motivating the development of methods to detach magnetic beads from purified cells. These limitations have led to the development of microfluidic cell sorting platforms as detailed below.

**Microscale Approaches.** Microfluidic devices are inherently advantageous cell sorting platforms compared to conventional methodologies for a variety of reasons. Miniaturized devices allow for reduced reagent consumption and portability.8 The devices can be made using standard micro fabrication tools and soft lithography,36 thereby lowering production cost. Laminar fluid dynamics in devices allows for predictable spatiotemporal control of flowing cells, thereby enabling oftentimes passive and label-free cell separation. Passive sorting translates to simplified device operation and reduces the high technical expertise required for FACS. Sorting is typically accomplished continuously in an enclosed device, minimizing contamination risk. The advent of microfluidic cell sorting technology has alleviated many of the limitations imposed by FACS and MACS. However, microfluidic cell sorting requires further development in key areas if it is to displace conventional technologies. A major drawback of microfluidic systems is lower throughput compared to commercial FACS machines. Thus, this has been a major research focus and is steadily gaining traction. Finally, microfluidic devices fabricated via soft lithography are prone to cell adhesion and clogging, limiting their long-term use. The body of literature regarding microfluidic enabled cell sorting is extensive and there are a number of excellent reviews of work in this area.37–41 Here, we discuss select cell separation modalities that have specific relevance to single cell analysis as a step in the sample preparation sequence. Typically, knowledge of the target cell type’s properties relative to the whole population guides the selection of an appropriate separation mechanism.

**Active Mechanisms.** Microfluidic cell sorting methods that rely on external force fields, such as electrical or magnetic fields, are discussed first. These force fields induce cell movement. The fields are generated by integration of specialized parts with a microfluidic chip such as electrodes or magnets. Active cell sorting methods are advantageous over passive sorting mechanisms due to increased cell control and specificity. However, fabricating microfluidic chips with specialized, integrated features is more complicated. This can limit chip fabrication to laboratories with prior experience or the ability to outsource. At the very least, chip production time may be increased. Furthermore, operation of devices with active sorting mechanisms generally requires power and control systems, thereby increasing cost. Nevertheless, a plethora of active cell
sorting devices have been reported and will be described in more detail below.

**Electrophoresis and Dielectrophoresis.** Most cells typically possess a negative surface charge at neutral pH. As such, suspended cells will move toward a positive electrode under a constant electric field. In a liquid, the cells will be directed with a velocity that can be derived from a force balance. The dominant forces acting on the cell are the Coulomb and drag forces. In order to separate different cell types, the cells must have a difference in charge or size. This separation mode is termed electrophoresis (EP) (Figure 4A). Takahashi et al. designed a device in which two laminar flow streams converge at the center of the device. Cells are introduced in one stream and imaged every 1/30th of a second as they pass the convergence point. A specific cell is identified via phase contrast and fluorescence, at which point a voltage is applied to electrodes connected between the two streams. The electrophoretic force causes the cell to jump from one stream to the other. Similarly, Guo et al. separated single cells encapsulated in an aqueous-oil droplet into different streams via a pulsed electric field. In general, publications utilizing EP to sort heterogeneous cell suspensions are few due to insignificant specificity in EP mobility among cell types.

Contrary to EP, dielectrophoresis (DEP) is used much more frequently for cell sorting because of higher specificity in dielectric properties among cell types. For example, it was shown via dielectrophoretic field-flow fractionation (DEP-FFF) that all 60 cell types of the NCI-60 tumor cell panel, an array of human cancer cell lines, have dielectric properties different from peripheral cell types (monocytes, T-lymphocytes, basophils, neutrophils, eosinophils, B-lymphocytes, erythrocytes) and therefore could be isolated via DEP. In DEP-FFF, a heterogeneous mixture of cells is flowed through a channel while electrodes at the channel bottom generate an upward DEP force that balances the downward gravity force (Figure 4B). Each cell type stabilizes at a unique channel height, at which there is a distinct flow velocity due to a parabolic velocity profile. For DEP, cells do not need to possess a surface charge. Instead, an alternating current typically polarizes the cell. The cell then moves toward or away from the area of highest electric field density. Thus, a spatially nonuniform electric field is required in order to impart a force on the polarized cell. The migration direction is dependent on the electrical permeability of the cell relative to the surrounding fluid. Negative dielectrophoresis (nDEP), movement away from the field maxima, results when the fluid has higher permeability. The opposite is true for positive dielectrophoresis (pDEP), movement toward the field maxima. DEP has a few advantages compared to EP, namely, greater cell sorting specificity and the use of an alternating current prevents electrochemical reactions from occurring at the electrodes and limits detriment to cell viability.

The enumeration of rare circulating tumor cells (CTCs), cells that have shed from a primary tumor and entered the bloodstream, for metastatic cancer prognosis demands CTC isolation from a dense heterogeneous cell suspension, blood. Gascoyne et al. utilized batch DEP-field flow fractionation (DEP-FFF) to separate CTCs from peripheral blood cells and achieved a 90% CTC recovery when a 0.5 mL sample containing approximately $10^6$ cells/mL was applied to the device. However, this sample size represented less than 5% of clinically relevant sample sizes. Improving upon this method, Shim et al. designed a continuous DEP-FFF device that was capable of processing $4 \times 10^7$ peripheral blood cells in less than 1 h, corresponding to a more realistic clinical sample size. Following isolation via the DEP-FFF device, the isolated cells were identified as patient derived cancer cells via a genotype array. Many other studies have reported CTC isolation from blood, focusing on increasing separation throughput, yield, and CTC purity. Moon et al. serially combined hydrodynamic and DEP separation modalities on one chip in order to enable high throughput and purity. The integrated separation resulted in label free CTC isolation at a flow-rate of 126 $\mu$L/min with 99% and 94% removal of red and white blood cells, respectively. This approach leveraged a hydrodynamic modality to remove blood cells in high throughput and a subsequent DEP force to precisely isolate CTCs. Serial combinations of separation
The use of DEP to sort live and dead cells is a common application and serves as a label free alternative to flow cytometry. Xing and Yobas\textsuperscript{54} utilized DEP to separate live and dead human colorectal carcinoma cells to achieve a 90% capture efficiency of live cells. In a recent work, Wei and co-workers\textsuperscript{55} utilized DEP based separation to demonstrate high transfection efficiency of neurons via electroporation while requiring fabrication of on chip electrodes, which adds complexity to chip manufacturing. Complications of secondary electrophoretic cell movement can accompany electro-osmotic driven pumping. Additionally, cell exposure to electric fields may result in reduced cell viability. Nonetheless, this method enables precise control of small volumes of reagents and size based cell separation.

**Acoustic.** Acoustic based sorting has recently emerged as an additional modality that has no impact on cell viability.\textsuperscript{65} At their core, acoustic separation modalities (i.e., acoustophoresis) work by inducing cell movement in response to an acoustically generated pressure wave. There can be several subdistinctions of acoustic cell sorting depending on the wave type: bulk standing waves, standing surface acoustic waves, and traveling waves.\textsuperscript{66}

Bulk standing waves are generated within microfluidic channels when the applied wavelength matches the spatial channel dimension. As a result, two distinct regions are generated across the channel, along the wave’s path. The first is termed a node, where there is no pressure fluctuation. The second is termed an antinode, where there is a fluctuating pressure alternating between a minimum and maximum (Figure 5A). Particles flowing through the channel will respond to the standing wave depending on their acoustic contrast factor, referring to fluid movement by inducing solvated ion transport under an electric field. Suspended particles accompany the secondary fluid movement induced by migrating solvated ions (Figure 4C). Dittrich and Schwille\textsuperscript{60} demonstrated a sorting microchip with pump driven primary flow and a fluorescence triggered EOF sorting mechanism. This approach leveraged pressure driven flow to achieve a fast and stable flow, resulting in high throughput. EOF has also been used to augment a size based separation technique termed hydrodynamic spreading. Wu et al.\textsuperscript{61} combined electro-osmotic flow with pump driven primary flow in order to augment hydrodynamic spreading and separate E. coli and yeast cells. Similarly, Kawamata and co-workers\textsuperscript{62} demonstrated purely EOF driven separation of 1.0, 2.1, and 3.0 \( \mu \text{m} \) particles. The advantage of EOF is typically the precise control of volumetric flow through various channels occupying the same microfluidic device. However, EOF requires fabrication of on chip electrodes, which adds complexity to chip manufacturing. Complications of secondary electrophoretic cell movement can accompany electro-osmotic driven pumping. Additionally, cell exposure to electric fields may result in reduced cell viability. Nonetheless, this method enables precise control of small volumes of reagents and size based cell separation.

**Electro-osmotic Flow.** Akin to the previously discussed EP and DEP sorting mechanisms, electro-osmotic based separation also results from an applied electric field. However, the process is phenomenologically different. Electro-osmotic flow (EOF) refers to fluid movement by inducing solvated ion transport under an electric field. Suspended particles accompany the secondary fluid movement induced by migrating solvated ions (Figure 4C). Dittrich and Schwille\textsuperscript{60} demonstrated a sorting microchip with pump driven primary flow and a fluorescence triggered EOF sorting mechanism. This approach leveraged pressure driven flow to achieve a fast and stable flow, resulting in high throughput. EOF has also been used to augment a size based separation technique termed hydrodynamic spreading. Wu et al.\textsuperscript{61} combined electro-osmotic flow with pump driven primary flow in order to augment hydrodynamic spreading and separate E. coli and yeast cells. Similarly, Kawamata and co-workers\textsuperscript{62} demonstrated purely EOF driven separation of 1.0, 2.1, and 3.0 \( \mu \text{m} \) particles. The advantage of EOF is typically the precise control of volumetric flow through various channels occupying the same microfluidic device. However, EOF requires fabrication of on chip electrodes, which adds complexity to chip manufacturing. Complications of secondary electrophoretic cell movement can accompany electro-osmotic driven pumping. Additionally, cell exposure to electric fields may result in reduced cell viability. Nonetheless, this method enables precise control of small volumes of reagents and size based cell separation.
which is dependent on cell density and compressibility relative to the surrounding medium. Cells with a positive acoustic contrast factor will migrate toward the nodes and cells with negative acoustic contrast factors will migrate toward the antinodes, allowing for cell separation via different outlets.

Johansson et al. designed the first FACS device to sort cells using standing waves generated by an automated fluorescence triggered transducer. The device offered a label free and gentle cell sorting technique. However, only a sort rate of 27 cells s$^{-1}$ was achieved, significantly lower than FACS. Jakobsson also developed a microchip FACS to achieve binary particle sorting based on fluorescence detection. The chip also featured an acoustic 2-D prefocusing zone that improved optical detection, sorting accuracy, and overall throughput to 150 particles s$^{-1}$. Jakobsson’s approach was only capable of binary sorting. To overcome this limitation, Grenvall et al. fabricated a microchip with a 2-D, acoustic focusing zone to sort cells into five outlets based on their size. The device was used to fractionate leukocytes into high purity fractions with high total recoveries. However, a throughput of 150 cells s$^{-1}$ was not a significant improvement compared to other groups. Since mammalian generally exhibit a positive acoustic contrast factor, they typically align at the nodes and most groups exploit this fact to sort cells. In an alternative approach, Shields et al. synthesized elastomeric particles with negative acoustic contrast and showed that bioconjugation of mammalian cells to these particles could be used to selectively move cells to the antinodes within a microfluidic channel. As such, labeling one cell type among a heterogeneous cell population would allow sorting without a fluorescence trigger.

Standing surface acoustic waves (SSAW) are standing waves formed along the bottom of a microfluidic channel using interdigital transducers (IDTs). The IDTs are patterned on a piezoelectric substrate, which is mounted to a microfluidic device. The wave changes modes from a transverse to a longitudinal wave from the substrate to the fluid, and these longitudinal waves create pressure nodes (Figure 5B). SSAWs are capable of deflecting an object in fluid flow independent of its contrast factor and thus are thought to be more flexible in separating cell populations that are small and inherently difficult to sort via bulk standing waves. Li et al. designed a SSAW based device capable of sorting water in oil droplets into five different outlet channels. This type of device could be utilized as an upstream sorting component for droplet based genomic and transcriptomic analyses.

Most reports of acoustically driven cell sorting rely on standing waves, but traveling waves have been used to sort cells as well. Standing waves require wavelengths comparable to microfluidic channel width, and therefore the sorting rate cannot be increased because the wave frequency is constrained. Traveling waves offer a workaround to this limitation. Recently, Schmid and co-workers designed a microfluidic device using fluorescence triggered traveling waves to sort cells into one of three channels (Figure 5C). Schmid reported a rate of 3000 cells s$^{-1}$ compared to 222 droplets s$^{-1}$ via SSAWs. Similar to electrically driven cell sorting, acoustophoresis typically requires integrated transducers. This in turn complicates chip fabrication, operation, and increases cost. Furthermore, cell sorting via bulk standing waves is unsuitable for sorting subpopulations of the same cell type with similar contrast factors. In this case, a fluorescent label may be necessary to distinguish between subpopulations, thereby negating the label free advantage. However, acoustophoresis mitigates concerns regarding decreased cell viability which is frequently observed with electrical cell sorters and therefore the benefit of delicate cell handling may override cost and time concerns. Many more examples of acoustic based cell sorting are available and we refer the reader to focused reviews for further discussion. In addition to acoustic and electrical forces, researchers have utilized alternative biophysical cues such as optical or radiation forces to sort cells.

**Optical.** The use of light to move particles dates back more than 45 years when it was discovered that a focused laser could propel microparticles in a liquid. About 15 years later, the same researchers achieved stable trapping via a tightly focused laser and this formed the foundation of contemporary “optical tweezers”. Optical forces result due to momentum exchange between incident photons of light and the irradiated object. Light scattering and absorption due to incidence with an object changes the light’s direction and magnitude and therefore the
associated photons’ momentum. To achieve large displace-
ments, many photons are called into action by using a focused
laser. The particle’s behavior is dependent on its refractive
index compared to the surrounding fluid, much like DEP is
dependent on electrical permeability. When the particle’s
refractive index is higher than the surrounding fluid, the
particle will migrate toward the region of highest light intensity
and vice versa. In both instances, the particle will move in
the direction of light propagation as well. More detailed discussions
on the physics of light induced forces can be found elsewhere.

Since its inception, optical manipulation has been used to
sort and manipulate cells on microfluidic chips (Figure 6A).
Optical approaches to cell sorting are minimally detrimental to
cell viability, in contrast to sorting via an electrical field.77
Bragheri et al.78 reported a device with a fluorescence triggered
optical mechanism which deflected cells toward a specific
outlet. However, the device could only sort approximately 100
cells per minute due to the lag in software communications as
reported by the authors. Low throughput limits the application
to smaller or less dense cell suspensions. Indeed, one of the
major drawbacks of all microfluidic FACS systems is the slow
throughput compared to conventional FACS that can sort at
10^6 cells per second. To address this issue, Chen et al.79
reported a microfluidic device capable of sorting 23,000 cells
per second with 90% purity and 45,000 cells per second with
45% purity. The device utilized a tightly focused laser to induce
a cavitation bubble in flow, which provided a mechanical force
for cell deflection. The key innovation to achieving high sorting
rates was a 3-D sheath focusing step made possible by a
multilayered device structure. Two disadvantages to this
approach was sample dilution due to the required sheath flow
and the complexity of building 3-D microfluidic channels. The
group improved on their initial design80 by using an inertial
based focusing step, therefore eliminating the need for a 3-D
device geometry. The devices discussed above utilized a laser
focused at a fixed position in a microfluidic channel and
therefore require focused single cell flows. In contrast, Wang
and co-workers81 eliminated the focusing step by integrating
traditional optical tweezers in laminar flow. Eliminating the
need for a focusing sheath flow simplifies device architecture
and fabrication and also minimizes sample dilution, as
previously discussed. Minimizing sample dilution is critical for
assays with high detection limits.

Magnetic. Many of the discussed microfluidic systems have
borrowed from conventional FACS systems in that a
fluorescent signal acts as a trigger for an external force field
to separate cells. Microfluidic magnetic based separations also
borrow from their conventional MACS counterpart.32,33 Cells
are bioconjugated to magnetic particles via a cell-specific
antibody on the magnetic particle. These specific cells can be
separated from a sample by passing the sample through a
microfluidic device possessing a magnetic field or magnetized
surface (Figure 6B). This method’s simplicity and ability to
separate via action at a distance is particularly advantageous
compared to electrical cell sorters, which require electrodes in
contact with the cell suspension. As previously discussed, this
can lead to electrochemical reactions at the electrode fluid
interface that may decrease cell viability or impact cell
phenotype downstream.

The magnetic field can be induced via an external or
integrated permanent magnet or an electromagnet. Capture via
an external permanent magnet is common because of ease. For
example, Wang et al.32 isolated CTCs from whole blood by
incubating whole blood with anti-EpCAM antibody function-
alized magnetic nanoparticles. The incubated sample was
introduced through a microfluidic device placed underneath a
permanent magnet. Similarly, Besant and co-workers83 isolated
CTCs with magnetic nanoparticles and a permanent magnet
but also integrated structures and channel design that captured
CTCs with varying surface expression into designated zones.
Chen at al.84 combined both a bulk permanent magnet and
patterned micromagnets in order to minimize the cell
aggregation at the capture site. In contrast to the immobiliza-
tion approaches discussed above, permanent magnets can
deflect magnetic particles to an alternative outlet46,52 for

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**Figure 7.** Passive microscale cell separation techniques. (A) A schematic of DLD based cell separation. An array of pillars directs cells with a radius greater than the critical radius toward a distinct outlet. (B) A schematic of inertial based cell separation in spiral microfluidics. The spiral channel design results in vortex formation perpendicular to the primary flow at two distinct regions, also known as Dean flow. Cells with different physical properties are focused at a particular vortex and the cells are separated via bifurcating outlets. Reproduced from Di Carlo, D. Lab on a Chip 2009, 9 (21), 3038–46 (ref 101), with permission of the Royal Society of Chemistry. (C) A schematic of filtration based cell separation, showing three filter configurations. From top to bottom: the weir filter, the pillar filter, and the cross-flow filter.
continuous flow sorting. Kim et al. integrated a ferromagnetic wire array into the bottom of a microfluidic device so that an externally applied magnetic field was deformed resulting in lateral displacement of magnetically labeled cells. An alternative to using a permanent magnet is to use an electromagnet. Electromagnets may offer an advantage in some separations because the magnetic field strength may be finely tuned by varying the current. However, device fabrication is more complicated and large currents can quickly generate Joule heating. The generated heat can increase sample temperatures above physiological temperatures and decrease cell viability. Furthermore, increased fluid temperatures can result in bubble formation, which is problematic for functionality in microfluidic devices. To overcome this limitation, one can dissipate Joule heating by integrating dedicated cooling channels or using a highly thermal conductive substrate such as silicon to construct the chip. Of course, both remedies add to the chip manufacturing cost. Implementing microfluidic magnetophoresis typically requires antibody labeling. Immunolabeling can be problematic for several reasons: labeling may negatively impact post cells' endogenous genetic expression, labeling may not be possible due to lack of surface markers, labeling may be undesirable because of clinical concerns, and antibodies are quite expensive. In some instances, the cell's native magnetic properties can be exploited for sorting as in the separation of red blood cells containing high levels of iron. Some cells can be chemically treated in order to impart the desired magnetic properties. For example, Sofla et al. devised a label free magnetic strategy to separate live cardiomyocytes by rendering the native myoglobin from diamagnetic to paramagnetic via oxidation with sodium nitrite. Microfluidic magnetophoresis based cell sorting has been applied extensively and the reader should refer to several recently published reviews.

**Passive Mechanisms.** The methods for microfluidic cell sorting discussed so far rely on an external force field to move cells. Methodologies that utilize intrinsic cell properties such as size, density, and deformability are discussed next. Passive mechanisms are particularly advantageous in that a fluorescent trigger to activate sorting is not required. Nor is external labeling as in magnetic based separations. **Deterministic Lateral Displacement.** Deterministic later displacement (DLD) sorting uses a pillar array to direct cells one way or another based on cell size. Cells with a size less than a critical radius move in the direction of primary fluid flow. Cells greater than the critical radius are deflected in a direction determined by array design (Figure 7A). The critical particle size is dependent on the pillar design. This has also been shown that cell shape and deformability can be exploited for DLD sorting. This study extended the capability of DLD separation to discriminate between cells by morphology and deformability, in addition to size. However, only one post geometry was investigated, thereby neglecting the role of post geometry on cell separation. Ranjan et al. explored the effect of the pillar shape in order to sort spherical particles, disc-shaped red blood cells, and rod-shaped bacteria. The study showed that rod shaped bacteria can be separated with 100% efficiency using "I-shaped" pillars and provided a method to separate nonspherical particles based on their longest dimension, adding to the microfluidic toolbox. DLD has also been used in other applications: to separate cancer cells from diluted whole blood, erythrocytes of different phenotype as determined by deformability, leucocytes from erythrocytes, and subpopulations of leucocytes. DLD is advantageous because it offers gentle and label-free cell sorting and requires relatively facile fabrication to implement. However, the cells must differ in size or shape. For more on DLD, we refer the reader to a recent focused review.

**Inertial.** Microfluidics, as the name implies, tends to occur in channels with micrometer sized channel dimensions. As a result, the dimensionless Reynolds number is typically small and inertial effects are often neglected. However, at modest channel dimensions or velocities, inertial effects manifest and can be exploited for cell sorting. One typical manifestation of inertial effects in microfluidics is Dean flow, which results in vortex formation perpendicular to the primary flow (Figure 7B). Cells with differing properties such as size, density, or shape respond differently to inertial effects and are focused at different channel locations. Cell sorting is achieved by splitting a single channel into multiple outlets. Recent reviews discuss the physics of inertial microfluidics in depth. Warkiana et al. exploited Dean flow in curved channels to sort label-free CTCs from blood with high throughput (1.7 mL/min) and conducted heterogeneity studies via DNA fluorescent in situ hybridization (FISH) post cell isolation. This method is advantageous because it is label free, and therefore can be used for various cancers lacking biomarkers. Furthermore, in contrast to magnetophoretic CTC separation that requires immunomagnetic labeling, this method may better preserve endogenous molecular expression and utilized a simpler workflow. In addition, a thorough mechanistic understanding of inertial microfluidics tends to be poorly developed leading to extensive trial and error during device development.

To address this limitation, Kim et al. empirically investigated the effect of several parameters (channel height, width, radius of curvature, and flow rate) on inertial sorting in spiral microfluidics and then implemented these principles to design a two stage device to isolate CTCs from leucocytes. Using the results from the parametric experiments, the device was designed in one attempt, demonstrating and providing a toolbox for other researchers designing spiral inertial microfluidics. Recently, isolation of CTCs via inertial microfluidics has been demonstrated extensively. Several sub categories of inertial microfluidic techniques are recognized such as field flow fractionation, pinched flow, and hydrodynamic spreading. A recent review discusses cell sorting via inertial microfluidics in depth. Inertial microfluidics enable passive and label free cell sorting for instances when the target and nontarget cells vary in size. Passive sorting simplifies device operation and fabrication, which is important for widespread adoption. Label free cell sorting is attractive for numerous reasons: minimized antibody related costs, applicability to cells lacking biomarkers, and maintaining integrity of endogenous expression. The last reason is critical for conducting high fidelity single cell molecular analyses. Furthermore, the methods are continuous, increasing throughput. A disadvantage is that most devices require diluted samples because cell-to-cell interactions manifest at high cell concentrations.

**Filtration.** In the chemical process industry, filtration is a well-established technique for size based separation. Several sub categories of filtration are in existence and are typically descriptive of the underlying process such as dead-end filtration, cross-flow filtration, microfiltration, ultrafiltration, etc. Likewise, scientists have long since implemented various filtration mechanisms in microfabricated devices for cell enrichment. Weir filters (Figure 7C) offer a simple design; a
single channel containing an obstruction that almost closes the channel permits only cells small enough to pass above the obstruction. Pillar filters (Figure 7C) feature regularly spaced pillars in a microchannel which obstruct the passage of larger cells. However, both filter types are only useful for low-density samples or small sample sizes because they are prone to clogging, akin to dead-end filters in the chemical process industry. To address this limitation, scientists have microfabricated cross-flow filters, which have filter elements parallel rather than perpendicular to the main flow direction. The main fluid flow sweeps across the filter in order to clear clogged cells. Cross flow filters (Figure 7C) have been used for the separation of myocytes and nonmyocytes, leukocytes, bacteria, and CTCs from blood. In another approach to eliminate clogging, McFaul et al. incorporated a periodic backflow to reduce clogging in a microfabricated cross-flow filter. Filtration based microfluidic cell sorters are relatively easy to fabricate, operate continuously, and are label free. However, they are only capable of sorting cells differing in size.

**Outlook for Microfluidic Cell Sorting.** Microfluidic cell sorting is a mature research field and was well established even prior to the paradigm shift toward single cell analysis. For brevity, we have only discussed the most prevalent modalities for microfluidic cell enrichment. Other approaches implemented include use of valves, transient cell adhesion, and permanent cell immobilization. Despite the field’s maturity, researchers continue to innovate and conceive new techniques for cell separation. In fact, researchers are enriching cells in creative ways that are not easily categorized. Wang et al. demonstrated a microfluidic device for cell enrichment on the basis of cell viscosity. The device demonstrated fractionation of two leukemia cell lines as well as leukemia cells from healthy leukocytes. Zhang et al. exploited the difference between surface free energy between two microbial cells to accomplish enrichment. As evidenced, we expect the field of microfluidic cell enrichment to continue growing.

Among the many discussed works, most were examples of standalone microfluidic cell enrichment. FACS remains a powerhouse for microfluidic enrichment prior to single cell analysis. This may be for several reasons; high throughput ($10^4$ cells per second), facile integration with single cell compartmentalization (well plates), time tested (invented in 1969), high purity (95−100%), multiple and simultaneous sorting criteria, etc. FACS itself is a means of single cell analysis in conjunction with hybridization techniques. In contrast, FACS has shortcomings that can be addressed via a microfabricated system such as large sample size requirements, low total recovery, large footprint, and high costs. So what factors prevent wider adoption of microfluidics? Limited throughput is a key factor. Modalities, which are amenable to continuous flow and trigger free enrichment, promise the highest throughput. If a single chip cannot offer competitive throughput, scaling strategies such as chip parallelization need to be implemented. Additionally, microfluidics is not readily reusable due to clogging and cell adhesion. Perhaps, microfluidic platforms will find a niche in the sterile single use market. To do so, investment in manufacturing and validation is required. We anticipate wider adoption as these issues are addressed and systems are commercialized.

**Single Cell Isolation. Conventional Approaches.** After obtaining an enriched cell suspension, the next step in the workflow is to isolate single cells from one another for analysis as discrete individuals. Single cell immobilization is inadequate on its own. Cells must be compartmentalized in such a way so lysate contents of one cell do not contaminate another, allowing true single cell analysis. As already discussed, FACS can be used as a method of both sorting cells and addressing single cells into an individual microwell for molecular analysis. FACS based cell compartmentalization is high-throughput and automated, and microwells can be easily accessed for reactant addition or product collection. The disadvantages, as previously discussed, are high cost and large sample volume requirements. Furthermore, wells open to the environment introduce a contamination risk. Nevertheless, FACS remains a staple, commercial technology.

Micromanipulation is a technique that involves manually selecting and transporting a single cell of interest to its own container for downstream workup. One way to do so is mechanically, via a pipet (Figure 8A). A particular advantage of micromanipulation is that cell enrichment is not required since cells may be visually identified prior to manual selection. Manual identification and selection under a microscope ensures a high confidence level that a single cell has been compartmentalized. Selection via micromanipulation can also be done in combination with FISH to discriminate cells by specific genetic features. Additionally, micromanipulation is well suited for small sample sizes of fragile materials. However, micromanipulation is low throughput and is also done in an open environment, thereby risking contamination. Citri et al. demonstrated picking up single neuron cells for quantitative PCR (qPCR) analysis. Recently, Marinov et al. quantified...
stochastic RNA expression in a lymphoblast cell line using micromanipulation for cell isolation and an RNA-sequencing technique. Pipet micromanipulation is commonly utilized due to its simplicity and low price, and commercially available automated systems can increase throughput.

Optical tweezers, as discussed above in regards to cell enrichment (Figure 6A), are also categorized as a micromanipulation technique and are readily used for compartmentalizing a single cell. This mechanism harnesses the momentum of refracted and scattered light photons to position a particle at the center of the light’s focus. Optical tweezing for single cell isolation prior to molecular analysis has also been demonstrated. The main advantage of utilizing optical tweezers compared to mechanical micromanipulation is volume reduction, equal to approximately the cell volume itself. This minimizes lysate dilution and therefore increases assay sensitivity. A second advantage is contactless cell compartmentalization, thereby minimizing contamination risk. However, optical tweezers suffers from low throughput akin to mechanical micromanipulation.

A crude but simple method for single cell compartmentalization is serial dilution (Figure 8B). This involves stepwise dilution of a cell suspension until single cells are obtained in individual vessels. Though cheap and simple, serial dilution can be laborious and unreliable, resulting in some fractions containing no cells, single cells, or several cells. Furthermore, batch wise processing introduces contamination risks.

Up until this point, all of the discussed techniques have been applicable for suspended cells. As a result, it is impossible to recognize the isolated cell’s spatial origin in the starting intact tissue. This information is instrumental if attempting to correlate genetic or other molecular expression to cellular spatial distribution within native tissue. The ability to spatially map single cell data would greatly increase our understanding of complex heterogeneous tissues, far beyond solely cell type enumeration. Coupled with a temporal analysis, researchers could study how molecular processes propagate throughout various tissues. Practical applications include studying embryonic development and comparing healthy and diseased tissue to identify new therapeutic targets. Laser capture microdissection (LCM) is a powerful technique, developed for isolating single cells from their heterogeneous, native tissue environment while preserving their spatial origin.

In the LCM technique, a thermoplastic film is attached to a tissue section and the tissue section is placed tissue-side down on a glass slide. The glass slide is positioned on an inverted microscope for imaging and a plastic cap is placed over the glass slide. The glass slide is positioned on an inverted microscope for imaging, and a plastic cap is placed over the glass slide. The glass slide is positioned on an inverted microscope for imaging, and a plastic cap is placed over the glass slide. The glass slide is positioned on an inverted microscope for imaging, and a plastic cap is placed over the glass slide. The glass slide is positioned on an inverted microscope for imaging, and a plastic cap is placed over the glass slide. The glass slide is positioned on an inverted microscope for imaging, and a plastic cap is placed over the glass slide. The glass slide is positioned on an inverted microscope for imaging, and a plastic cap is placed over the glass slide. The glass slide is positioned on an inverted microscope for imaging, and a plastic cap is placed over the glass slide. The glass slide is positioned on an inverted microscope for imaging, and a plastic cap is placed over the glass slide.

The cap attached to the film. Once cells of interest are identified via microscopy, a focused infrared laser melts the plastic film over the cells, thereby adhering the cells to the film (Figure 8C). When the cap attached to the film is removed, the cells of interest are sheared from the intact tissue and transferred to the cap. There are several commercial systems available with different laser types, infrared (IR), ultraviolet (UV), and combined IR/UV. The ultraviolet systems operate on a slightly different principle; a cell of interest is removed via UV laser by cutting around the cell rather than adhering to plastic. As mentioned, LCM permits single cell isolation with knowledge of in situ origin. The technique allows direct visualization and morphological cell identification. Tissue are typically flash frozen and then cryosectioned, thereby providing a stable snapshot of gene expression for downstream molecular analysis. LCM is particularly advantageous when the number of cells to be isolated is too low to use FACS. As such, LCM has been used extensively for single cell molecular analysis. With continued method development, systems such as the Bio-Rad Clonis can now isolate live cells, rather than exclusively frozen or fixed samples. Despite its wide adoption, LCM is not without disadvantages. The technique is low throughput compared to FACS and an instrument can cost around $150,000. The protocol is performed at least partially in an open environment, thus again, risking contamination. Morrison et al. compared LCM and FACS in regards to isolating and measuring single cell gene expression in the avian embryo and showed FACS to be advantageous. The study showed that 96% of FACS isolated single cell generated usable gene expression profiles compared to ~60% of LCM isolated cells. Furthermore, with an optimized FACS protocol, native gene expression can be preserved and is highly correlated with that from LCM isolated cells. Despite some drawbacks, LCM remains the only technique capable of in situ single cell isolation.

Many challenges of single cell analysis are encountered in compartmentalizing single cells. Ideal methods would be both high-throughput and low cost, two requirements that usually oppose each other. At the same time, single cell compartmentalization must remain efficient for smaller samples. Dilution must be minimized in order to enable assays such as the analysis of low protein or transcript quantities. Furthermore, contamination effects become amplified at low analytic quantities and must be avoided. Extreme care should be taken at this juncture to ensure a high fidelity and unbiased result. In the next section, we discuss how microfluidic devices are employed for single cell compartmentalization.

Microscale Approaches. Microfluidic devices offer many advantages to achieving single cell compartmentalization. Single cell compartments are miniaturized to reduce lysate dilution. This is critical for assaying low-abundance biomolecules such as mRNA which can be present at 0.01–2.5 picograms per single cell. Reagent volumes are minimized, thereby decreasing cost. A high surface area to volume ratio ensures high heat transfer and temperature control, critical for PCR assays requiring temperature cycling. Compartmentalized cells can be closely arrayed allowing for facile visualization via microscopy. Furthermore, many microfluidic solutions are completely hands-free, closed systems, minimizing contamination risk. For example, Fluidigm’s C1 Array is a state of the art, commercial microfluidic product for automated single cell isolation. Cells are introduced into the chip, in which they follow a serpentine pattern through each available capture site. Once a cell occupies a capture site, subsequent cells bypass the occupied site and are trapped at the next capture site. Trapping can be followed by reagent addition for single cell reverse transcription and amplification. Marcy et al. reported microfluidic DNA amplification to be highly advantageous due to reduced amplification bias, higher analyte specificity, and better economies of scales in nanoliter volumes. In the following sections, we review microfluidic compartmentalization for single cell analysis. The focus is predominantly on designs for molecular single cell analysis. We briefly review trapping methods that do not isolate single cell lysates but can be used for microscopic analysis of single cell dynamics. For a discussion on additional cell trapping mechanisms, we refer the reader to a previously written review.

Valves. On chip valves offer an easily conceptualized system for single cell trapping. Additionally, valves can be used to both
regulate fluid flow and control flow direction. Quake et al. popularized the commonly used microfluidic valve architecture, frequently termed the Quake valve.\textsuperscript{138,139} Using Quake valve architecture, Streets et al.\textsuperscript{140} investigated gene expression in mouse embryonic cells and mouse embryonic fibroblasts using RNA-sequencing on a microfluidic chip. Single cells were trapped between two pressure actuated valves in a chamber of only 0.86 nL. Subsequent steps were also performed in similar chambers of 1.35–128 nL. The authors attributed the high experimental reproducibility to the use of microfluidics, which reduces stochastic variation due to pipetting errors and handling. Reagent mixing was accelerated by actuating the valves independently in a back and forth fashion. Shi et al.\textsuperscript{141} designed a chip containing 120, 2.0 nL microchambers isolated by push-down valves. The chambers were used to trap single cells and analyze each cell’s protein content via a patterned antibody array within the chamber. Fan et al.\textsuperscript{142} utilized a microfluidic chip to isolate a single cell, lyse the cell, and partition the resulting chromosome suspension into 48 different cell chambers for amplification, allowing for genome wide haplotype analysis. The cell and chromosomes were compartmentalized in discrete chambers by pressure actuated valves on each chamber. Similarly, White and co-workers\textsuperscript{8} designed a chip single cell digital PCR analysis. The device was used to analyze 200 single cells on a 10 cm\textsuperscript{2} chip and was operated by controlling 12 pneumatic valves independently. Recently, Sun et al.\textsuperscript{143} designed a chip for single cell reverse transcription quantitative PCR (RT-qPCR) to investigate the effects of methylmethanesulfonate on human cancer cell (MCF-7) gene expression. The chip trapped a single cell between two pneumatically controlled valves. The microfluidic method increased assay sensitivity and gene upregulation was detected after 27 amplification cycles.

While on chip valve systems are easily conceptualized, all of the above examples were two layer pneumatic valves or Quake valves. The bottom microfluidic layer houses channels for cells, buffers, and reagents and the top microfluidic layer houses larger channels that perpendicularly intersect the underlying channels. The larger channels are pneumatically pressurized and force the bottom channels to close (Figure 9A). In addition to two-layer architecture, these devices typically employ computer controlled pneumatics. Both characteristics compli-
cate device fabrication, operation, and increase cost. Finally, single cells may be loaded via pipet injection or syringe pumped flow but require a user to confirm single cell trapping via microscopy, limiting throughput. Microvalve performance has advanced significantly in regards to cost, leakage, minimizing volume, and fabrication options. Work toward automatic feedback controlled valves coupled with microscopy would enable completely hands off single cell analysis and increase throughput significantly.

Dielectrophoretic. Dielectrophoretic methods to compartmentalize single cells have been applied with great success. In fact, the DEPArray System (Figure 9B) from Silicon Biosystems is a commercial microfluidic system for single cell isolation. The system includes a single use cartridge and analysis platform. The cartridge is an array of individually controllable electrodes enabling single cell trapping via DEP cages. Post trapping, cells are visually identified and manipulated to other traps or isolated off chip. The system has been recently used for isolating single lung and breast cancer cells from patient blood samples in order to perform sequencing and RT-qPCR. Using the DEPArray, Carpenter et al.147 isolated single patient derived neuroblastoma cells for genome sequencing. In this approach, the researchers moved single cells into a microtome or other container post on chip DEP trapping and identification. Thus, strictly speaking cells are not compartmentalized on chip. For the same reason, the method is relatively low throughput, limiting applications to smaller samples and increasing labor costs. Furthermore, multiple single cell measurements are necessary to distinguish true cell heterogeneity from experimental noise.

Microwells. Microwells provide a facile way to isolate single cells using physical boundaries, akin to how multiwell plates physically isolate cell groups (Figure 9C). The key difference between microwells and conventional multiwell plates is smaller well size and volume. Microwells for single cell isolation have been previously reviewed in detail.148,149 Cells are typically seated into individual microwells by gravitational and remaining outside of the wells are flushed away. One drawback is that typically single cells will occupy only a fraction of the wells; the rest may contain more than one cell or no cells. However, adjusting well size, shape, and cell concentration can optimize the single cell seeding efficiency. Large arrays can be fabricated to ensure enough single cells are trapped for meaningful analysis. Some experiments demonstrated simultaneous use of DEP and microwells to control cell seating.150,151 Despite facile fabrication and operation, microwell approaches are more suitable for microscopic single cell studies rather than molecular analysis, since cell lysates may mix.

Hydrodynamic. Single cell trapping can be accomplished by passive, hydrodynamic mechanisms. This approach is attractive because it does not require sophisticated experimental systems such as DEP or valve based systems. There are several subcategories to hydrodynamic trapping. The terms “eddies” and “vortices” are interchangeably used to describe trapping induced by recirculating fluid flow. In one example, it was demonstrated that four recirculating eddies induced by an audible frequency fluid oscillation around a cylinder can be used to trap single cells at the eddy centers.152 The effect was termed “hydrodynamic tweezers” (Figure 9D). The same laboratory investigated noncylindrical geometries and showed that device geometry and oscillation frequency controls the eddy number, shape, and strength.153 Tanyeri and Schroeder demonstrated controllable, 2-D, hydrodynamic particle steering by adjusting flow rates at a junction of four buffer streams. Additionally, a permanently stable stagnation point was demonstrated wherein the trapped particle is not displaced by Brownian motion. Cell lysates are not isolated via this method; therefore, the utility of hydrodynamic tweezers is geared toward microscopic analysis. However, Hayakawa et al.155 used vibration induced flow around spirally arranged micropillars to capture a single cell in a patterned thermoresponsive gel. After a single cell is captured at the gel center, the chip is cooled and the expanding gel closes over a single cell, resulting in compartmentalization. However, the method reported only a 60% cell capture success rate with 61% of captured cells remaining viable. For further discussion of vorticity based cell trapping, we refer the reader to a recent review.156

A second passive hydrodynamic trapping method uses physical obstructions such as U-shaped cups to physically isolate single cells on chip (Figure 9E). The obstructions may have cutaways allowing fluid flow through an unoccupied trap, thereby increasing trapping occurrence. An early example was demonstrated by Di Carlo157 who used an array of physical U-shaped traps to isolate 100 single human cervical carcinoma cells and performed on-chip for 24 h. This approach has been modified and implemented for various applications such as trapping and culturing single E. coli cells,158 human dermal fibroblasts,159 trapping and selective exposure of single hepatocytes via coflowing streams,160 and lymphoma cell capture for microscopic analysis of cellular markers via antibody staining.161 Recently, Guan et al.162 published a comprehensive design procedure for designing devices for single cell hydrodynamic trapping. They demonstrated the design of a device with 100% capture efficiency and confirmed their finite element simulation results with experiments. All of the experiments discussed above utilized a flow perpendicular to the trap. Another derivative of this technique utilizes flow tangential to traps.163,164 Espulgar et al.165 designed a device for trapping single cardiomyoctyes to observe cell growth, coupled, and beating. Uniquely, the cell loading method did not require a pump to drive cells into traps; instead cells were pipetted into the device and the device was centrifuged to trap cells. Another format for hydrodynamic single cell trapping was recently demonstrated by Jin and co-workers.166 The authors demonstrated 100% cell trapping with HeLa cells. The device design provides deterministic single cell trapping akin to that demonstrated by Guan et al.162 However, this format does not require long bypass channels to achieve optimum flow resistances. The isolation methods utilizing hydrodynamics and physical obstructions as discussed above are beneficial because they are passive and can be adopted for various cell sizes and shapes. However, almost all works used trapped cells for culturing and transient imaging analysis, rather than single cell lysate analysis. Nevertheless, these methods can be coupled with FISH for single cell genomics.167

Droplets. Droplets have emerged as a popular means for single cell compartmentalization (Figure 9F). Monodisperse droplets are generated by coflow of two immiscible fluids.168 This methodology is especially useful for molecular analysis of single cell lysates, as each droplet can function as an independent chemical reactor.169,170 Using droplets eliminates complex fabrication requirements such as two layer valve systems or integrated electrodes for DEP systems. Droplet generation and cell encapsulation is done in continuous flow, enabling high throughputs, typically up to several kilohertz.169,171 Pico-liter169,170,172 or femtoliter173 droplets are achievable, thereby

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Droplet based approaches compartmentalize cells according to a Poisson distribution, meaning that more than half of droplets will be empty. Some studies have reported approaches to beat Poisson statistics and increase single cell compartmentalization efficiency. For example, Ramji et al. developed a device with an initial stage in which lung cancer cells are disaggregated, focused, and uniformly spaced through a series of expanding and contracting channels. Then, single cells are combined with reagents and encapsulated in droplets. Their experiment resulted in a single cell encapsulation efficiency of 55% and only 25% of droplets were empty. The platform was utilized to study single cell activity of the epidermal growth factor receptor, an indicator of cancer metastasis. Kemna et al. utilized Dean flow in a spiral microchannel for cell prefocusing and reported a single cell encapsulation efficiency of 77%. The preceding two examples optimized cell focusing and spacing prior to the compartmentalization junction in order to increase efficiency. Jing et al. noted that these approaches require cell high cell densities (10^7 cells/mL), potentially limiting their clinical application. The group designed a two stage device consisting of droplet generation and DLD droplet sorting. The droplet generator produced empty 14 μm diameter droplets, which increased to 25 μm after single cell encapsulation. The DLD stage then sorted empty droplets from those containing single cells. The device generated droplets at 5 kHz with 80% single cell encapsulation efficiency. Additionally, only a 100 μL low-density (10^7–10^8 cells/mL) sample is required. The device was used for assaying single lung cancer cell matrix metalloproteinase secretion. Metalloproteinase secretion was investigated using a predetermined calibration curve, spanning enzyme concentrations from 5 to 64 nM.

As evidenced above, increasing single cell compartmentalization in droplets can be achieved via prefocusing or postsorting steps. In fact, facile integration of droplet formation with other steps enables applications other than molecular assays. Schoeman and co-workers demonstrated single cell droplet encapsulation followed by fusion of two droplets to produce a hybridoma, a fusion of myeloma and B-cells. The device featured two inlets for introducing the two different cells. Each cell traversed an “alternating spiral microchannel” for cell prefocusing. Then, each cell was encapsulated in a droplet at a T-junction, operating such that the cells are encapsulated by alternating cell type. The two paired droplets then pass on chip electrodes which induce droplet coalescence. Next, the coalesced drop is reduced in size through contact with a “pitch-fork” structure to promote cell-to-cell contact. Finally, the two cells are electrosurfed via on chip electrodes. This study demonstrated the versatility of droplet microfluidics, beyond cell assay applications.

Droplets have emerged as a powerful platform for single cell biology. Single cell molecular assays such as PCR and fluorescence based protein assays have been miniaturized and compartmentalized into picoliter droplets. The yearly publication number regarding single cells and droplet microfluidics has more than doubled over the last 5 years. As such, we can expect considerable growth in this research field. For more applications and information regarding single cell droplet microfluidics, we refer the reader to several focused reviews.

**Single Cell Lysis. Conventional Approaches.** Once a single cell of interest is compartmentalized, and the biomolecule under investigation is identified, the next step is quantifying the target. Transmembrane proteins are frequently quantified via fluorescent antibody probes. In contrast, biomolecules within the cell are typically extracted for assay. To extract the molecule, the cell plasma membrane must be disrupted. The nature of the plasma membrane varies across cell types and organisms. Conventional methodologies for membrane disruption can be classified as detergent and nondetergent based. Nondetergent based approaches include mechanical agitation, liquid homogenization, temperature cycling, and sonication. Traditional mechanical cell lysis relies on rotating impeller blades, a mortar and pestle, bead beating, or a homogenizer to break open cells. However, these methods are not suitable for single cell lysis, but rather are appropriate for many cells in suspension or larger tissue samples. Gunerken et al. recently reviewed these and other lysis techniques as they apply to lysing microalgae for biofuel production. Sonication disrupts the cell membrane via high frequency sound waves generated by an immersed piezoelectric transducer. Cheow et al. lysed single liver cancer cells confined in nanoliter wells by immersing the well plate in an ultrasonic bath. However, sonication typically requires 50 s for single cell lysis and in that time, can generate enough heat to denature proteins. Pretreatment with a nonionic detergent shortened the required time to only 3 s. Additionally, heating can be prevented by utilizing short discontinuous bursts instead of continuous sonication and by sonicating in an ice water bath. A freeze–thaw cycle can also be used to lyse cells. Ice crystals formed during freezing contract during thawing to break cells. Kim et al. evaluated freeze–thaw lysis of single lymphocytes for DNA extraction where the cell was frozen in 10 μL of purified water via liquid nitrogen and thawed at room temperature. The cycle was repeated twice. Multiple cycles are typically necessary and as such, the freeze–thaw approach can be lengthy.

Detergent based cell lysis is a potentially milder and quicker approach compared to mechanical, sonication, and freeze–thaw cell lysis and can be scaled down for single cells. Detergents lyse a cell by solubilizing proteins associated with the cell plasma membrane and disrupting interactions between lipids and proteins. A variety of detergents are available, characterized by the nature of their hydrophobic tail and hydrophilic head. Detergent selection is empirical. However, general rules are useful; nonionic or zwitterionic detergents are less denaturing compared to ionic detergents and are therefore used when maintaining native protein structure or function is important. Though, a milder detergent will require longer exposure times for cell lysis and could confound transient biomolecule expression in single cell studies. For example, Han et al.

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tested various detergents and concentrations for their ability to lyse a single cell while maintaining the nucleus intact. A nonionic detergent, Triton, at 2% was able to lyse the cell membrane within 10 min of exposure and maintain an intact nucleus for up to 1 h. Shoemaker et al.187 lysed single cells with 0.1% of Triton X-100 for subsequent enzyme activity analysis. As evidenced above, detergent based lysis protocols require precise liquid handling for accurate data interpretation and careful selection of optimal lysis media.

A frequent goal of cell lysis is to minimize alteration of a molecule’s native structure and expression. This means that the approach must be both gentle and rapid, normally confounding requirements. Sonication, freeze–thaw, and detergent based methods are applicable for single cells, though each may have their disadvantages: excessive heat generation, long protocols, and arduous implementation. Microfluidic technology has enabled new cell lysis approaches, specifically suited for single cells.

**Microscale Approaches.** Microfluidic devices provide an ideal platform for single cell lysis. Devices can be manufactured with unique geometries and precise dimensions and operated in the laminar flow regime. This allows for finely tuned mechanical or chemical single cell perturbation. Microfluidic dimensions on the length scale of single cells minimize lysate dilution increasing assay sensitivity. Laminar flow characteristics minimize convective transport of lysate. Most devices are optically transparent, allowing for visualization. Devices are typically contained, minimizing contamination for sensitive molecular assays such as PCR. There are several microfluidic single cell lysis methods and each has its own merits. Selecting the appropriate technique is dependent on many factors akin to conventional cell lysis and is crucial for achieving the desired result. In the next sections, we discuss select single cell lysis modalities highlighting recent experiments. For further discussion of microfluidic cell lysis, we refer the reader to two focused reviews.188,189

**Mechanical.** Mechanical cell lysis punctures cell membranes via a mechanical force. The force can be induced by shear, compression, collision with sharp features, and other methods. Kim et al.190 devised a fabrication method enabling spatio-specific and reversible channel formation on a microfluidic chip, following mechanical strain application and release. The group demonstrated single cell lysis using the new technology. Briefly, channels were created via applied strain and a single cell was placed in the newly formed channel. Following strain release and channel collapse, the single cell was lysed via compression. However, this early phase demonstration required manual handling and placement of single cells into the channels. Hoefemann et al.191 demonstrated single cell lysis in continuous microfluidic flow. Cells passing over an integrated heater were lysed by heat generated bubbles which compressed the cell against the channel ceiling (Figure 10A). Single cell

![Figure 10. Microscale single cell lysis techniques. (A) A schematic illustrating mechanical single cell lysis. A channel of flowing fluid is heated via an integrated heater on the channel bottom, thereby generating a bubble above the heater. Single cells passing above the heater are lysed by the rising bubble. Reprinted from Sensors and Actuators B: Chemical, Vol. 168, Hoefemann, H.; Wadle, S.; Bakhitina, N.; Kondrashov, V.; Wangler, N.; Zengerle, R. Sorting and lysis of single cells by BubbleJet technology, pp. 442–445 (ref 191). Copyright 2012, with permission from Elsevier. (B) A schematic illustrating chemical single cell lysis. A single cell is trapped in chamber defined by flanking Quake valves. Post cell capture, lysis buffer is introduced into the chamber and the reagent is mixed via actuating Quake valves to facilitate lysis. Reproduced from White, A. K.; Heyries, K. A.; Doolin, C.; Vaninberghe, M.; Hansen, C. L. Analytical Chemistry 2013, 85 (15), 7182–90 (ref 8). Copyright 2013 American Chemical Society. (C) A schematic of a microfluidic device for single cell lysis in adherent cell culture. The device tip is positioned at the target cell and a hydrodynamically focused lysis buffer selectively lyses the single target cell. The single cell lysate is captured on chip for fluorometric assay. Reprinted by permission from Macmillan Publishers Ltd.: Nature Communications (ref 215), copyright 2014.](image-url)
lysis in less than 20 ms was achieved with 100% efficiency. However, the experiment did not include a single cell assay, and therefore lystate diffusion or compartmentalization should be considered for single cell molecular analysis via this approach. The review by Nan and co-workers discusses mechanical lysis further, though specificity to single cells is rare.

**Thermal.** Thermal cell lysis relies on heat induced denaturation of cell membrane proteins, thereby opening the cell. Thermal, single cell lysis for single cell PCR has been executed off chip using a standard thermocycler and PCR tubes. The lysis step required a 95 °C hold for 90 s. Thermal lysis can be preferable compared to chemical lysis when enzymatic or detergent contamination of the intracellular biomolecules is to be avoided. However, careful consideration and precise control of the temperature is required for heat sensitive molecules. As such, thermal cell lysis is rarely used for protein analysis. Instead, it is most frequently used for parallel, sensitive molecules. As such, thermal cell lysis is rarely used for biomolecules is to be avoided. However, careful consideration and precise control of the temperature is required for heat sensitive molecules. As such, thermal cell lysis is rarely used for protein analysis. Instead, it is most frequently used for parallel, sensitive molecules.

**Chemical.** Given its long history of application to bulk cellular analysis and facile scalability, chemical lysis is a popular technique for single cell lysis. Treutlein et al. captured single cells for RNA sequencing and qPCR using the commercially available Fluidigm C1 system, which uses a lysis buffer to open cells. Fluidigm provides a proprietary lysis buffer or refers their customers to other commercial detergents such as Thermo Scientific’s nonionic NP-40 detergent. Similarly, Streets et al. fabricated their own microfluidic chip for single cell RNA sequencing. After trapping a single cell in a reaction chamber, the cell was lysed by addition of buffer containing 10% NP-40. Selecting the appropriate lysis protocol can be done by consulting manufacturers’ guides for bulk cell applications, typically made available by companies providing lysing buffers. Furthermore, a plethora of scientific literature can guide appropriate reagent selection for mammalian, bacterial, and tissue lysis.

Lysing speed can be dependent on many factors such as the detergent or enzyme being utilized, the concentration, and the contact efficiency. The former two parameters are partly constrained by the biomolecule of interest. Ionic detergents such as sodium dodecyl sulfate can lyse cells rapidly compared to nonionic detergents such as Triton X-100 but will also denature proteins. In contrast, it is always desirable to maximize contact efficiency for faster cell lysis. Doing so can be particularly challenging in microfluidic systems. Microscale flows typically occur in the viscous flow regime and, as such, molecular transport and mixing is dominated by diffusion. Shi et al. utilized cell lysis buffer from cell signaling to lyse single cells isolated in microfluidic chambers. The lysis buffer was brought into contact with cells strictly via diffusion. The authors allowed 20 min for lysis buffer diffusion, and another 20 min for incubation. As already discussed, the length of the lysis process can limit the study of faster occurring intracellular events. To overcome this limitation, peristaltic pumps, syringe pumps, actuating valves, or manual pipet injections are used to sequentially move reagents across cells and facilitate mixing.

The protocols discussed so far applied chemical lysis in discrete on chip chambers, formed by flanking pneumatic valves. This approach limits single cell analyses to “batch” wise operation. However, chemical lysis can be utilized for continuous single cell analyses by compartmentalizing the lysis buffer along with a single cell into picoliter droplets as already discussed. Hence, cell compartmentalization and lysis are performed simultaneously. For example, DeKosky et al. encapsulated single lymphocytes along with lithium dodecyl sulfate in ~73 μm diameter oil droplets by using a flow focusing nozzle and reported 100% lysis efficiency. Many more studies have reported the use of droplets for single cell compartmentalization and chemical lysis with application to RNA-sequencing.

Electrical. The cell plasma membrane is composed of a lipid bilayer that serves to protect the cell from its exterior environment. Upon exposure to an electric field, the lipid bilayer undergoes molecular reorientation and thermal phase transitions and new pores are formed. If the electric field is mild (0.2–1 V) and the exposure time is short, pore formation is reversible. This is termed electroporation and is frequently used for loading therapeutic and genetic materials into cells. In stronger electric fields or prolonged exposure, the pore formation is permanent. As the osmotic pressure between the cytosol and the surrounding media becomes unbalanced, cells swell and rupture. The electric field can also be high enough to cause rapid cell rupture. Electrical cell lysis offers a distinct advantage that in the electric field can be tuned for rapid cell lysis without denaturing target biomolecules. In fact, the difference between trans cell-membrane potential and trans organelle-membrane potential can be exploited to selectively rupturing the cell membrane while leaving organelles intact.
Mellors et al.\textsuperscript{207} built a chip for single cell analysis of erythrocyte proteins via capillary electrophoresis and electrospray ionization mass spectrometry. All flows on the chip were driven via electroosmotic pumping. An injected cell suspension traveled to a T intersection where an increase in the electric potential (4 kV) lysed cells. The authors found single cell lysing events occurring at a rate of 0.2 cells s\textsuperscript{-1}. Young and co-workers\textsuperscript{208} demonstrated a microfluidic device for EOF positioning and electrical lysis of a single lymphoma cell. Their method reported a success rate of 80% while taking approximately 60 s on average from cell injection to cell lysis. The method described by Young processed a single cell at a time. Bahi et al.\textsuperscript{209} fabricated a chip capable to trap an array of single cells via pDEP at 1 V potential, followed by lysis via a 60 V potential. The method reported 100% trapping and lysing efficiency. Despite reliable trapping and lysing of an array of single cells, the chip did not provide a way to compartmentalize single cell lysates. Thus, all extracted RNA was collected via pipet post the cell lysis step. To realize single analysis post electrical lysis, Kim et al.\textsuperscript{210} integrated microwell compartmentalization, DEP immobilization, and electrical cell lysis all on one chip. The group reported 95% of 3600 wells were loaded with single cells due to DEP facilitated trapping. Following DEP immobilization, reagent exchange was done rapidly in 30 s via pressure driven flow, without perturbing the cell positioning. To confine lysates, the wells are physically closed by pressing a PDMS membrane on top of the wells. The group reported 100% of trapped cells were lysed simultaneously via a series of 30 V electrical pulses. Similarly, another group designed a microfluidic chip to load single cells into 30 μm diameter wells via electrosomotic driven flow and then lyse the cells under a 30 V potential.\textsuperscript{211}

Electrical approaches offer rapid cell lysis, without damaging an assay’s target biomolecule, as opposed to thermal lysis. Furthermore, electrical lysis avoids target contamination contra to chemical lysis methods. Nevertheless, electrical lysis sees limited application for single cell molecular analyses. This may partly be because electrical lysis requires integration of electrodes and respective control systems on a microfluidic chip. Thus, the manufacture and operation of these chips is not trivial and is a limiting factor to clinical adoption. Though not discussed here, optical lysis methods face a similar hurdle. Optical lysis or laser lysis is rapid and therefore ideal for analyzing intracellular events on shorter time scales. However, laser equipment can be quite expensive and process integration complicated. The previously referenced reviews further discuss optical lysis.\textsuperscript{188,189} Currently, the predominant methods of single cell lysis for molecular single cell analysis are chemical. As already discussed, this may be due to researchers’ familiarity with bulk chemical lysis methods and the facile scale down to single cells.

**Single Cell Analysis.** Up to this point, this review has discussed tissue dissociation, cell sorting, single cell compartmentalization, and single cell lysis. The next and final step in the workflow is the single cell assay. The growing interest in single cell assays has been fueled by many sources. For example, studies have shown high heterogeneity among intra tumor cancer cells,\textsuperscript{212,213} spurring a debate as to whether bulk cell assays are appropriate tools for studying cancer cell genomes and transcriptomes. A plethora conventional single cell assay technologies are commonly used. Their selection is dependent on the biological application. For example, does one want to investigate the genome, transcriptome, or proteome?

Flow cytometry (FC) is one of the most well-known techniques for single cell analysis. We have already discussed a flow cytometer derivative, the FACS machine. A sample cell suspension is injected into a cone shaped cavity, filled with pressurized sheath fluid. The pressurized sheath fluid constrains the injected sample to a single laminar flow stream. This fluidic system ensures that individual cells flow past a laser. As the laser interrogates each cell, the light scatter is recorded in a direction directly opposite of the laser and a direction perpendicular to the laser incidence. These two parameters are forward scatter and side scatter, indicative of cell size and granularity. Cells labeled with fluorophores will also generate a fluorescence intensity signal.\textsuperscript{214} As such, FC allows the phenotypic characterization and sorting of single cells from cell suspension. FC and FACS are instrumental tools in single cell characterization, due to their high throughput (10^4 cells s\textsuperscript{-1}) and multiple analyte capability.\textsuperscript{30} While traditionally reserved for protein based profiling via fluorophore conjugated antibodies, new labeling strategies have enabled various FC and FACS applications. For example, FISH is typically used for detecting specific nucleic acid sequences in single cells via fluorescence microscopy. However, several groups have adopted this labeling strategy for flow cytometric RNA detection, enabling transcriptome analysis of larger sample sizes.\textsuperscript{115,216} Already discussed, Lim et al.\textsuperscript{72} developed a technique to sort single cell droplets via FACS on the basis of an in-droplet PCR assay, enabling genomic sequence cell characterization and sorting. FC and FACS do how some limitations as discussed previously, namely, cost, large sample size requirements, and required expertise. Furthermore, time-course single cells analyses are not feasible with FC or FACS.

Another technique for single cell genome and transcriptome analysis is quantitative PCR.\textsuperscript{217} One challenging aspect of implementing single cell qPCR is single cell isolation. Common techniques for doing so include micromanipulation, LCM, and FACS, each with respective advantages and disadvantages. We discussed single cell qPCR in combination with inkjet-like printing for facile single cell isolation.\textsuperscript{218} Microscopy is also frequently used for single cell analysis. As opposed to FACS or FC, microscopy has capability for time-course monitoring of a single cell. In combination with fluorescent labeling strategies, fluorescence microscopy allows for intracellular investigation against a variety of targets.\textsuperscript{218–220} Other microscopy variants, such as confocal microscopy, allow higher resolution 3-dimensional imaging for sub single cell studies. Monks et al.\textsuperscript{221} utilized confocal microscopy to study spatial distribution of surface receptors and intracellular proteins in T cells during interaction with antigen presenting cells. The study showed proteins to be spatially segregated rather than evenly distributed. Some microscopy throughput limitations have been addressed by automating both imaging and data analysis. However, a limited field of view and difficulty discerning cell boundaries can present challenges in studying intact tissues or cultures. A variety of other conventional techniques for studying single cells are discussed elsewhere.\textsuperscript{115,136,222–224}

Interest in single cell biology is abundantly clear from the literature. However, continued discovery in this field will require new tools possessing higher sensitivity and throughput. Given the size of single cells, implementing single cell assays on microfluidic platforms is particularly attractive. Upon lysis, intracellular contents diffuse and dilute. Many target biomolecules such as RNA or proteins are present in low quantities; therefore, dilution is detrimental to detection. As
already discussed, microfluidic devices can be used to confine cells and their lysates into pico or femtoliter volumes, thereby prohibiting diffusion and limiting dilution. Cells, lysates, and reagents are easily confined or manipulated across these tiny volumes via predictable laminar flows. Robust microfabrication procedures can be used to tailor device dimensions to various specimens for wide applicability. Conventional single cell assay methods are frequently plagued by the laborious task of isolating and compartmentalizing a single cell, for example, via LCM or micromanipulation. This issue is frequently contrasted by sample size requirements, e.g., FACS analysis offers facile cell isolation but requires large sample size. As discussed previously, microfluidic platforms are capable of high-throughput cell compartmentalization even with small sample sizes.

The field of on chip single cell analysis is rapidly expanding, and for further discussion, we refer the reader to several recently written reviews.\textsuperscript{16,225–230} In the next sections, recent experiments regarding on chip single cell analysis for genome, transcriptome, and proteome analysis are reviewed, particularly highlighting approaches for high-throughput, multiplex analysis.

\textbf{Genome.} Sequencing technologies have significantly advanced decoding the human genome. However, these decoded genomes do not describe haplotype structure on homologous chromosomes. Haplotypes are combinations of single nucleotide polymorphisms that are inherited together. Haplotype structure has been linked to drug resistance and disease susceptibility and thus has significant importance for human health.\textsuperscript{231,232} While a variety of methods for reconstructing haplotypes have been demonstrated, many of these cannot determine their relative location along the chromosome or are low-throughput and expensive. As such, whole genome haplotyping for an individual has not been demonstrated. Fan et al.\textsuperscript{142} demonstrated whole genome haplotyping via a microfluidic device. The device captures a single cell, where it is microscopically identified to be metaphase. Next, the cell is moved to a chromosome release region and the enzymatically released chromosomes are partitioned into 48 separate chambers. Chromosomes are individually amplified in the chambers and the amplified products are individually collected for downstream sequencing. This approach allows for targeted gene amplification and sequencing across all chromatin and thus determination of haplotypes. The same group utilized the microfluidic device for the first ever single sperm cell genomic analysis to characterize meiotic recombination events occurring during gametogenesis.\textsuperscript{233} The device was used for loading single sperm cells into the 48 microfluidic chambers, conducting lysis and whole genome amplification. Data from 91 single sperm cells from the same individual was used for the analysis and revealed regions where chromosome recombination occurred. The authors attributed higher throughput and reduced contamination to microfluidic implementation.

The two approaches above utilized a microfluidic platform for conducting cell compartmentalization, lysis, and DNA amplification. However, sequencing was done off chip. In contrast, Abate et al.\textsuperscript{234} conducted on chip sequencing using two DNA probes, labeled with a dye pair exhibiting fluorescence resonance energy transfer (FRET). FRET is a distance dependent phenomenon, where excitation energy is transferred from a donor dye molecule to an acceptor dye molecule, thereby preventing photon emission that one would observe with a single fluorescent dye. Upon adjacent annealing to a target DNA molecule, the FRET dye pair exhibits quenching, indicating the presence of the target DNA. By varying the probe pair, various DNA targets are assayed. To conduct the analysis, the two probes are conjugated with the FRET dyes in solution, and 50 μm diameter oil-aqueous drops are generated via microfluidic drop generator. This process is performed for various DNA probes and the drops are consolidated in one vessel. DNA for sequencing is injected on chip into each drop and incubated for 1 h, followed by optical detection of the FRET signal. The system was able to distinguish instances of perfect DNA complement and single base pair mismatch.

Most single cell genomic analyses require DNA material amplification. PCR and its variants such as digital PCR (dPCR) have been implemented in microfluidic devices extensively.\textsuperscript{235,236} The benefit of microfluidic implementation is usually higher throughput. For example, Heyries et al.\textsuperscript{237} implemented dPCR in one million picoliter reactions on chip, representing an assay density of 440 000 cm\textsuperscript{-2}. Despite wide implementation, PCR based amplification can introduce amplification bias. New amplification methods, such as multiple annealing and looping based amplification cycles (MALBAC), demonstrate better amplification uniformity. Yu et al.\textsuperscript{238} implemented MALBAC in a microfluidic device for the parallel processing of 8 cells in less than 4 h, enabling higher throughput and reduced contamination risk. An advantage of microfluidic DNA amplification is reduced reactor volumes and increased surface areas. Both features result in reduced cycle time due to reduced thermal mass and increased heat transfer. Sample heating can be accomplished in several ways including resistive heaters, thermoelectric devices,\textsuperscript{238} or laser irradiation. Lagally et al.\textsuperscript{239} used a resistive thin film heater directly underneath the PCR chambers and reported cycle times as short as 30 s. Another study accomplished contactless heating via infrared irradiation.\textsuperscript{240} Isothermal DNA amplification methods utilizing enzymes have also been developed and a recent review discusses their microfluidic implementation.\textsuperscript{241} For further discussion of microfluidic DNA amplification, we refer the reader to additional focused reviews.\textsuperscript{242,243}

The microfluidic platforms for DNA analysis discussed above typically executed only one step on chip, such as chromosome manipulation\textsuperscript{142} or single cell compartmentalization and lysis.\textsuperscript{233} Sequencing was performed off chip. Similarly, experiments reporting microfluidic DNA amplification usually utilize an external microscope for quantifying PCR products.\textsuperscript{236,237} Integration of processing and detection is a separate challenge in microfluidics research and will be briefly discussed later. Nevertheless, microfluidic systems confer significant advantage over conventional technologies. The reduction in size allows for efficient heat transfer, shorter DNA amplification cycles,\textsuperscript{239} and thus faster detection. Similarly, reducing reaction volume enables accurate single cell\textsuperscript{238} or single molecule\textsuperscript{245} DNA amplification and quantification, because the relative amount of target DNA compared to amplification reagents is increased. Finally, we have seen that massively parallel single cell analysis can be achieved on cm\textsuperscript{2} chips\textsuperscript{237} for high throughput.

\textbf{Transcriptome.} Single cell gene expression analysis has been broadly applied to fields such as immunology,\textsuperscript{244} neurobiology,\textsuperscript{245} developmental,\textsuperscript{53} and cancer biology.\textsuperscript{246} Amplifying RNA is a key step in gene expression analysis and microfluidic integration has enabled present in picogram quantities. The low volumes achievable via microfluidics provide easier RNA detection, typically present in picogram quantities. Early microfluidic systems utilizing milliliter sized chambers for
The field of microfluidic single cell gene expression analysis has experienced tremendous growth over the past decade. Early systems mostly performed on-chip RT and off qPCR or vice versa. However, systems capable of single cell detection, high throughput, and full integration are now common. Recently, Zhu et al. reported a droplet RT-qPCR single cell assay with integrated single cell compartmentalization, lysis, RT, qPCR, and fluorescence detection. In their work, an automated robot was used to print 2 nL single cell droplets onto a hydrophilic patterned microchip. The hydrophilic spots ensured droplet immobilization within an oil cover layer, which prevents droplet evaporation. After cell counting and thermal cell lysis is performed in 2 nL droplets, the automated robot adds 18 nL of RT reagents. After RT, 30 nL of PCR reagents are added. The significant dilution prevents RT-PCR reaction inhibition via cell lysate and buffer. The automated robot allowed for droplet generation at a rate of 20 droplets min−1. The system showed repeatability, with a 2.7% relative standard deviation for fluorescent intensity across 100 droplets and a lower detection limit of 6 copies per droplet. In contrast to automated robotics, Sun et al. reported a microfluidic device with pneumatic valves for integrated single cell compartmentalization, lysis, mRNA purification, RT, and qPCR. Purification was enabled by the use of oligo(dT) labeled beads. Similarly, Han et al. utilized oligo(dT) functionalized magnetic beads for mRNA capture, RT, and gene amplification on a microfluidic chip. Cell lysis, however, was performed off chip. The high-throughput capability of microfluidics for single cell gene expression analysis was demonstrated by White and colleagues who built a chip for integrated cell capture, lysis, RT, and dPCR. Complementary DNA from a single cell is partitioned into a 1020 chamber dPCR array. Each chamber is 25 nL in volume. Two hundred single cells are analyzed each run, totaling to 204 000 PCR reactions, corresponding to 118 900 reactions cm−1. In comparison to other methods, this study utilized single cell dPCR (in contrast to qPCR), which is better suited for quantifying lower abundance transcripts and does not require reference standards. Microfluidic integration lends better economy of scale and a streamlined workflow to dPCR.

The preceding discussion focused on qPCR for single cell transcriptome analysis. Despite its long history, qPCR remains an indispensable tool in genomic and transcriptomic research. However, whole transcriptome single cell RNA sequencing, developed in 2009, has shown great potential in transcriptomic research. As we saw with qPCR; after an analytical technique is established, researchers are often interested in multiplex and high-throughput implementation. That is, how can we apply the technology to thousands of cells, many target RNAs, while minimizing labor and financial cost? Single cell RNA sequencing technology is no different and microfluidics enables its high throughput application. Recently, Klein et al. developed a microfluidic platform, termed “inDrop”, for multiplex RNA analysis of thousands of single cells simultaneously. Single cells, lysis buffer, RT reagents, and barcoded primers are encapsulated in droplets on-chip. Within each droplet, a single cell is lysed, and complementary DNA (cDNA) is synthesized using the barcoded primers. Then, all of the droplets are ruptured and the cDNA material from all single cells is combined for amplification and sequencing. The key innovation is the barcoded primers. Each droplet contains a diverse set of primers for in-depth mRNA sequencing, but each droplet’s primers are uniquely labeled with one of 147 456 barcodes. The current library of barcodes allows for 99% of 3 000 single cells to be uniquely labeled and the library can be easily expanded. Therefore, sequencing reads can be tracked back to the cell of origin. Using inDrop, the researchers processed single cells at a rate of 4 000–12 000 cells h−1. The authors also note that this technology should be adaptable to RNA processing and integration with droplet sorting chips. However, the method did boast a low mRNA capture efficiency, making it unsuitable for detection of low abundance transcripts (<20–50 transcripts/cell). Furthermore, the random barcoding strategy does not associate a barcode to cell type, size, location, or any other identifier.

Similarly, Macosko et al. developed “Drop-seq”, a microfluidic droplet approach for single cell gene expression analysis. The two approaches diverge in their barcoded bead library and its preparation. Drop-seq boasts approximately 16 million unique labels compared to inDrop’s 147 456. Therefore, Drop-seq is capable of processing larger samples. However, inDrop has a higher single cell capture efficiency, enabling analysis of small samples, which are hard to come by. Furthermore, inDrop accomplishes RT in droplets while Drop-seq does so in bulk after the droplets are broken. Rotem et al. demonstrated another microfluidic droplet based approach to labeling mRNA prior to sequencing. This method electrically coalesced two adjacent droplets, each containing either the mRNA from a single cell lysate or the unique labels. Reverse transcription reagents were injected post droplet coalescence.

The microfluidic methods discussed above are addressing the current challenges in single cell gene expression analysis. One challenge is increasing sensitivity and accuracy. This is especially important for low-abundance transcripts where it is difficult to differentiate signal and experimental noise. We previously discussed a microfluidic platform for single cell RT and amplification; the authors attributed efficiency and reproducibility to low volume, microfluidic liquid handling. Another challenge is increasing throughput. Transcriptional profiling of rare cell types, e.g., tumor cells, that are present in larger cell populations requires rare cell type enrichment and/or processing larger samples of single cells. Furthermore, in order to accurately capture stochastic effects, it is likely that many cells need to be analyzed simultaneously. Several recent droplet microfluidic platforms show great promise for increasing throughput.

Proteome. The central dogma of biology describes the movement of hard-wired genetic information from the genome to the transcriptome and translation to the proteome. To understand the holistic flow of information, researchers often need to study the proteome. Furthermore, given that the proteome defines the cell’s functional state, its study is a high priority. Additionally, high mRNA abundance does not always correlate to high protein abundance, justifying the need for single cell proteomics. Microfluidic devices have been used for measuring single cell protein expression. Lab on a chip technology confers several advantages for single cell protein analysis: decreased sample loss and contamination and lower detection limits. All are critical attributes for single cell protein
assays because unlike DNA or RNA, proteins cannot be amplified.

Recently, several microfluidic platforms have been reported for studying single cell enzyme activity and function. For example, Xu et al.\textsuperscript{254} created a microfluidic, multifunctional pipet to study alkaline phosphate (AP) activity within a single cell as a function of local cell temperature. The device is used to hydrodynamically confine a single cell in an aqueous environment, while an IR laser manipulates the environmental temperature. A confined cell was permeabilized and exposed to an enzyme substrate, fluorescein diaphosphate, which undergoes reaction with AP to produce a fluorescent product. The fluorescence signal indicated enzyme activity. In contrast to traditional enzyme activity assays, this approach allows for studying enzyme activity without cell lysis or enzyme extraction. Son et al.\textsuperscript{255} used a microfluidic device with a micropatterned, antibody functionalized hydrogel array to capture single cells and study the activity of secreted protease. The group used protease cleavable FRET peptides for measuring proteins secreted by single circulating tumor cells confined in microwells. While the proteomic approaches discussed above measured one target protein, Hughes et al.\textsuperscript{257} developed a novel microfluidic platform for multiplex single cell protein analysis. The platform is an innovative application of traditional Western blotting. A 30 μm thick polyacrylamide gel is molded with 6720, 20 μm diameter, wells. Single cells are seeded into the wells via gravity and the cells are lysed to release intracellular proteins. Electrophoresis for each cell is achieved via an electrode that spans the entire gel. Then, antibody probes are diffused through the gel followed by imaging. The group demonstrated multiplexed measurement of 48 protein targets.

Since cascading events lead to protein production, there is growing interest in simultaneously quantifying multiple target types: proteins, metabolites, DNA, and RNA. The challenge to do so is because of the targets' chemical diversity, each requiring different preparation and detection protocols. Xue et al.\textsuperscript{258} developed a “single cell barcode chip” (SCBS) for quantitative, single cell protein and metabolite measurement. The SCBS chip houses 310 1.5 nL chambers, which are used for loading and lysing a single cell via a valve to an adjoining lysis buffer compartment. Each chamber also contains both the metabolite and protein binding assays in a barcode format. The chip was tested on 200 single cells from a neurosphere tumor model. The test compared metabolite and protein levels and their interactions following 24 h drug exposure. Wu et al.\textsuperscript{259} developed an automated microfluidic platform for detecting miRNAs, mRNAs, and proteins via in situ hybridization, immunostaining, and on chip flow cytometry. The entire chip is capable of testing 10 different conditions in parallel, requiring only 270 nL of reagent per condition, and the protocol is performed in less than 8 h.

Currently, microfluidic single cell protein analysis is scarce when compared to single cell analyses of the genome and transcriptome. The microfluidic platforms discussed, with one exception,\textsuperscript{257} are not multiplexed. In contrast, traditional mass spectrometry proteomics allow for detection and quantification of many target proteins. Some groups have packaged traditional proteomics into a microfluidic format, but single cell analysis yields limited proteome coverage due to processing difficulties.\textsuperscript{260,261} Therefore, microfluidic platforms that measure multiple targets at the single cell level are of great interest.

Several recently published reviews further discuss microfluidic single cell proteomics.\textsuperscript{259,262,263}

## CONCLUSIONS AND FUTURE OUTLOOK

Single cell biology is a relatively new and rapidly expanding field. Therefore, it is difficult to predict the field’s future focus and applications as new technologies emerge. Nevertheless, it is clear that innovative microfluidic platforms have and will continue to play an important role in both single cell biology and biology in general. What can we expect from next generation microfluidics? This review has discussed microfluidic devices used for tissue dissociation and tissue dissociation’s relative nascent compared to cell sorting applications. Therefore, it is likely that more microfluidic devices for tissue dissociation will emerge. However, one would not expect microfluidic tissue dissociation to reach the maturity of microfluidic cell sorting in the near future. This is because conventional tissue dissociation is cheap and effective for most research applications utilizing animal tissues. An exception may be the processing of rare clinical tissue samples, which could benefit from tightly controlled microscale processing. In contrast, cell sorting was and continues to be driven by economic factors and the need to isolate rare cell populations such as CTCs and those lacking biomarkers, tasks unfulfilled for conventional methods. Rapidly growing research and clinical applications, such as CTC based diagnostics and stem cell therapies, require cell sorting. In turn, these applications generate a necessity for facile, low cost, and high-throughput cell sorting systems. These are key advantages of microfluidic devices, and as such, we expect microfluidic cell sorting to continue growing. To drive adoption of microfluidic cell sorting over traditional FACS, the focus should be on increasing throughput and multiplexing capability, while simultaneously maintaining facile operation. Microfluidic cell sorters with competitive throughput (10⁴ cells s⁻¹) are more frequent and further increases should promote wider adoption. As discussed, microfluidic cell sorters are being coupled to single cell genomic and proteomic assays allowing for identification, isolation, and cloning of rare cells. This may have important implications for production of biologics. Microfluidic CTC isolation is a rapidly growing derivative of cell sorting. Commercializing devices offering higher throughput for clinically relevant specimens will enable rapid and cheaper CTC isolation, propelling cancer research and personalized therapy. Droplet microfluidics have experienced tremendous growth and have proven to be highly advantageous and flexible for single cell compartmentalization. In the future, one would expect droplet microfluidics to become a laboratory standard, enabling high throughput and multiplex drug screening, clonal selection, and whole genome sequencing.

This review discussed genomic analysis on microfluidic chips, and it was observed that for sequencing experiments, the workflow is performed on chip up to DNA amplification. Sequencing is usually accomplished off chip via standard protocols. Nevertheless, one study was discussed that attempted on chip sequencing via a FRET based detection scheme. This approach, however, is limited in depth. Therefore, one can anticipate that future efforts will be directed at full workflow integration, from sampling to sequencing. In fact, this has been a long sought goal in microfluidics research, the micro total analysis system (μTAS). Microfluidic transcriptome analysis is in a similar position. While RT-qPCR transcriptome analysis has been fully miniaturized, transcriptome wide studies
via sequencing are not completely integrated. We discussed two droplet microfluidic devices, which enable very high throughput and multiplexed cDNA preparation from single cells. The massive amount of data generated by these approaches will allow mapping of complex heterogeneous tissues and will most likely uncover previously unrecognized cell types and states. However, computational biology and computer science will have to keep pace with these developments in order to decipher the data. Proteomics at the single cell level has been demonstrated in diverse formats. However, the methods highlighted in this review target only one protein or a few proteins of interest. Similarly, microfluidic packaged mass spectrometry has not attained proteome coverage equivalent to conventional mass spectrometry techniques. Therefore, future efforts in microfluidic proteomics should look to expand multiplex capabilities.

The modern era of single cell biology is particularly exciting. Technological advances in amplification, sequencing, and microfluidics have allowed us to probe the fundamental unit of life, the cell, in unprecedented ways. Single cell biology is sure to have a lasting impact in cancer, immunology, developmental biology, and stem cell research. Even though this paradigm shift in biology is relatively recent, we are curious to know what the next frontier is. Currently, researchers study single cells by removing cells from their native microenvironment and into alien buffers and tools. As such, the impact of the cell’s native tissue is completely lost and the cell’s behavior is irreversibly changed. It is widely accepted that cells’ spatial context has profound implications. For example, tumor microenvironments are known to impact therapeutic response. This missing dimension has not gone unnoticed. Recently published experiments describe transcriptome profiling of single cells in their native context via various methods; in situ hybridization, photoactivated mRNA capture tags, in situ single cell RNA sequencing, spatial tissue sampling via LCM, computational approaches, tomography, and combinatorial RNA labeling with sequential imaging. Nevertheless, these pioneering methodologies have their limitations. Tomography is high throughput with respect to the number of cells and genes but lacks cellular resolution. LCM, mRNA capture, and in situ sequencing achieves single cell resolution but is low throughput with respect to the number of cells. Automated imaging and in situ hybridization are higher throughput at single cell resolution but do not provide the depth of sequencing. In this sense, computational approaches integrating both sequencing and imaging may be able to balance depth and throughput. Still, all the aforementioned techniques were demonstrated exclusively for transcriptome analysis. If the past is indicative of the future, these works only mark the beginning of a new frontier; spatial single cell omics and innovative microfluidic instrumentation will open the floodgates.

AUTHOR INFORMATION

Corresponding Author
*E-mail: a.koppes@neu.edu.

Notes
The authors declare no competing financial interest.

Biographies
Sanjin Hosic earned his Bachelor’s degree in Chemical Engineering from Northeastern University in 2011. From 2011 to 2013, Sanjin developed carbon nanotube (CNT) materials and CNT manufacturing techniques for next generation nonvolatile memory applications at Nantero, the first company to introduce carbon nanotubes into a semiconductor fabrication facility. From 2013 to 2014, Sanjin developed purification processes for preclinical, clinical, and commercial stage drugs and supported discovery efforts via impurity purification and scale-up at Cubist Pharmaceuticals. In 2014, Sanjin returned to Northeastern University to pursue a Ph.D in Chemical Engineering. His current research focuses on developing new micro fabricated systems for single cell analysis.

Shashi K. Murthy is a Professor of Chemical Engineering and Faculty Fellow at the Barnett Institute of Chemical and Biological Analysis at Northeastern University where he also serves as the Founding Director of the Sherman Center for Engineering Entrepreneurship Education. His current research applies microfluidic methodologies to address problems in immunology, stem cell biology, and proteomics. Murthy received his B.S. from The Johns Hopkins University, his Ph.D. from the Massachusetts Institute of Technology, and performed postdoctoral research at the Harvard Medical School and Massachusetts General Hospital. He currently serves on the Features Panel of Analytical Chemistry.

Abigail N. Koppes is an Assistant Professor of Chemical Engineering at Northeastern University. Her research focuses on creating new platforms for autonomic nervous system control, with a particular focus on the impact of the enteric nervous system on the intestinal crypt-niche. Dr. Koppes received her B.S. and Ph.D. from Rensselaer Polytechnic Institute and performed postdoctoral research as an NSF ADVANCE Fellow with Northeastern University, Harvard Medical School, and the Massachusetts Eye and Ear Infirmary. She is also currently a visiting scientist at the Massachusetts Institute of Technology, Department of Bioengineering.

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