In Vivo Fluorescence Imaging and Tracking of Circulating Cells and Therapeutic Nanoparticles

A Dissertation Presented

By

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ABSTRACT

Noninvasive enumeration of rare circulating cells in small animals is of great importance in many areas of biomedical research, but most existing enumeration techniques involve drawing and enriching blood which is known to be problematic. Recently, small animal “in vivo flow cytometry” (IVFC) techniques have been developed, where cells flowing through small arterioles are counted continuously and noninvasively in vivo. However, higher sensitivity IVFC techniques are needed for studying low-abundance (<100/mL) circulating cells.

To this end, we developed a macroscopic fluorescence imaging system and automated computer vision algorithm that allows in vivo detection, enumeration and tracking of circulating fluorescently labeled cells from multiple large blood vessels in the ear of a mouse. This technique –“computer vision IVFC” (CV-IVFC) - allows cell detection and enumeration at concentrations of 20 cells/mL. Performance of CV-IVFC was also characterized for low-contrast imaging scenarios, representing conditions of weak cell fluorescent labeling or high background tissue autofluorescence, and showed efficient tracking and enumeration of circulating cells with 50% sensitivity in contrast conditions degraded 2 orders of magnitude compared to in vivo testing supporting the potential utility of CV-IVFC in a range of biological models.

Refinement of prior work in our lab of a separate rare-cell detection platform - “diffuse fluorescence flow cytometry” (DFFC) - implemented a “frequency encoding”
scheme by modulating two excitation lasers. Fluorescent light from both lasers can be simultaneously detected and split by frequency allowing for better discrimination of noise, sensitivity, and cell localization. The system design is described in detail and preliminary data is shown.

Last, we developed a broad-field transmission fluorescence imaging system to observe nanoparticle (NP) diffusion in bulk biological tissue. Novel, implantable NP spacers allow controlled, long-term release of drugs. However, kinetics of NP (drug) diffusion over time is still poorly understood. Our imaging system allowed us to quantify diffusion of free dye and NPs of different sizes in vitro and in vivo. Subsequent analysis verified that there was continuous diffusion which could be controlled based on particle size. Continued use of this imaging system will aid optimization of NP spacers.
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Chapter 1 – Introduction

1.1 Dissertation Overview

This dissertation details the candidate’s contributions in two major areas of research: i) development and validation of methods for detection, enumeration and tracking of rare circulating cells in the peripheral blood of small animals, and ii) measurement and quantification of nanoparticle diffusion from functionalized spacers in diffusive media. The relevant introductory material for these two research areas are described in this section. In section 1.2, fundamentals of optical imaging of biological tissue are presented. In sections 1.3-6, we describe existing technologies for circulating cells enumeration, their limitations, and the motivation for the present work. In section 1.7, background related to implantable spacers and the motivation for the near infrared imaging of nanoparticle diffusion is discussed.

1.2 Biological Tissue Optics – Red and Near Infrared Sensing and Imaging

Fluorescence imaging is advantageous in small animal preclinical research because of the relatively low cost of the instrumentation involved, the ability to perform serial imaging on individual animals, and the broad availability of biocompatible cell labeling techniques (red fluorescent proteins, Alexa-fluor dyes, and Cyanine dyes) and fluorescent nanoparticles. All of the optical instruments described in this dissertation
employ optical imaging at red or near infrared (NIR) wavelengths, which has significant advantages over visible or mid-infrared imaging [1]. As shown in figure 1, red and NIR light experiences significantly less absorption and scatter - primarily due to hemoglobin, water, and melanin absorption - in biological tissue compared to visible and infrared light [2-4]. For this reason, the red and NIR region (corresponding to approximately 650 nm to 1.3 μm) of the electromagnetic spectrum is often referred to as the “diagnostic window.”

![Figure 1](http://spie.org/x35504.xml)

Figure 1- The relative absorbance of major chromophores in biological tissue, displaying the optical window where light absorbance is minimal. (source: [http://spie.org/x35504.xml](http://spie.org/x35504.xml))

In the 650-850 nm range, the mean absorption length of a photon is on the order of several centimeters, making NIR fluorescence imaging applicable for small animal preclinical imaging studies. However, the mean distance between scatter events in tissue
is on the order of only 100 μm, so that the albedo of biological tissue is close to 1. This presents a major challenge for imaging with light in bulk tissues in vivo.

1.3 Preclinical and Clinical Applications Involving Circulating Cells

The primary area of research for this dissertation is development of new methods for detection, enumeration, and tracking of rare circulating cells in the bloodstream. There are many areas of preclinical biological research where this is of interest and, more generally, clinically. Circulating tumor cells (CTCs) are a widely studied example where CTC concentrations are between 1-100 cells/mL during cancer metastasis. Other important examples include hematopoietic stem cell transplants, immunology, reproductive medicine, and tissue and organ transplants. These are described in more detail here.

1.3.1 Circulating Tumor Cells

Circulating tumor cells (CTCs) are cells that have shed from primary tumors and have entered the bloodstream (“intravasation”). This is a critical step in cancer metastasis, which is responsible of >90% of cancer deaths in humans. CTCs are emerging as an important cancer biomarker, since CTC numbers correlate with metastatic progression, prognosis, and treatment efficacy for many types of cancer [5-14]. For example, two clinical trials (M. Cristofanilli et al and S. Riethdorf et al) reported that
over 61% of metastatic breast cancer patients had at least 1 CTC in 7.5 mL of blood compared to 1% of control patients [14, 15]. A number of other studies have demonstrated self-renewal capabilities of CTCs, as well as resistance to anti-cancer treatment [7, 12, 16]. However, the exact role of CTCs as the initiator, indicator, or by-product of metastasis has yet to be determined [6]. Therefore, detection, enumeration, and profiling of CTCs are critical for study of the basic biological process of metastasis, and as a biomarker in cancer staging and management. The extremely low (1 CTC per billion blood cells) number of CTCs in circulation during metastasis presents a significant detection problem, both for basic enumeration, as well as for more advanced characterization of CTCs [17-23].

Animal models of cancer are used frequently in the basic study of disease development, progression, and for preclinical testing of new treatment modalities. Specifically, enumeration of CTCs in small animal models has allowed staging of metastasis in mice, tracking of the development of metastasis with different cancer models (e.g. breast cancer, prostate cancer, and melanoma), and evaluation of treatment strategies [24-26]. Biomolecular processes of metastasis are continually being studied in order to better understand the mechanisms responsible for tumor cell intravasation, circulation, and extravasation [15, 18-19]. For example, it is still unclear if the release of individual cells into circulation is sufficient for metastasis, or if clumps of CTCs - with a combination of epithelial-to-mesenchemal and mesenchemal-to-epithelial properties – is
required [6, 24]. Through better understanding of metastatic processes, new drug targets delivery methods, and treatment strategies may be developed.

1.3.2 Multiple Myeloma Cell Mobilization

Multiple Myeloma (MM) is a cancer of plasma cells that originate in the bone marrow. When they become malignant, MM cells accumulate through (re)circulation and (re)homing in the bone marrow and interfere with normal blood cell production. Chemotherapeutics (e.g. bortezomib or doxorubicin) are normally used to treat MM to remission, but MM is currently incurable, since MM cell reside in protective bone marrow niches leading to eventual relapse. As such treatment of MM “minimal residual disease” is an active area of research. One strategy (currently under clinical trials) is to co-treat with a mobilizing agent such as AMD3100 (“Mozibil”), which interferes with homing receptors (CXCR4) of MM cells. This prevents homing of circulating MM cells to the protective (therapeutically resistant) bone marrow niche and encourages MM cells in the niche to egress into circulation where they are more vulnerable to treatment. This mobilization effect has been studied and quantified in small animal models. In preclinical models with MM.1s cells, combination treatment of MM with bortezomib and AMD3100 resulted in an increased number of circulating apoptotic cells and an increased survival time for mice [27-29]. However, extremely rare MM circulating cells associated with Minimal Residual Disease are on the order of ~100 cells per mL of
circulating blood and are currently extremely difficult to detect with flow cytometry or in vivo flow cytometry methods (see below). As such, new methods for detecting and enumerating circulating MM cells are needed for development of better treatments for MM.

1.3.3 Hematopoietic Stem Cell Transplants

Hematopoietic stem cells (HSCs) differentiate into blood and immune cells. HSCs are found primarily in the bone marrow and in low concentrations (i.e. 1 in 100,000 blood cells) in the spleen and peripheral blood [30]. Patients with cancers of the blood or bone marrow (e.g. Multiple Myeloma (MM) and Non-Hodgkin Lymphoma) are the most common recipients of HSC transplants (HSCTs) due to the lack of immune system strength and resistance to radiation or chemotherapy. HSCT patients undergo bone marrow ablation through high-dose chemotherapy (often combined with radiotherapy) followed by a HSC graft. The graft can either be HSCs collected from the patient prior to the ablation (an autologous graft which nearly eliminates the rejection of the HSCT) or from a healthy donor (an allogenic graft which prevents reintroduction of cancer cells). Traditionally, grafts are harvested from the bone marrow in a painful procedure where the donor must be under general anesthesia, but more recently peripheral blood stem cells collected via apheresis has become widely used. A limitation to the collection of peripheral HSCs is the small circulating population; therefore,
mobilizing agents (e.g. AMD1300) are used to encourage HSCs into circulation from the bone marrow niche. Enumeration after mobilization is needed when evaluating whether a donor is a “good mobilizer” and ready for apheresis [27, 28, 31].

1.3.4 HIV/AIDS

Another area of medicine where detection of specific populations of circulating cells is important is in enumeration of T-Lymphocytes in HIV patients. T-cells in circulation are known to decline as HIV progresses to later stages of the disease. This is problematic for the health of the patient because it indicates weakening of the immune system and reduction in the ability to fight infection. As such, a major therapeutic goal in management of HIV/AIDS is restoring normal T-cell levels. The efficacy of treatments is in part determined by measurement of changes in T-cell population. M. Hellerstein et al used T-cell numbers to assess the efficacy of highly active antiretroviral therapy (HAART) for patients with low levels of T-cells [31]. The decline in T-cell count correlated with the decline in patient health, but additional markers, such as HIV-1 RNA, were found to more accurately predict disease prognosis and efficacy of treatment [32].
1.4 Cell Enumeration Techniques

Standard methods for detecting and counting circulating cells is by removal of peripheral blood (PB) samples which are analyzed with a variety of assays including flow cytometry (FC), hemocytometry, microfluidic devices, and immunomagnetic techniques [14, 33-36]. In flow cytometry, purified peripheral blood samples containing cells of interest are passed single-file through a flow stream and illuminated with laser(s) in the FC field of view (fig. 2). The sensor, usually a photo multiplier tube (PMT), detects fluorescence or forward and side cell scatter, which are affected by cell size and composition. Fluorescence is also commonly used in flow cytometry. This can be used to detect and quantify, e.g., fluorescent protein expressing cells, fluorescent labeling of specific cell receptors, fluorescent membrane dye of a population of cells of interest, and fluorescence emission according to cell states (i.e. normal, apoptotic).
Figure 2- Flow cytometer diagram showing single file analysis of cells in suspension. Properties of interest are measured for each cell, for example fluorescence or forward and side scatter. (source: http://withfriendship.com/user/mithunss/flow-cytometry.php)

In hemocytometry, small (1-10 mL) PB samples are analyzed, under a white-light microscope. The sample is placed on a slide which contains gridlines that allow the user to count cells either by eye or automatically by software.

Microfluidic devices have become more widely used in recent years as another method for circulating cell identification but are still largely limited to experimental applications. The blood sample is run through a set of micro-devices which capture and isolate the cells of interest. For example, circulating tumor cells can be captured and
isolated on a microfluidic device through nucleic acid (RNA) based detection, physical properties i.e. size or density, or antibody attraction [37] and then counted on a microscope slide [38]. Additional examples have included quantification of endothelial progenitor cells for cardiovascular disease [39], circulating tumor cells from the lung on iChip (fig. 3) [33], and T-cells for HIV diagnostics [40].

Figure 3- iChip adhesion of a CTC captured by the microfluidic device (SEM image) [33].

Last, the CellSearch system utilizes immune-magnetic techniques by attaching antibodies and ferrofluids to separate CTCs from the blood sample [14]. This method is the first FDA-approved CTC assay and provides an automated and standardized separation and counting technique for CTC counting in the clinical setting. CTCs are currently being used as biomarkers for prostate cancer [15], breast cancer [41], and colorectal cancer [42].
1.4.1 Limitations of Ex Vivo Analysis of Peripheral Blood Samples

In summary, many techniques involve PB extraction, enrichment, and subsequent ex vivo analysis and have proven to be useful in studying cell populations. Despite their utility, these techniques suffer from a number of fundamental limitations:

1. The blood sampling procedure itself may cause damage to the cell structure, biomarkers, and morphology.

2. The blood volume sampled is small which may result in the rare cell of interest to be missed. These methods then cannot effectively observe and treat early stage metastasis, septic shock, or sickle cell crisis where there is a very low concentration (1 cell/mL) of the cells of interest in the PB [43-45].

3. Extraction, transport and purification of PB are labor intensive and time consuming procedures that take hours to days which limit the timely feedback about cell counts or treatment efficacy. Moreover, during each of these steps cells can be lost or damaged.

4. In small animals such as mice, the amount of blood that can be extracted is ~100 μL per day for non-terminal studies [46] which does not allow for monitoring changes in cell populations that occur on the timescale of minutes or hours. In the case of studying very rare cells (<100/mL), the entire peripheral blood volume is typically extracted and studied after sacrificing
the mouse, thus eliminating the possibility of serial study on the same animal over time.

(5) Observation of cells in their natural environment is not possible when the blood is removed from the blood stream, for example, observation and analysis of aggregation, rolling, and adhesion.

(6) Mislabling and false counting during blood sample processing can cause many false positive errors due to contamination, amplification, and illegitimate transcription [23, 47] and false negatives errors due to the supplies used to identify a circulating cell.

1.5 \textit{In Vivo Flow Cytometry}

These limitations have motivated development of techniques that do not require drawing PB samples. “\textit{In vivo} flow cytometry” (IVFC) approaches – first developed in 2004 by Lin \textit{et. al.} - are rapidly gaining acceptance since they allow continuous, noninvasive optical detection of circulating cells in situ. Example IVFC technologies that have been reported are described here.

1.5.1 \textit{Fluorescence In Vivo Flow Cytometry}

J. Novak \textit{et. al.} developed a fluorescence microscopy IVFC system which uses a laser line focused across a small arteriole (~35 µm in diameter) in a mouse ear [48]. As
fluorescently-labeled cells pass through this excitation beam, a transient fluorescence “spike” is detected with a photomultiplier tube. Through continuous data collection, changes in cell populations that occur over time can be measured. The system has been used to study red and white blood cell circulating kinetics, prostate cancer cell kinetics, apoptotic cell clearance, and MM cell populations [22, 48-50]. The system schematic and example data are shown in fig. 4.

Figure 4- Schematic of fluorescence microscopy IVFC system. Examples of sample data "spikes".[48]

Due to the size (~15 μm diameter) and flow speed (~3-5 mm/s) of these arterioles, only about 1 μL of blood per minute is sampled. Therefore, it would take over 33 hours to monitor the total (~2 mL) blood volume of a mouse. In practice, (given an approximately 30 minute acquisition time) sensitivity of microscopy IVFC is therefore in a range of $10^3$ cells/mL.
1.5.2 Retinal In Vivo Flow Cytometry

In order to improve the sensitivity of fluorescently detected cells in vivo, C. Alt et al. developed the ‘retinal in vivo flow cytometer” that allowed sampling of blood vessels in the mouse retina [51]. In order to sample a larger blood volume, multiple vessels were sequentially monitored by implementing a circular sweeping technique of the laser spot around the optical nerve. Confocal line-scanning microscopy was used to direct the laser onto the blood vessels in the retina and define the field of view, and fluorescent light was measured with a filtered PMT. The schematic for the retinal IVFC is shown in fig. 5.

![Schematic of the Retinal Flow Cytometer.][51]

The raw data output of the retinal flow cytometer is a set of vertical lines that are mapped from the circular scanning method shown in fig. 6b. From these vertical lines (each line is one circular scan, with the vertical axis corresponding to time); analysis of
the signal corresponding to each blood vessel allowed detection transient increases in fluorescence corresponding to individual cells. Automatic cell detection and counting was also employed by extracting fluorescence differences between the background signal and transient changes.

Figure 6- Confocal fluorescence image of mouse retinal vessels visualized with the fluorescent dye Evans Blue. a) Circular scan drawn on the fluorescence image. b) Vessels appear as vertical, fluorescent structures.[51]

The system was first validated with vibrant DiD-labeled lymphocytes in mice. Retinal IVFC was later modified to allow multicolor detection of multiple cell populations. Sampling of the five artery-vein pairs simultaneously resulted in a system with approximately five times detection sensitivity improvement versus fluorescence microscopy IVFC.
1.5.3 Two-Photon In Vivo Flow Cytometry

Multiple research groups have developed two-photon fluorescence IVFC. Y. Chang et. al. developed a minimally invasive procedure where optical fibers are inserted into blood vessels [52]. The optical fiber probe employed a two channel detection system with ability to detect two different color fluorescent markers. The advantage of this system was the reduction of scattering and absorption of tissue resulting in high signal strength of fluorescent cells in whole blood to be similar to fluorescent cells in phosphate buffered saline. C. Zhong et. al. alternatively used a noninvasive approach with a microscope objective focused on a mouse ear vessel 50 μm in diameter as shown in figure 7 [53]. The system was able to detect fluorescence from two different cell populations using a femto-second NIR laser focused to a volume in the blood vessel selected to be the size of a single cell. Two beam scanning aided in the calculation of cell size and speed by using distance between fluorescence intensity peaks to normalize flow parameters. The drawback of two-photon IVFC is the high cost of the femto-second NIR laser, the area interrogated is often very small with low blood perfusion, and the possibility of missing cells circulating in larger blood vessel is high [52-54].
1.5.4 Photoacoustic and Photothermal *In Vivo* Flow Cytometry

Photothermal (PT) and photoacoustic (PA) IVFC methods are two emerging methods which reply on the principle nonradiative relaxation (thermal) of absorbed laser energy. A schematic of processes involved in these methods is shown in fig. 8 (V. Zharov *et al*).
Figure 8- Principle illumination and output signals of photothermal and photoacoustic in vivo flow cytometry.[20]

Photothermal IVFC detects the thermal signature given off by single circulating cells (e.g. red blood cells or melanoma cells) after being probed by a laser pulse. Since this signature is related to differential absorption (pigmentation) the signal is often small. Antibodies or nanoparticles are therefore frequently needed to amplify the effect [7, 20, 55, 56]. The system is then able to detect the temperature changes due to variations in the refractive index induced by the laser. This allowed for detection of white blood cells with low absorption (μₐ around 10⁻² cm⁻¹) traveling at slow speeds [7] and other cells labeled with a PT nanoprobe. Sensitivity of the PT effect is a major limitation.

PA IVFC also takes advantage of the PT effect. A high repetition rate, pulsed laser heats cells which expand the surrounding tissue leading to the generation of acoustic
waves. The signals are detected by ultrasound transducers on the skin, and the circulating cells are differentiated by the difference in background signal generated by red blood cell signals [7, 20, 43, 57-61]. The instrumentation of integrated PT and PA systems is shown in fig. 9.

**Figure 9**- Schematic of integrated flow cytometer *in vivo* combining photothermal and photoacoustic techniques. [57]

PA IVFC works better in deep tissues than PT IVFC, which is most effective in thin tissues. PA is also not sensitive to light scattering and tissue autofluorescence and can operate with relatively low laser power [7, 20]. Galanzha *et. al.* exploited the overexpression of melanin clusters in melanoma cells and a focused transducer on the
mouse aorta (where the flow rate is on the order of 1–2 mL/min) to detect circulating melanoma cells with a reported sensitivity of 0.5-1 CTCs/mL [60, 62]. While the identification of melanoma and red blood cells without labeling is a major advantage of PA IVFC, it is not effective for other, less pigmented cell types. As such, labeling of cells with gold nanoparticles has been explored, but toxicity levels and quick cleaving of NPs from the cells were both concerns. PA IVFC is also a more expensive system than traditional fluorescence methods since it requires pulsed lasers and ultrasound transducers.

1.5.5 Drawback of Current In Vivo Flow Cytometry Approaches

Despite great progress in IVFC technology development, detection sensitivity due to the low flow rate of the sample blood vessels (~1 μL/min) is a persistent limitation. For rare cells, this results in impractically long sampling times (e.g. 33 hours for whole blood circulation through a 35μm diameter blood vessel, 10 hours for whole blood circulation through a 50μm diameter blood vessel) [63]. Second, techniques require some labeling of cells of interest and further toxicity studies must be completed for many dyes or NPs. Last, most IVFC methods generate simple “spikes” when cells of interest are detected. Therefore, minimal or no information about the cells movement and behavior in vivo are recorded.
1.6 Development of New, High-Sensitivity IVFC Technology

These limitations were a major motivation for the techniques developed in this dissertation. In particular, a major focus of this work was the development of new, high-sensitivity IVFC techniques, and techniques which could yield information on cell homing and docking *in vivo*. These techniques are introduced briefly here, and are described in detail in chapters 2-4 and 5.

1.6.1 Computer Vision *In Vivo* Flow Cytometry

The technical principle behind Computer Vision IVFC (CV-IVFC) is to increase cell detection sensitivity by interrogating a larger fluorescence imaging field of view (for example, a larger region of the ear) so that more blood vessels and correspondingly larger blood volumes are optically sampled. CV-IVFC uses an EMCCD camera to image a wide field of view and generate fluorescence image sequences of circulating cells. We were able to enumerate and track circulating cells in a 5 x 5 mm$^2$ field of view in multiple frames of an image sequence. The use of “macroscopic” fluorescence imaging with a wide field of view presented two significant technical challenges. First, as we discuss, this requires relatively high laser illumination intensity and high applied detector gain which results in detection of substantial nonspecific tissue autofluorescence. Individual
cells became small relative to the total image (1–5 pixels in dimension) and of comparable intensity to noise on autofluorescence becoming difficult to distinguish from background autofluorescence and noise in a single image. We approached this detection problem by utilizing a simple *a priori* feature of circulating cells, that is, that they are in motion. Second, at low circulating cell concentrations (as we use in the experiments described herein) cells pass through the imaging field of view very infrequently, for example, on the order of one cell per minute or less. As such, a method for automated detection and counting of cells to assist a human operator was developed.

After initial proof-of-concept CV-IVFC testing and validating *in vivo*, we characterized system performance with low contrast. For example, CV-IVFC could be used with a fluorescently labeled cell line with a constitutively expressed fluorescent protein such as GFP, YFP or mCherry and antibody targeting of fluorophores both which generally yield lower contrast than Vybrant-DiD used in our *in vivo* work. These methods would yield lower contrast imaging scenarios, both from labeling and due to increased autofluorescence and reduced light penetration from the use of visible excitation and emission wavelengths.

To study this, we developed a phantom model to control the contrast in a systematic way. The contrast conditions of the phantom material were controlled by the amount of added fluorescent dye (Alexa-fluor 680, to mimic tissue autofluorescence) and added fluorescent microspheres to mimic stationary autofluorescent tissue components
(e.g. sebaceous glands) in the mouse ear. We analyzed image sequences obtained with these phantoms and showed that we were able to closely replicate conditions observed *in vivo*.

We also changed the brightness of microsphere intensities to mimic the range of labeling intensities expected *in vivo* with other labeling techniques in optical phantoms with varying levels of autofluorescence and embedded with fluorescent microspheres. The performance of CV-IVFC with 27 phantom and microsphere combinations was characterized and compared to the cell and mouse results. Overall, we determined the CV-IVFC retains 80% detection sensitivity when contrast was 38 times lower than our proof-of-concept contrast with Vibrant-DiD labeled cells. These results imply that CV-IVFC can be used with many other cell models.

### 1.6.2 Diffuse Fluorescence In Vivo Flow Cytometry

An alternative method for increasing the blood sampling volume in IVFC was first developed by E. Zettergren *et. al.* in our lab. In this work, a system to interrogate larger circulating blood volumes in a limb of a mouse, where circulating blood volumes are on the order of 0.2-0.5 mL per minute, was developed. This technique was termed “diffuse fluorescence flow cytometry” [64], and the first prototype system is shown in fig. 10. A ring with an array of optical fibers encircled a mouse limb or tail (2-3 mm in
diameter) and allowed detection of diffuse fluorescent light from individual circulating cells.

Figure 10- a) Schematic and b) photograph of the DFFC.[64]

Given the high blood flow rate in the tail or leg [64], in principle the whole mouse blood volume could be sampled in less than 10 minutes. As cells moved through the field of view, a “spike” (similar to that in the fluorescence microscopy IVFC) was measured on 6 detector channels (fig. 11). In addition to detection, axial localization of the cell was important to avoid double counting cells that are returning through the vasculature in adjacent blood vessels of the mouse tail (ideally, the system would determine cell counts per blood vessel, as well as yield cell direction and speed[65]). The locations of the detectors and lasers were used to determine the location of the cell by observing the difference between cell spike heights between detectors. This was
performed successfully in phantoms and in vivo with fluorescently-labeled Multiple Myeloma cells as shown in fig. 11.

Figure 11- Localization of the fluorescent microsphere in phantoms. A) and b) are two example traces displaying spikes as microspheres passing through the field of view. C) is the phantom tested with two labeled locations of tygon tubing where microspheres are pumped through. d) and e) show the tomographic reconstruction of microspheres location in the DFFC field of view.[65]

A second DFFC prototype was developed by N. Pestana et. al. in our group and further improved the sensitivity of the system using a multi-wavelength design [66]. The instrument also used an “open-optics” configuration that removed the optical fibers to improve geometric collection efficiency. The schematic is displayed in fig. 12.
The multi-wavelength design also allowed removal of motion artifacts that are frequently observed in mouse limbs. By choosing two filters, one that matches the peak of fluorescent dye and one “out of band”, the background noise could be subtracted (after being weighted appropriately) from the fluorescent signal and motion was removed. Fig. 13 shows the resulting data spikes. The sensitivity of the multi-wavelength, open optics DFFC system was 1 cell/mL or less in circulating blood. Part of this dissertation has continued development of a frequency modulated open optics DFFC and is introduced in section 1.6.3 and chapter 5.
Figure 13-Example data trace acquired at 700 nm (a) from a flow phantom oscillating at 1.8 Hz with 2 mm amplitude. The effects of movement artifacts obscure fluorescent spikes from the microspheres. (b) the background signal acquired at 800 nm, (c) weighted subtraction of the out-of-band signals allowed effective removal of the movement artifacts, allowing better visualization of spikes, and (d) replotting the 700 nm data trace with a 1.8 Hz-bandpass filter applied in post-processing, demonstrating the accuracy of the motion artifact removal approach.[66]

1.6.3 Frequency Modulated Open Optics DFFC Instrument

We continued to build on prior work by Pestana et. al. related to DFFC. The frequency modulated (FM) -DFFC has 4 open-optic detector arms with frequency modulated lasers [66]. Each arm contains an optical filter, a PMT, and filtration and amplification circuitry. The demodulation circuitry was optimized to include signal filtering and amplification and was assembled for each of the four detector arms to
cleanly separate the two frequencies. Two lasers were modulated at different frequencies and illuminated the sample from opposite sides. This will allow simultaneous detection and discrimination of fluorescence generated from each laser.

1.7 Near-Infrared Fluorescence Imaging of Prostate-Cancer Nanoparticle Functionalized Spacers

A separate focus of this dissertation was performed in collaboration with Prof. Sridhar et. al. and the IGERT Nanomedicine program. We developed new NIR imaging and image processing techniques to support development and optimization of new drug delivery spacers. Specifically, we developed an imaging platform that allowed repeatable imaging and measurement of nanoparticle diffusion \textit{in vitro} and then from functionalized spacers \textit{in vivo}. This section introduces the motivation behind the implantable spacers and how observing the diffusion assists in finding an optimal NP solution.

1.7.1 Implantable Spacer Applications

Implantable drug delivery systems are an appealing approach to delivering a number of different classes of drugs that cannot be effectively delivered via more common routes such as intravenous, oral, or topical administration. An implantable drug reservoir can increase the bioavailability of drug without the need for multiple injections [67]. Many current drug reservoirs must be removed through a second procedure, but
more recently biodegradable properties have been applied to the drug delivery field. The unknown degradation rate of the implant combined with the diffusivity of the drug makes predicting the release profile of the drug in vivo a challenge.

For example, polymer-Dexamethasone implants are used for intravitreal dosing for persistent macular edema, but detection of drug levels was performed using common techniques such as liquid chromatography-tandem mass spectrometry, which greatly limits the amount of data collected to a single time point, i.e. when the animal is sacrificed [68]. Similar post-sacrifice analysis techniques were used in determining diffusion profiles of clinically used biodegradable implants in delivering insulin, steroids, chemotherapeutics, antibiotics, or analgesics [67]. Another important application of local dosing using an implant is brachytherapy. During brachytherapy, clinicians space brachytherapy seeds with inert biocompatible spacers for controlled dosing of the tumor. Localized delivery of a chemotherapy agent in a functionalized drug reservoir would eliminate the high systemic toxicities while allowing for an effective and concentrated dose directly at the tumor site. The “Implantable Nanoplatform for Chemo-Radiation Therapy” (INCeRT) spacer fabricated by Sridhar et. al. is loaded with nanoparticles (NP) that encapsulate a chemotherapy drug and provides slow-release of the drug (e.g. Docetaxel) [69].
1.7.2 Nanoparticle Diffusion Quantification with NIR Imaging

In order to better understand the release profiles of drugs from nanoparticles and implantable spacers, an imaging system that allows quantification of the diffusion \textit{in vivo} is needed. Nanoparticle (NP) diffusion is of interest because it provides a dual drug release system of the nanoparticle from the spacer followed by the drug from the nanoparticle. We believe this release system would allow for drug delivery internally over long periods of time, but the optimal size for long term release is not known. We built a near-infrared fluorescence imaging system to analyze the diffusion of a fluorescent dye (drug model) over time from nanoparticles and spacers to better understand the diffusion kinetics in phantoms and in mice. Near infrared fluorescence imaging was chosen in order to take advantage of the minimal light attenuation in tissue at these wavelengths. Fluorescence imaging also reduces the exposure to harmful radiation and provides a low-cost approach compared to MRI, PET, and SPECT.

The broad-field fluorescence imaging system we developed allowed us to observe and systematically quantify free dye and NP diffusion both in phantoms \textit{in vitro} and in mice \textit{in vivo}. We first performed \textit{in vitro} studies with free dye and NPs of different sizes (30 nm and 200 nm) in agar gel phantoms and analyzed the diffusion over time. We then performed studies for 2 weeks in mice tracking and analyzing the diffusion \textit{in vivo}. We were able to observe size dependent diffusion characteristics and provide a platform for future study and optimization of nanoparticle diffusion.
1.8 Thesis Organization

This dissertation is organized as follows:

In Chapter 2, the design of the CV-IVFC instrument and the computer vision algorithm is presented. The results of initial testing and validation in phantom models in vitro and in mice \textit{in vivo} are presented. These findings were published in Cytometry A in 2013 [70].

In Chapter 3, we describe development, validation, and characterization of improved, tissue-mimicking flow phantom models for CV-IVFC. These studies were presented at the International Conference of the IEEE Engineering in Medicine and Biology Society 2014 [71].

In Chapter 4, we used phantoms and fluorescent microspheres to characterize performance of CV-IVFC in low contrast conditions. This study was published in the Journal of Biomedical Optics in 2015 [72].

In Chapter 5, work on the FM-DFFC instrument development and demodulation circuit testing is presented.

In Chapter 6, instrumentation and image processing methods to quantify nanoparticle diffusion using fluorescence imaging in phantoms and \textit{in vivo} is presented. This work was presented and published in proceedings at the Northeast Bioengineering Conference 2014 [73]. Results from these studies were published (along with the
description of the INCeRT platform) in the Journal of Radiation Oncology, Medicine and Physics in 2015 [69]. A second manuscript presenting experiments where diffusion properties of NPs and spacers are investigated has also been submitted to the International Journal of Nanomedicine.

Finally, in Chapter 7, conclusions and future work from this dissertation will be presented.
Chapter 2 – Computer Vision In Vivo Flow Cytometry

Tracking and enumeration of rare circulating cells in small animals is important in many areas of preclinical biomedical research (see section 1.3 in the Introduction). Noninvasive and highly sensitive detection is needed in the study of cancer cell metastasis, immunology, and stem cell therapeutics. Normally, peripheral blood (PB) samples are drawn from the tail and analyzed with conventional techniques such as flow cytometry, hemocytometry, and microfluidics. These methods are limited by errors associated with handling, purification, and storage of PB samples. Moreover, PB removal from mice is limited to ~100μL per day which does not allow rapid changes in cell populations to be measured. In contrast, in vivo flow cytometry (IVFC) allows continuous and noninvasive monitoring of circulating cells. Fluorescence microscopy IVFC, for example, excites fluorescently labeled cells in a small blood vessel (arteriole) with a focused laser slit, and fluorescence “spikes” are detected with a photon multiplier tube detector. While useful, the sensitivity limit of these systems is about $10^3$ cells/mL which is insufficient for many applications involving rare circulating cells. The computer vision (CV) - IVFC approach described in this chapter solves this problem by imaging a 5x5mm$^2$ field of view, so that 3-4 large artery-vein pairs and the surrounding capillary bed are optically sampled. Two technical challenges arose with this wide field imaging approach: 1) high intensity laser illumination and detector gain resulted in significant levels of background autofluorescence, and 2), as a result, imaged cells were small (1-5
pixels in size) and of similar intensity and size to noisy background pixels. Since we were primarily interested in using this system for low abundance cells, in the in vivo studies below, we injected only small numbers of circulating cells. At these concentrations about 0.5 cells/min or less passed through the imager field of view. This motivated development of an automatic detection algorithm.

In this work, we approached this problem by utilizing a simple a priori feature of circulating cells, that is, that they are in motion. Circulating cells appear in multiple frames of an image sequence. As we demonstrate, this simple property could be exploited to identify cells in noisy image sequences. It is important note that the idea of computer vision “cell tracking” or “cell counting” is not novel [75-80]. However, previously reported methods typically identify clearly defined objects with strong background contrast, for example, of cells in culture on a microscope slide. In this case, our objective was to image circulating cells in situ with a widefield imager so that they appear as only a small cluster of pixels with comparable intensity to the noise on the autofluorescence background. Therefore, existing software packages for identifying or tracking cells (e.g. Imaris, Bitplane [81-83] or Volocity, Improvision [84-86]) were generally not suitable for tracking small moving cells in widefield fluorescence image sequences such as those presented here. This motivated us to develop a new computer vision algorithm as described in this work.
In this chapter, we describe and validate our rare-cell “computer vision in vivo flow cytometry” (CV-IVFC) method, first in flow phantom models and then in nude mice in vivo. As we demonstrate, this method allowed us to sample and detect circulating cells at very low concentrations. Based on reported blood flow rates of vasculature in the ear [49, 63, 87], we estimate that the instrument samples about 10–12 μL of PB per minute. We tested CV-IVFC in nude mice injected with Vybrant-DiD-labeled multiple myeloma (MM) cells. As we quantify in detail, this algorithm enabled high detection sensitivity with a small false alarm rate (when compared to human operator), yielding an overall estimated system sensitivity of 20 circulating cells/mL. CV-IVFC represents an entirely new, high-sensitivity but easily implementable approach to rare cell sensing and enumeration in preclinical small animal models.

2.1 Materials and Methods

2.1.1 Fluorescence Macroscope Design

A schematic diagram and photograph of the video-rate fluorescence macroscope used in these experiments are shown in figure 14. The sample, either a tissue-mimicking flow phantom or a mouse ear (see below), was placed on an adjustable imaging platform and was trans-illuminated with the output of a 660 nm solid-state diode laser (DPSS-660; Crystalaser, Reno, NV). The output beam was expanded to ~5 mm full-width at half
maximum using a simple plano-convex lens pair ($f = 50$ mm and 200 mm; Edmund Optics, Barrington, NJ). A 660 nm “clean-up” filter (d660/20x Chroma Technology, Rockingham, VT) was also used in front of the laser to remove a small amount of out-of-band near infrared (NIR) output from the laser. The light intensity at the sample was 10 mW/cm². At this intensity, a small amount of tissue autofluorescence photobleaching (about 0.8% per minute on average) was observed during the *in vivo* experiments.

![Diagram](image)

**Figure 14** - (a) Schematic diagram and (b) photograph of the fluorescence macroscope used to acquire image sequences for this work (see text for details). M, mirror; Lin Pol, linear polarizer; Obj, 2X objective. (c) Photograph of a mouse ear positioned on the imaging stage for *in vivo* experiments. As shown, a large region of the ear is illuminated and imaged.

We used a high-sensitivity, 14-bit electron multiplied charge coupled device (EMCCD) camera (iXonEM + 855 Andor Technology, Belfast, Northern Ireland) fitted with a low-magnification objective with NA = 0.055 (2X Mitutoyo Plan Apo Infinity-Corrected Long WD Objective Edmund Optics, Barrington, NJ) and 200 mm 1X tube
lens (Mitutoyo MT-1, Edmund), so that the imaging field of view was about $5 \times 5$ mm$^2$. The depth of focus of this objective was 91 μm and we were able to resolve cells within 75 μm above or below this region (although cells were slightly blurry) so that the effective working depth of field was about 241 μm in tissue. This is well matched to the mouse ear, since the thickness is about 250–300 μm and most of the blood vessels are located within 100 μm of the ear surface [88, 89]. As such, essentially all cells traveling in blood vessels in the field of view were detectable. Fluorescence images were acquired with a 710 nm filter with 50 nm bandpass filter in place (et710/50m; Chroma) while the laser was illuminating the sample. The filter was mounted in a motorized six-position filter wheel (FW102, Thorlabs, Newton NJ). Crossed linear polarizers (25 mm diameter, Edmund) were also placed between the laser and the sample, and between the sample and the EMCCD to further reduce leakage of the laser light into the imager. In principle this polarizer pair was not required but was experimentally found to reduce laser leakage into the fluorescence channels, although this had the drawback of reducing the detected fluorescence by 50%. White light images of the sample were acquired by removing the fluorescence filter from the optics train (i.e., moving to an open position of the filter wheel) so that all wavelengths were detected by the camera and by back-illuminating the sample with an LED ring (Digi-Slave L-Ring 3200, Edmund). We chose to use a red laser and a near-infrared fluorophore for these experiments since tissue autofluorescence is markedly reduced compared to, for example, the blue-green wavelength region. Other
lasers in the near-infrared range (such as a Titanium:Sapphire laser or diode-pumped solid state lasers) could similarly be used with near-infrared dyes such as Vybrant DiL, Alexafluor750 or Cyanine-7. In principle visible lasers and fluorophores could also be used (as is done routinely in microscopy IVFC [49, 52]), but as we discuss this would result in an increase in tissue autofluorescence and potentially greater attenuation of light through the ~250–300 μm thick ear.

The exposure time for fluorescence images was typically 0.05 s, resulting in a frame rate of ~19 Hz (allowing time for data transfer to on-board memory). We also rebinned the 1,024 × 1,024 imaging array to 128 × 128 pixels in on-board camera hardware, which also increased the frame rate on the camera. Given our optics and camera configuration, a 10 μm diameter cell occupied an object only about 1–5 pixels in dimension in each image (rapidly moving cells were often recorded as a streak that were up to 5 pixels in length). Further, given the large imaging field of view relative to cell size, detection of fluorescently-labeled circulating cells required application of gain to the EMCCD camera. This could be configured with a personal computer running Andor software, and typically we operated this between 10 and 90 out of a maximum of 300 (arbitrary units). This resulted in substantial imaging noise (as opposed to background intensity) on the order of the detected cell intensity which necessitated the development of the imaging algorithms described here. Use of a higher magnification objective with a larger NA would have resulted in greater sensitivity and required less camera gain to
resolve cells; however, this would have come at a cost of smaller imaging field of view and depth of focus, so that the overall cell detection sensitivity of the CV-IVFC instrument would have been reduced. During data collection we acquired 1,000 frames per imaging sequence (52 s), but this could be repeated an arbitrary number of times with only about 0.5 s lag between image sequence acquisition to allow transfer of image sequences to the PC.

2.1.2 Computer Vision Algorithm

We developed a two-step algorithm to analyze image sequences and extract the tracks of moving cells from noisy widefield fluorescence images as shown schematically in figure 15. The overall strategy was as follows: in the first step we identified candidate cells in individual images in the sequence, and in the second step we connected cell candidates in multiple image frames into cell tracks. We first performed basic flat-field correction of the image (for the laser beam profile), followed by pixel-by-pixel background subtraction. This was done by taking the mean value $m_{ij}$ of each image pixel $p_{ij}$ in all $N$ image frames in the sequence,

$$m_{ij} = \frac{1}{N} \sum_{k=t}^{N} p_{ij}$$  \hspace{1cm} (1)
Figure 15- Flow chart of the two-step automated computer vision analysis of fluorescence image sequences as well as example images obtained after each step. The algorithm identified cell candidates (Step 1) from image sequences, and then connected them dynamically into cell tracks (Step 2). The position of the cell is indicated with a red arrow and dotted circle.

Next we converted the 14-bit image sequence into a binary image sequence by subtracting this mean value (pixel-by-pixel) and comparing the resulting value to a threshold value $\tau$ as follows:

$$b_{ij} = \begin{cases} 
1 & \text{if } |p_{ij} - m_{ij}| > \tau \\
0 & \text{otherwise}
\end{cases}$$

(2)
Therefore, in general there was no condition that a cell be a specific size or shape. Rather at least 1 pixel must have exceeded the threshold for a cell to be identified as a candidate. As we discuss, selection of the particular threshold $\tau$ for each sequence was very important since it altered the performance of the algorithm with respect to overall sensitivity and false alarm rate (see Performance Metrics in section 2.1.5 and Performance Metrics Results in section 2.2.3 given below for more details). We observed substantial inter-experimental variability in overall image intensity ($\sim$50%) and therefore determined that a fixed threshold across all experiments was not suitable. Instead, we set the threshold as a function of percentile of all pixel intensities (ranging from 0 to 16,383) in the image sequence; as we demonstrate, threshold percentiles in the range of 99.93–99.99 were empirically determined to work well in mice in vivo. We further note that the raw image intensity data sets directly from the EMCCD were processed by the algorithm and no prior image manipulation filters (such as edge, sharpen, contrast, etc.) were employed since these can often exacerbate existing noise or introduce additional artifacts in the image sequence.

Following this operation, the resulting binary image sequence contained the set of circulating cell candidates which in practice greatly outnumbered the actual number of cells. Specifically, many post-threshold cell candidates were due to image noise (as we show, for in vivo data this was about 97% of candidates). Therefore, in Step 2 cells were distinguished from background noise through dynamic analysis of image sequences to
identify cell “tracks.” First, (Step 2A) cell candidates were connected into tracks in consecutive frames. For each cell candidate, a search $S_k$ was performed on pixels $p_{ij}$ in the next image frame inside a radius equal to two times the dimension (e.g. diameter) of the candidate cell $\Phi_k$ from the center of the candidate cell $P_{ij}$:

$$S_k : p_{ij} \text{ where } |p_{ij} - p_{ij}| < (2\Phi_k)$$

Typically this diameter was about 1–3 pixels, but could be slightly longer for fast moving cells where $\sim$5 pixel long “streaks” were observed. This search was performed for all candidates in the image sequence. When multiple cell candidates were observed inside this radius, the closest candidate was selected to merge to the track. Because cells were relatively dim and the intensity could intermittently drop below the threshold, gaps in a given cell's track (of up to $\sim$10 frames) were often observed after Step 2A. As such, in Step 2B these were connected together by merging individual tracks that occurred relatively close together in space and time as follows: when the final position of one track (identified in Step 2A) occurred within a radius of 15 pixels and within 15 image frames from the first position of the next identified track in the image sequence, the tracks were merged. We then applied a second condition for merging cell tracks in Step 2C that combined the start and end points of consecutive identified tracks $S_{m}^{T}_{\text{start}}$ and $S_{n}^{T-t}_{\text{end}}$ observed at times $T$ and $T-t$, respectively,
where \( M \) was the condition for merging the tracks. The search area was determined by analyzing the final position and velocity \( v \) of the previous track \( S_{m}^{T-t} \),

\[
M = \begin{cases} 
1 & \text{if } S_{n}^{T}(\text{start}) - S_{m}^{T-t}(\text{end}) \in (S_{m1}, S_{m2}, S_{m3}) \\
0 & \text{otherwise}
\end{cases}
\]  

(4)

where \( \tau \) was the inverse of the frame rate (in this case 0.052 s) and by extrapolating the final position assuming a speed in the range of 0.5\( v \) to 2\( v \) as follows:

\[
v = \frac{S_{m}^{T-t}(\text{end}) - S_{m}^{T-t}(\text{end}-1)}{\tau}
\]  

(5)

where \( t \) was the elapsed time since the end of the previous track. The three search regions \( S_{m1}, S_{m2} \) and \( S_{m3} \) centered on \( P_{m1}, P_{m2}, \) and \( P_{m3} \) with a radius of 0.5\( vt \), \( vt \), and 2\( vt \), respectively; in practice, this produced a cone-shaped search area. Therefore, Steps 2B and 2C differed in the search region for merging as follows: Step 2B used a circular search radius whereas Step 2C used a cone oriented in the direction of cell movement. At
the end of Step 2, remaining cell candidates that did not connect to any track (i.e. were observed in only single frames) were discarded from the analysis. As we show, this was the case for about 97% of cell candidates that were identified in Step 1. As such Step 2 was critical in the rejection of false positive counts. Last, for visualization, cell tracks were overlaid onto the white light image of the sample (mouse ear vasculature). A total cell count in an image sequence was obtained by simply counting the total number of identified cell tracks detected in an image sequence.

2.1.3 Phantom Measurements

We first tested our imager and algorithm in an optical flow phantom model which was intended to mimic a blood vessel in a mouse ear. Disk shaped phantoms (fig. 16a) ~2 mm thick by 20 mm in diameter were made from polyester resin (Casting Craft, Fields Landing, CA) with titanium oxide (TiO$_2$; Sigma-Aldrich, St. Louis, MO) and India ink added at 50 parts per million to yield optical properties similar to biological tissue at near-infrared wavelengths. Specifically, based on previously published characterization of similar phantoms [88] the final optical properties at 700 nm were estimated to be as follows: reduced scattering coefficient $\mu'_s = 15$ cm$^{-1}$ and absorption coefficient $\mu_a = 0.1$ cm$^{-1}$. Strands of microbore Tygon tubing (250 μm internal diameter, TGY-010C, Small Parts, Seattle, WA) were embedded in the phantom in either an arc or in a straight line ($N = 2$ each) before hardening to mimic a blood vessel. The position of the clear tubing is
indicated with an overlaid dotted line in figure 16a. These were attached to a syringe mounted on a precision microsyringe pump (70-2209, Harvard Apparatus, Holliston, MA), so that solutions of fluorescent microspheres suspended in PBS could be passed through the phantom at controlled linear flow rates between 0.5 and 10 mm/s, therefore approximately matching the blood flow rates in large ear blood vessels reported in the literature [49, 58]. We used 6 μm diameter fluorescent microspheres with absorption maxima at 645 nm, and emission maxima near 695 nm (Peakflow Claret, P-24670, Invitrogen, Calsbad, CA), to match commonly used Cyanine5.5 and Alexafluor-680 organic fluorophores. Microspheres were suspended at a concentration of 3,000 spheres/mL. For these experiments, the EMCCD gain was set to 10 (arbitrary units; out of a maximum of 300). Image sequences were acquired for a total of 5 min for each phantom.
Figure 16- (a) Photograph of an ear-mimicking phantom with curved length of embedded Tygon tubing. The position of the clear tube is indicated with the dotted black line. (b–f) Raw fluorescence image sequence (separated by 0.25 s) showing a fluorescent microsphere (red arrows and dotted circles) flowing in the phantom. The stationary white points were autofluorescent inclusions in the flow tube, simulating stationary tissue autofluorescence observed *in vivo*. (g–k) the same image sequence is shown after application of Step 1 of the algorithm. (l–p) The CV-IVFC algorithm successfully connected the sphere path in a trajectory over the arc. This trajectory was over-laid on the white light image of the phantom (green line).

### 2.1.4 *In Vivo* Measurements

We performed *in vivo* testing of our system and algorithm using six nude (nu/nu) mice injected intravenously with Vybrant-DiD-labeled multiple myeloma (MM) cells. All mice were handled in accordance with Northeastern University's Division of Laboratory Animal Medicine rules on animal treatment and care. MM cells were grown in culture
and suspended in RPMI with 0.1% bovine serum albumin (BSA) at a concentration of $1 \times 10^6$ cells/mL. Cells were labeled with 1 μmol/L of Vybrant-DiD and incubated for 30 min at 37°C and then spun down and re-suspended at a final concentration of $5 \times 10^4$ cells/mL prior to injection. Mice were anesthetized with a combination of ketamine (100 mg/kg) and xylazine (5 mg/kg). About 100 μL of the cell suspension were injected intravenously via the tail vein, so that the injected cell population was $5 \times 10^3$ cells, or $\sim 2.5 \times 10^3$ cells/mL of PB (assuming about 2 mL of mouse blood). Mice were then placed on the translation stage and positioned so that the ear was flat on a microscope slide in the imager field of view. A glass cover-slip was also added (with a drop of water) to keep the ear flat during imaging. For in vivo experiments, we increased the EMCCD gain to 90 (arbitrary units; out of a maximum of 300) due to the relative dimness of the labeled MM cells compared to the fluorescent microspheres. As above, image exposure times were set to 0.05 s, yielding a frame rate of about 19 Hz. Images were acquired for $\sim 30$ min for each of the six mice.

### 2.1.5 Performance Metrics

We used two standard metrics to assess the performance of our imaging system and cell detection algorithm, specifically, sensitivity $= TP/(TP + FN)$ and false alarm rate (FAR) $= FP/min$. Here TP is “true positive” cell count, FN are the “false negative” counts (in this case, cells that were undetected by the algorithm) and FP are the “false positive”
counts. As noted in Computer Vision Algorithm section, we tested a range of histogram threshold percentiles from 99.93 to 99.99, and quantified each performance metric at each level. To determine the “true” cell counts, a human operator manually counted the circulating cells appearing in the image sequences and compared them with those identified by the algorithm. Therefore, these metrics do not reflect circulating cells that may have been missed if they were not sufficiently bright to be visible on image sequences. However, the number of detected cells was generally in good agreement given the low concentration of injected cells and PB volume in the imager field-of-view.

2.2 Results

2.2.1 Optical Flow Phantom Testing

We first tested our CV-IVFC approach in tissue-mimicking optical flow phantoms (fig. 16a) with fluorescent microspheres. We tested a range of flow speeds (0.5–10 mm/s) as well as straight and curved flow channel geometric configurations. An example set of fluorescence images acquired during the experiment is shown in figures 16b–16f, along with the corresponding mean background subtracted and thresholded image sequence (after Step 1) in figures 16g–16k. In this case, a phantom with a curved flow channel and linear flow speed of 5.1 mm/s is shown. By inspection of figures 16b–16f, fluorescence contrast was excellent in the optical flow phantom model and this allowed us to verify
that the algorithm could correctly identify and track small (~1–3 pixels diameter) fluorescent targets (indicated by red arrows and dotted circles), even when significant directional changes were observed. The extracted microsphere track (green curve) was overlaid on the white light images of the phantom as shown in figures 16l–16p. It is important to note that the algorithm successfully distinguished moving fluorescent targets from stationary bright pixels or pixel groups. For example, two stationary bright pixels can be clearly observed in the raw fluorescence images in figures 16b–16f, but these points were rejected (primarily) during Step 1 of the CV-IVFC algorithm (figs. 16g–16k). Residual stationary pixels that were not rejected in Step 1 (for example due to photon counting noise as in figs. 16g and 16h) were rejected in Step 2, since they did not form trajectories in subsequent image frames (see Computer Vision Algorithm section). In general, microspheres were successfully tracked at speeds up to 10 mm/s, which exceeds the expected flow speeds in blood vessels in the mouse ear *in vivo* of 0.5–5 mm/s from literature values [49, 58]. Since fluorescence contrast was extremely high in the flow phantom models, it was not necessary to adjust the threshold value in Step 1 (as opposed to the *in vivo* experiments where this was necessary; see below) so that we used a single threshold value of 99.96% of maximum. The overall system performance over all four phantoms and flow speeds tested was sensitivity = 0.993 and the false alarm rate = 0.074/min.
2.2.2 Testing in Mice In Vivo

We next tested the CV-IVFC in nude mice injected with very low concentrations (injected concentrations of ~2,500 cells/mL PB) of fluorescently-labeled multiple myeloma (MM) cells. An example fluorescence image sequence acquired in vivo is shown in figures 17a–17e. By inspection, the noise and background autofluorescence (for example, from sebaceous glands on the ear [89]) observed were significantly higher than in phantoms, and made discrimination of cells in individual frames (red arrows with dotted circles) more difficult. As is evident in figures 17f–17j, a much larger number of “cell candidates” (i.e. pixels that exceed the threshold after background subtraction) were identified after Step 1, which greatly outnumbered the true numbers of cells. However, the vast majority of these cell candidates did not form cell tracks as defined in Step 2 and were therefore rejected by our algorithm (please see Performance Metrics in Results section). The trajectories of the subset of cell candidates that did form tracks (in Step 2) were stored and counted by the CV-IVFC algorithm. Example tracks of two circulating cells are shown in figures 17k–17o, overlaid on white light images of the mouse ear vasculature. In this case (and in about 95% of cells observed), these cell tracks appeared to correlate to the large blood vessels indicated by dark regions in the white light image. This specific example also demonstrates the ability of the algorithm to distinguish between multiple circulating cell tracks in the same image sequence (indicated by green and yellow tracks). Likewise, figure 18 shows an example case where a single cell
changed speed and direction rapidly as it moved from one blood vessel to another (figs. 18c, 18h, and 18m). It is possible that a given volume of PB may pass twice through the imager field of view so that this could potentially result in over-counting of cells. In the future, this effect could be corrected by considering the direction of movement (distal or proximal) and adjusting the cell count accordingly.

Figure 17- (a–e) An example fluorescence image sequence obtained from the ear of a mouse, where each image is separated by 0.4 s. Two fluorescently-labeled MM cells (red arrows and dotted circles) passed through the field of view, with the second cell appearing in (c) and the first disappearing in (e). By inspection, these were difficult to discriminate from autofluorescence in a single image. (f–j) The corresponding image sequence is shown after the background subtraction and thresholding operation performed in Step 1. The relatively large number of “false alarm” cell candidates in the sequence are evident, but these were rejected by dynamic analysis of the image sequence in Step 2.
(see Computer Vision Algorithm section for details). (k–o) The extracted tracks of the two cell candidates are shown (here indicated by green and yellow curves), overlaid on the white light image of the mouse ear vasculature.

Figure 18- (a–e) Example fluorescence image sequence of a single MM cell (red arrows and dotted circles) that was observed to rapidly change direction in two blood vessels in the mouse ear. Images shown were separated by 0.4 s. (f–j) Corresponding images sequence after thresholding operation performed in Step 1, and (k–o) the full track of the MM cell that was correctly recovered and overlaid on the white light image of the mouse ear.

Cells that were moving rapidly were often recorded as “streaks” rather than single points. In general this had the effect of reducing the recorded intensity, that is, since the intensity was divided over the pixels in the streak. However, as long as the intensity exceeded the
threshold (Step 1B) and the object appeared in multiple frames of an image sequence (Steps 2B and 2C), it was recorded as a cell track. Moreover, the ear was easily immobilized (by securing the mouse and using a drop of water on the ear) so that image sequences were generally free of breathing artifacts. It is also worth reiterating that the injected cell concentrations used here were extremely low and were generally below the operating range of other reported optical IVFC techniques. Therefore, occurrences of circulating cells passing through the imager field of view were very rare compared to the total length of video sequences (specifically about 0.5/min on average) thereby underscoring the importance of the automated detection algorithm.

While it is difficult to exactly quantify the number of cells in circulation during these experiments (as opposed to the injected concentration), we estimate this concentration as follows: we injected on average $5 \times 10^3$ labeled MM cells suspended in 100 μL of media, which were diluted in the $\sim 2$ mL blood volume for a total injected concentration of $\sim 2.5 \times 10^3$ cells/mL of PB. From previous analysis of extracted PB samples, it is estimated that about $\sim 10$–20% of injected cells were retained in circulation 5 min after injection, with the balance either trapped at the site of the tail vein injection, or trapped rapidly in the lungs and in the spleen after injection of the initial bolus. Moreover, MM cells are known to home to the bone marrow continuously during circulation, so that we estimate that overall there was 250–500 cells/mL in circulation during these experiments. At this concentration, we observed 0.5 cells/min over all
experiments performed. Assuming that the lower limit of sensitivity for our system would occur when only 1 cell observed in a 60-min period, this is about 17 cells/mL. Conservatively then, we estimate that the practical lower limit of detection sensitivity of our approach is about 20 cells/mL. To better quantify the true circulating cell population in the future, we could extract PB samples and analyze them using conventional flow cytometry. However, the low concentrations of circulating cells used here would necessitate euthanizing the mouse and analyzing PB volumes on the order of 1 mL. Moreover, measurement of cell populations at multiple time points in the 30-min period following injection would be required because MM cells are known to clear rapidly from circulation [48, 52].

### 2.2.3 Performance Metrics

In figures 17 and 18, the threshold value \( \tau \) used in Step 1 [Eq. (2)] was arbitrarily selected to be the 99.96th percentile of all measured pixels in the image sequence. However, the overall performance of the algorithm could be controlled by adjusting this threshold. The concept is shown in figure 19a, where the distribution of background (autofluorescence) noise is shown for a typical mouse (blue curve), along with the distribution of fluorescence intensities of circulating cells (red curve). Here, all pixel values for all image frames of full 30 min acquisition for a single mouse are represented. Data is presented following mean background subtraction, so that that mean value for the
background is zero but the standard deviation was 980 counts. Likewise the mean and standard deviation of the cell intensities were 7,304 counts and 1,955 counts, respectively. The dotted vertical line indicates the value of the 99.96th percentile (i.e. the threshold) which in this case was 4,200 counts. Although only a small overlap between the blue and red curves is evident by inspection, it should be emphasized that the number of points in the blue distribution outnumbers the number of points in the red distribution by a factor of $10^4$; as such, this small overlap results in many potential false positive cell candidates that are removed in Step 2 of the algorithm. We therefore quantified the performance of our CV-IVFC method over all six \textit{in vivo} data sets according to sensitivity and FAR as a function of the selected threshold. As shown in figure 19b, use of a lower threshold (i.e. 99.93rd percentile) resulted in a sensitivity of better than 0.9, but resulted in a relatively high FAR of 1.5/min (fig. 19c). Increasing this threshold (e.g. to 99.99th percentile) reduced to false alarm rate to only 0.04/min on average, but likewise this resulted in a reduction of sensitivity to 0.65. Therefore, it is possible for the end user to adjust this threshold to trade-off sensitivity and FAR depending on the specific cell-counting application. This is illustrated explicitly in the operating curve shown in figure 19d. As we have noted (and by inspection of the image sequences) the use of simple background subtraction and thresholding (in Step 1) was not sufficient to identify circulating cells in the image sequences. This is shown in figure 19e, where the FAR realized after Step 1 (threshold step) and Step 2 (merging step) as described in detail
in figure 15 and Testing in Mice \textit{In vivo} section shown above. As indicated, the dynamic merging analysis performed in Step 2 reduced that FAR by at least 97\% for all threshold values used. As such, the second step was critical for the CV-IVFC approach, as noted primarily due to substantial tissue autofluorescence and EMCCD imager noise. Moreover, we note that less brightly labeled cells (e.g. with a fluorophore with smaller extinction coefficient or quantum yield) would result in a shift of the dotted curve to the left, so that more overlap between the distributions and a greater potential increase in the false alarm rate would be observed. Similarly, an increase in autofluorescence noise (e.g. if a visible fluorescent dye were used) would result in increased overlap. In this case, the threshold in Step 1b could be increased, or the radius of merging (Steps 2b and 2c) decreased to reduce the FAR, but this would most likely come at a reduction in sensitivity. Likewise, a microscope objective with greater magnification could be used to reduce the autofluorescence noise, but this would come at a cost of a smaller imaging area and less overall sensitivity.
Figure 19-Performance metrics of the computer vision algorithm as a function of the threshold level (as a function of maximum pixel intensity) over all six mice studied. (a) Example distributions of background signal (blue curve) and cell fluorescence signal (red curve) following mean background subtraction. Data shown is for all pixels in a 30-min image sequence from a single mouse. The dotted vertical line indicates a threshold of 99.96th percentile, which in this case was 4200 counts. (b) Sensitivity of the CV-IVFC method in vivo as a function of threshold level. (c) The corresponding false alarm rate (FAR) as a function of threshold, and (d) the sensitivity and FAR operating curve. (e) The overall average FAR as a function of threshold, obtained over the six mice studied after Steps 1 and 2 of the detection algorithm. As shown, the dynamic analysis in Step 2 resulted in rejection of >97% of false alarms over all thresholds tested.

Finally, we note that in addition to detection and counting, our computer vision IVFC approach allows us to automatically extract cell behavior information. For example, as shown in the histogram in figure 20, we extracted the speed of circulating cells from the detected cell tracks. These data were generated from a total of 85 tracked cells, and the mean speed from each cell is displayed (i.e. since cells frequently changed speed during the track). A wide range of speeds were observed, and the mean speed was about 1.2 mm/s over the experiments performed which is in good agreement with literature values. Rapid changes in speeds in a single cell were observed, for example one cell speed went from 7.4 mm/s to 1.5 mm/s, presumably as it entered a smaller region of the blood vessel. Generally the range of cells speeds observed were consistent with previously reported literature values in the mouse ear. For example, Novak et. al. [49] reported flow speeds of 1–3 mm/s, and V. Zharov et. al.. [58] reported flow speeds of
0.5–4 mm/s. It is possible that faster moving cells were recorded less frequently than slower moving cells due to the streak effect described above. However, observed tracks consistently coincided with the location of larger blood vessels with larger blood flow rates so that we do not believe this was a major effect. MM cells were also observed to stop at a site in the tissue, which we interpret to have been a possible docking event at the blood vessel wall. In the future, we could modify our algorithm to automatically identify and characterize homing and extravasation events for example, for immune cells in response to inflammatory injury [90]. As such, this approach can yield information that cannot be obtained with existing IVFC techniques that simply count cell events.

Figure 20-Incidence of cell speed range observed from all tracked cells in vivo, normalized to the total number of cell observations. The average cell speed was about 1.2 mm/s.
2.3 Discussion and Conclusions

In summary, we have developed a new computer vision IVFC method for detection and enumeration of very rare circulating cells. The CV-IVFC algorithm utilized a fairly straightforward “detect-and-connect” methodology that nonetheless resulted in a powerful IVFC instrument. The main advantage of the CV-IVFC approach is the high detection sensitivity, owing to the relatively large imaging area (5 × 5 mm\(^2\)) and correspondingly large blood sampling volume. In the experiments shown here, three to four large artery-vein pairs in the ear and the surrounding capillary bed were typically simultaneously sampled. To our knowledge, this has never been implemented previously. In future work, it could also allow us to automatically characterize cell behavior in vivo, for example, in studying the adhesion, rolling and extravasation of immune cells in response to insult. Moreover, although we have chosen to focus on rare circulating cells in this work in principle the algorithm would also work with higher circulating cell concentrations. As shown in figure 17, multiple cells could be tracked simultaneously in an image sequence, so that operation with several orders of magnitude greater cell concentrations is feasible.

While in principle the automated computer vision algorithm is “optional” (since a human operator could manually count cells in a fluorescence video sequence) in the case of the rare circulating cell populations shown here the frequency of cell detection events was 0.5/min or less on average, thereby making this extremely tedious in practice.
Another advantage of our computer vision IVFC approach is that false positives identified by the algorithm can easily be rejected by a human operator, simply by reviewing the video sequence and checking the identified cells (again, this is not possible with many existing IVFC systems). The use of the dynamic analysis (Step 2) rejected >97% of false positives, making this a much less time consuming activity.

Although we have chosen to use a cell membrane-labeling fluorophores (Vybrant-DiD) for these proof of concept experiments, in principle the CV-IVFC system could be used for a wide range of biological models using, for example, constitutively expressed red fluorescent proteins (RFPs), although to date we have not explicitly tested this. RFPs would most likely yield less-brightly labeled cells than the Vybrant-DiD dye used here [91-93]. Comparison of the extinction coefficients quoted in the product literature, for example, for Turbo-FP650 (Evrogen, Moscow, Russia) and Vybrant DiD (Life Technologies, Carlsbad, CA) suggests that RFP-MM labeled cells would be on the order of three times less brightly-labeled than the cells used here (although a literature search failed to reveal the relative quantum yields and expected local fluorophore concentrations of labeled cells which also contribute to brightness). The fluorescence distributions presented in figure 19a imply that cells three times less bright would be detectable, but with a greater false alarm rate (or decreased sensitivity). Again, this could be accommodated by adjustment of the merging algorithm in Step 2 or use of a higher powered objective with smaller field of view but better contrast. Likewise, receptor
targeted near-infrared fluorescent nanoparticles [64,94,95] in principle could be used with the instrument. Moreover, we have chosen to use a near-infrared dye here but in principle the CV-IVFC approach could be used for virtually any visible or near-infrared fluorophore, simply by changing the laser and filter combinations of the instrument. Again, we have not yet explicitly tested this and we anticipate that the associated increase in background autofluorescence noise may result in increased false alarm rates, which would also necessitate optimization of the instrument and cell tracking algorithm. Optimization of the collection optics, for example, through alternative selection of filters or removal of the second linear polarizer may also improve collection of fluorescence light and further improve sensitivity of the instrument in future versions.

Finally, we note that the false alarm rate could be reduced simply by restricting the area under consideration to regions corresponding to large artery-vein pairs (i.e. the dark regions) on white light images. We chose not to do this here since we observed a small fraction of cells (about 5%) moving between these larger vessels, presumably in the capillary bed in the ear. As such this improvement in FAR would have come at a penalty of about 5% in sensitivity, which was deemed to be more important for the CV-IVFC approach. Moreover, MM cells were intravenously injected immediately prior to imaging in the case and therefore were generally circulating in larger blood vessels. In the case of, for example, a metastatic cancer model this fraction could be significantly higher and have even greater effect on the overall instrument sensitivity.
**Chapter 3 – Toward Lower Contrast CV-IVFC with Modified Optical Phantoms**

In Chapter 2, we showed proof-of-principle validation of CI-IVFC *in vivo*. We next extended this work to study the performance of CV-IVFC under lower-contrast imaging conditions than the NIR dye used in the experiments in Chapter 2 [72]. If, for example, CV-IVFC could successfully track cells labeled with GFP, this would be extremely useful for many applications due to the ubiquity of GFP labeled cell lines. However, use of green (versus NIR) laser light would result in increased tissue autofluorescence and potentially degraded system performance. Likewise, anti-body targeted injected contrast agents are extremely attractive for study of CCs *in vivo*, but these are likely to yield worse contrast than membrane-bound labels (lower fluorescent probe uptake). Therefore, understanding performance of CV-IVFC with lower contrast could significantly increase the utility of the instrument in biomedical research.

To achieve this, we developed optical phantoms with varying levels of background contrast and structure. We analyzed image sequences obtained with these phantoms and showed that we were able to closely replicate conditions observed *in vivo* and then systematically reduce the image contrast. In Chapter 3, we evaluated the performance of the CV-IVFC algorithm with respect to sensitivity and false alarm rate (FAR) due to modifications in the optical phantoms. In Chapter 4, we compare the performance of CV-IVFC due to both modifications in optical phantoms and fluorescent
microspheres with varying intensities (seen with different cell labeling approaches). Chapter 4 also discusses the range of sensitivity of the system at varying levels of contrast.

3.1 Methods and Materials

CV-IVFC instrument and algorithm were described in detail in sections 2.1.1-2.

3.1.1 Phantom Preparation

Tissue-mimicking phantoms were made with polyester resin, TiO$_2$, and India Ink added to simulate approximate optical properties of tissue at 700 nm with reduced scattering coefficient $\mu'_s = 15$ cm$^{-1}$ and absorption coefficient $\mu_a = 0.1$ cm$^{-1}$ [88]. These were placed in a mold to mimic the approximate size of a mouse ear (20 mm diameter, 2 mm thick), with a strand of Tygon tubing inserted to simulate a large blood vessel (fig 21).

![Figure 21- a) Photograph of an example ear-mimicking phantom with a dotted line showing the Tygon tubing position. B) Photograph of an example the mouse ear for comparison.](image-url)
Inspection of *in vivo* image sequences (fig 17a-e, fig 18a-e) showed that sebaceous glands in the ear of the mouse appear as discrete point autofluorescent objects. Specifically, an average of 65 sebaceous glands per image was present over a set of 12 mice that were tested previously. To replicate this in phantoms, 6 μm diameter fluorescent microspheres were added to the resin before hardening; either 0, 850 or 1,700 spheres/mL were added to a volume of 10 mL of phantom material. In order to mimic the background autofluorescence, 0.4 μM of Alexafluor-680 dye was added to the resin. To simulate blood flow, a solution of PBS and the microspheres, representing fluorescent “cells” matching the spectra of Cyanine 5.5 and Alexafluor-680 dyes, were pumped through the tube via a syringe pump at a flow speed of 1.7 mm/s. Tests were run with microspheres at concentrations of $2 \times 10^3$ spheres/mL, EMCCD gain of 10-30 (of a maximum of 300), exposure time 0.05s, and frame rate of 19 frames/s. Data was collected for 20 minutes for each phantom.

### 3.1.2 Performance Metrics

Background and sphere intensities from image sequences for each phantom were analyzed. Histograms of pixel intensities were plotted after pixel-by-pixel background mean subtraction. Microspheres were tracked with our CV-IVFC algorithm as we have described in Chapter 2, and sensitivity and FAR performance was quantified for each phantom. Here, sensitivity was calculated as $TP/(TP + FN)$, here TP were the true
positives count (determined by a human operator) and FN were the false negative counts, i.e. cells missed by the algorithm. The FAR was calculated as the number of false positives (FP) incorrectly detected by the algorithm per minute. The performance was recomputed for a range of threshold percentages in Step-1 of the CV algorithm, specifically, 99.93 to 99.99 of maximum.

3.2 Results

An example set of fluorescence images for a phantom with high contrast (no embedded spheres or AF-680 dye added) and low contrast (1,700 spheres/mL and 0.4 μM AF-680 added) are shown in figs. 22a-c and 22j-l, respectively. By inspection, addition of the contrast materials resulted in an image sequence that qualitatively better matched that observed in vivo. The effect of this contrast on Step-1 (cell candidate identification) is clearly observed in figs 22d-f and 22m-o, respectively; a significantly higher number of false-candidates are indicated. However, the CV algorithm was successful in identifying the moving microspheres for both cases as shown in figs 22g-i and 22p-r.
Figure 22- Example fluorescence image sequences for a high-contrast (a-c) and low-contrast (j-l) phantom, respectively, with frames separated by 0.25 s. (d-f, m-o) corresponding thresholded image sequences are shown, indicated microsphere candidates identified in Step-1 of the algorithm. (g-i, p-r) extracted tracks and white-light overlay images for the high- and low-contrast phantoms, respectively.

To better quantify this effect, we computed the pixel-intensity histograms and algorithm tracking performance (sensitivity vs. FAR) for all of the phantoms we tested. Example data is shown in figure 23. Intensity histograms for a high-contrast, medium contrast and low-contrast phantom are shown in figs 23a-c, respectively. For the high-contrast phantom, the background mean and standard deviation were 0 and 103 counts while the spheres had a mean of 12,583 counts and standard deviation of 4,496 counts (fig 23a). For the low-contrast phantom the background intensity had a mean of 0 and standard deviation of 763 counts and sphere intensities with a mean of 5,500 and standard
deviation of 1,462 counts (25c). Comparison of the fig 19c verifies that the low-contrast phantom approximated what was observed in mice *in vivo*.

Last, the performance of the CV-IVFC algorithm with respect to sensitivity and FAR for two example phantoms are shown in fig 22, along with corresponding *in vivo* data. As such the overall system performance observed in the low-contrast phantom approximately replicated the *in vivo* performance of the algorithm. For example, for one operating threshold (99.96th percentile), the low contrast phantom had sensitivity of 0.89 and FAR of 0.4 spheres/min whereas the *in vivo* performance had similar sensitivity of 0.81 and FAR of 0.24 cells/min.
Figure 23- Pixel-intensity histograms for example a) high-contrast, b) medium-contrast, and c) low-contrast phantom. (d) Sensitivity versus FAR performance for an example high- and low-contrast phantom, as well as in vivo data.

3.3 Discussion and Conclusion

Chapter 2 reported the development and validation of a novel high-sensitivity computer vision system and algorithm for detecting, tracking and counting rare CCs at concentrations below 20 cells / mL in vivo. In this chapter, we were successful in developing a phantom model that closely replicated both the qualitative (appearance) and quantitative (intensity histogram, Sensitivity and FAR) properties of previously measured
in vivo data. As noted, the fluorescent labeling (contrast) of MM cells in our previous in vivo studies was relatively “bright” compared to that expected, e.g. with constitutively expressed fluorescent proteins or receptor targeted fluorescent dyes. Therefore, development of lower-contrast phantoms and characterization of the performance of the CV-IVFC algorithm was the subject of work performed in Chapter 4.
Chapter 4 – Performance Of Computer Vision In Vivo Flow Cytometry With Low Fluorescence Contrast

Our initial “proof-of-principle” validation of CV-IVFC represented a relatively high-contrast imaging model (Chapter 2) and building on the work from Chapter 3, we next tested the CV-IVFC instrument and algorithm with a series of tissue-mimicking optical phantoms with varying levels of autofluorescence and with calibrated cell-simulating microspheres with varying fluorescent brightness.

As we show, depending on instrument parameters, CV-IVFC allowed robust detection of microspheres with very low contrast, for example, detecting at least 50% of microspheres even with over two orders of magnitude worse contrast than our previously reported in vivo conditions. Occurrence of “false positive” detection events were found to correlate with temporal and spatial clustering of high-background pixels. As we discuss, these results support the significant potential utility of CV-IVFC for a wide range of in vivo biological models, for example, using fluorescent proteins or antibody targeted fluorophores.
4.1 Methods and Materials

The CV-IVFC instrument and algorithm were described in detail in section 2.1.1-2.1.2.

We developed a set of tissue (ear) mimicking optical phantoms and used a series of cell-simulating fluorescent microspheres. Our goal was to produce imaging conditions with equal or lower contrast than the in vivo work in Chapter 2. Example images from these studies where Vybrant-DiD labeled MM cells were tracked in mice ears in vivo are shown in figure 24 [71]. The variation in contrast (defined as $C = \frac{(I(s)-I(b))}{I(b)}$, where $I(s)$ is the microsphere intensity, and $I(b)$ is the mean background intensity in an image) is evident, and ranged between 0.18 to 1.59 (average of 0.73) between experiments. As we quantify in detail, the phantom experiments performed here yielded contrast with significantly lower contrast, allowing us to study the performance of the CV-IVFC with increased background autofluorescence or weaker cell labeling.

![Figure 24 - Example images of circulating cells obtained from our previous in vivo studies ([71]) in which fluorescently-labeled MM cells were detected and tracked with CV-IVFC, along with the corresponding cell contrast.](image)


4.1.1 Optical Phantom Preparation

Tissue-mimicking optical phantoms were prepared by mixing polyester resin (Castin’ Craft, Environmental Technology Inc., Fields Landing, CA), TiO2 powder (Sigma Aldrich, St. Louis, MO), and India Ink (Higgins, Leeds, MA) to approximately match the reduced scattering coefficient ($\mu'_{s} = 15 \text{ cm}^{-1}$) and absorption coefficient ($\mu_a = 0.1 \text{ cm}^{-1}$) of tissue at 700 nm [88]. To replicate the approximate dimensions of the mouse ear (figs. 25a,b), the resin was placed in a 20 mm diameter and 2 mm thick mold. The phantom material alone exhibited only modest autofluorescence, so we added AlexaFluor (AF)-680 dye (Life Technologies, Carlsbad, CA) to simulate the background autofluorescence present in biological tissue. Likewise, sebaceous glands are a prominent feature of in vivo fluorescence images in the ear and appear as bright spots (fig. 25c) that are occasionally falsely identified as circulating cells by the CV-IVFC algorithm. To mimic this, we added 6 μm fluorescent microspheres (Peakflow Claret cytometry beads, P-24670, Life Technologies) to the phantoms. In total, three sets of phantoms (N = 3 for each) were fabricated with either i) P1; 0.4 μM microspheres and 850 microspheres per mL of resin material added, ii) P2; 0.4 μM microspheres and 1700 microspheres/mL added, or iii) P3; 0.6 μM microspheres and 1700 microspheres/mL added. Phantom type P2 corresponded to approximate in vivo imaging conditions observed in our previous work, and type P3 represented significantly elevated
background fluorescence. Before hardening, a strand of Tygon tubing with 250 μm internal diameter (TGY-010C, Small Parts, Inc, Seattle, WA) was placed in the resin to simulate a blood vessel.

Example white light images of a phantom are shown in figs. 25d and e. Two example fluorescence images are also shown from a phantom without fluorescent material added, and with fluorescent material added (phantom type P2) in figs. 25f and g, respectively, showing the qualitative similarity to the in vivo fluorescence image shown in fig 25c.

Figure 25- Example images of a mouse ear (a-c) and optical phantoms (d-g). (a) Photograph of mouse ear with red arrows indicating large blood vessels. (b) White light image of the mouse ear taken with the CV-IVFC camera, (c) example fluorescence image of the same ear region with sebaceous glands and EMCCD shot noise appearing as groups of bright pixels. (d) Photograph of a phantom with a dashed black line indicating the position of the embedded Tygon tubing. (e) White
light image of the phantom taken with the EMCCD camera showing position of the Tygon tubing. (f) Fluorescence image of a phantom without fluorescent dye or microspheres added. (g) Fluorescence image of an example P2 phantom type, showing qualitative similarity to (c).

4.1.2 Fluorescent Microspheres

We used calibrated fluorescence microspheres suspended in PBS to simulate circulating cells and flowed them through the phantoms using a microsyringe pump (70-2209, Harvard Apparatus, Holliston, MA) configured so as to produce a flow speed of 1.7 mm/s in the phantom tube. To simulate the effect of different cell labeling (e.g. due to different fluorophores or labeling techniques), we used a set of near-infrared microspheres with varying fluorescence intensities and with similar excitation and emission spectra to, e.g. Cyanine 5.5 Alexafluor-680. These are denoted as follows for the balance of this paper: MS1 (Peakflow Claret cytometry beads, Life Technologies), MS2 (Flash Red Intensity Standard FR5, Bangs Laboratories, Inc., Fishers, IN) and MS3 (Flash Red Intensity Standard FR4, Bangs). As shown in figure 26, these 3 microspheres types had fluorescence intensities equal to 200%, 62.8% and 6.8% of the Vybrant-DiD labeled Multiple Myeloma cells that we used in our previous work. Therefore, this combination of spheres allowed us to test the performance of CV-IVFC with almost 2 orders of magnitude variation in fluorescence labeling.
Figure 26- Comparison of average fluorescence intensities of the three microsphere types (MS1, MS2 and MS3) used in these studies and Vybrant-DiD labeled MM used in our previous work, normalized to MS1 intensity.

4.1.3 CV-IVFC Testing

4.1.3.1 Data Acquisition

We tested each of our 3 different phantom types with each of the 3 different fluorescent microspheres for a total of 9 different contrast conditions. For each, microspheres suspensions of 2,000 spheres/mL were used. The EMCCD camera exposure time was 0.05 s and the gain was set in the range of 10-30 (out of camera maximum 300) to fill the dynamic range of the camera. We collected approximately 5 minutes of video with a minimum of 26 spheres viewed for each phantom type.
4.1.3.2 Performance Metrics

Video sequences were analyzed with the CV-IVFC algorithm and cell numbers and trajectories were recorded. Performance metrics for the algorithm were then calculated as follows: i) Sensitivity = TP/(TP+FN) where TP was the true positive count matching the ground truth and FN were the false negative counts (i.e. cells missed by the algorithm), ii) False alarm rate (FAR), which as simply the number of false positive counts per minute. The ground truth was taken as the result of a manual count by a human operator on the same image sequence. The metrics were computed for a range of threshold percentages from 99.90-99.99 of the maximum pixel from Step 1 in the algorithm.

4.2 Results

4.2.1 CV-IVFC with Varying Background Intensity

Example data generated by the CV-IVFC instrument are shown in figure 27. Here, example fluorescence image sequence for phantom type P2 and MS1 is shown (figs. 27a-c) where each image frame is separated by 0.25 ms. After microspheres candidates were identified in Step 1 (figs 27d-f), they were merged into trajectories in Step 2 and then overlaid on white-light images (figs 27g-i). As shown, even though significant background autofluorescence (AF680 and embedded fluorescent
microspheres) was present in the phantom, the algorithm was capable of robustly distinguishing moving spheres from stationary background.

Figure 27- Example image sequence of a single microsphere traveling through a phantom, separated by 0.25s. Fluorescence (a-c) and (d-f) binary images obtained after the thresholding operation (Step 1) of the algorithm are shown. Red circles and arrows indicate the position of the microsphere. In Step 2 dynamic analysis of image sequences was performed, and cell candidates were merged into trajectories, rejecting the stationary or spurious noise evident in (d-f). The recovered microsphere trajectory was overlaid (g-i) on the original white light image.

We systematically tested the CV-IVFC algorithm with phantoms with varying levels of background autofluorescence (phantom types P1-P3) with a single microsphere intensity (MS2). We analyzed image sequences and applied multiple detection thresholds in Step 1 of the algorithm as described in section 2.1.2 and in reference [71]. Example
representative results from 3 individual phantoms are summarized in figure 28. As indicated (and consistent with our previous work), use of a lower threshold increased overall detection sensitivity (fig. 28a) at the cost of increased FAR (fig. 28b), whereas use of a higher threshold reduced both parameters. In general, adjustment of this threshold allowed us to trade-off sensitivity and FAR with the system.

Figure 28-(a) Sensitivity and (b) false alarm rate (FAR) as a function of threshold level (Step 1) for three different phantom types (P1, P2 and P3) and MS2 microspheres.

4.2.2 CV-IVFC Performance with Varying Microsphere Intensity

Next, we tested a single phantom (P2) with different three microsphere types with decreasing fluorescence intensities (MS1-MS3). Again, we computed sensitivity and FAR for a series of threshold values for each case, and the data are summarized in figure
29. Generally, this followed the same trend as above, in that increasing the detection threshold resulted in lower sensitivity (fig. 31a) and lower FAR (fig. 31b).

![Figure 29](image)

**Figure 29**-(a) Sensitivity and (b) FAR as a function of threshold level (Step 1) for three different microsphere types (MS1, MS2 and MS3) and a P2 phantom type.

We also note that, in general, shorter trajectories were extracted by the CV-IVFC algorithm in lower-contrast conditions. Specifically, the average microsphere track lengths were 2.14 mm, 1.42 mm, and 0.82 mm for MS1, MS2 and MS3 types, respectively (fig. 30). Although circulating microspheres were visible for only brief periods in low contrast conditions, CV-IVFC was nevertheless capable of robustly detecting them.
Figure 30- Example microsphere trajectories obtained in phantom models for (a) MS1, (b) MS2, and (c) MS3 microsphere types for a single phantom type P2. (d) The average trajectory length for each of the microsphere types.

4.2.3 CV-IVFC Performance Summary - All Conditions

The overall performance of the CV-IVFC algorithm for the phantom and microsphere tests performed here is summarized in figure 31. We first considered the overall sensitivity of the algorithm for all 27 combinations (3 microsphere intensities x 9 phantoms) as a function of the image contrast. These data are shown in figure 31a. (Here, we selected a threshold of 99.95\textsuperscript{th} percentile for the algorithm in Step 2, although this curve could be re-generated for any other threshold). All phantoms and microsphere combinations that we tested are shown, where the triangle markers correspond to MS1,
squares to MS2, and circles to MS3. As indicated, although 3 sets of nominally identical phantoms were fabricated (P1-P3), significant inter-phantom variability was observed. For this reason we plotted all individual data points (rather than means and standard deviations). As shown, the range of average contrasts (C) for different phantom-microsphere combinations was between 0.23 and 1.54, although the contrast of individual microspheres was as low as 0.06. For comparison, the range obtained from our earlier in vivo experiments is indicated in red (fig. 24 and [71]), where an average contrast of 0.73 was observed. By inspection, C was a poor predictor of algorithm performance for low contrast phantom-microsphere combinations (i.e. when C < 0.5), which, as we have noted was our primary interest in this study. Review of our data suggested that the algorithm yielded highest tracking sensitivity when the background autofluorescence was relatively homogenous, and could be effectively removed by subtraction. Therefore, as an alternative metric we next considered the heterogeneity of the background intensity, which we defined as the number of “bright pixels” per frame in the image sequence (fig. 31b). Here, “bright pixels” was defined as the number of pixels per image after background subtraction that exceeded the mean intensity of a microsphere. In other words, these are the number of pixels where the noise on the pixel was comparable to the intensity of a microsphere; the higher this value. For the phantom experiments, a range of 0.2 to 518.3 bright pixels per image was observed, whereas on average 2.86 bright pixels per image was observed in our previous in vivo data. By inspection, the in vivo data
generally agrees with the phantom data, suggesting that our phantom-microsphere model mimicked *in vivo* imaging conditions well. Somewhat surprisingly (as shown in fig. 31c) the number of bright pixels correlated only weakly with the false alarm rate (FAR), since the CV-IVFC algorithm (Step 2) was efficient at rejecting bright pixels that were physically well separated. Further analysis revealed that a more complex quantity - the number of “bright pixel *clusters*” per minute – correlated well with FAR as shown in fig. 31d. Here, “bright pixel clusters” was defined as the number of occurrences for which two bright pixels were detected in subsequent image frames within a fixed radius (6 pixels). Analysis of our data indicated that in general these led to erroneous formation of microsphere trajectories. As above, our previous *in vivo* data was co-plotted with the phantom data showing good general agreement. The implications of these results are discussed below.
Figure 31 - (a) Sensitivity as a function of contrast for all phantoms and micosphere combinations tested. The mean and standard deviation sensitivity and bright pixel numbers for our previously acquired in vivo data is also plotted for comparison (red line). (b) Sensitivity as a function of “bright pixels” per frame (see text for definition) for experimental combinations tested. Unlike sensitivity, (c) FAR correlated poorly with “bright pixels” but correlated well with (d) “bright pixel clusters” per minute (see text for definition). Previous in vivo data is also shown for comparison in red. Dotted lines in (b) and (d) are linear fits to the data.
4.3 Discussion and Conclusion

CV-IVFC is a new technology for detection, enumeration and tracking of rare circulating cells in small animals in vivo. As we have discussed, the major advantage of CV-IVFC compared to previous IVFC designs is the relatively large circulating blood volume that is sampled, leading to improved detection of low-abundance cells. We previously showed that this instrument was capable of detecting circulating cells in the range of 20 cells / mL. The purpose of the studies performed here was to characterize CV-IVFC under conditions of low imaging contrast compared to our previous in vivo studies.

The data in figure 31 summarizes the effects of reduction in contrast from either mechanism. In combination, figs. 31a and b show the effect of loss of contrast (homogenous and heterogeneous) on detection sensitivity, and illustrates that CV-IVFC can effectively detect and track fluorescent targets with significantly lower contrast than in our previous in vivo studies. To quantify this, we considered the heterogeneous contrast data shown in fig. 31b. Although the parent function for these data is unknown, for comparison purposes we fit a linear function (dotted line on fig 31b; note that this appears as a curve due to the logarithmic x-axis). This fit implies that CV-IVFC would retain 80% detection sensitivity for the case corresponding to 109 bright pixels per image, which is equivalent to 38 times lower contrast versus our in vivo studies, where 2.86
bright pixels per image were observed on average. Likewise, 50% of microspheres would still be detected for the case of 457 bright pixels per image, corresponding to 159 times lower contrast. In practice, this reduction in contrast could come from either less-efficient fluorophore cell labeling, lower fluorescence quantum yield, or increase in background autofluorescence. This greatly increases the potential utility of CV-IVFC since it can be used with a wide range of fluorophores and labeling techniques. Absolute quantification of the mean equivalent soluble fluorochrome (MESF) units for the microspheres was not available from the manufacturer, but comparison with our previous work and literature values suggests that the intensity of the MS2 type would be comparable to a “typical” well-labeled cell. For example, comparison of extinction coefficient and emission quantum yield data suggests that cells labeled with the TurboFP650 red fluorescent protein (RFP; Evrogen, Moscow, Russia) [92-98] would be about 3 times less-brightly labeled than with Vybrant-DiD. Likewise, use of a green fluorophore would reduce contrast by approximately a factor of 10 due to increased autofluorescence [99-101], which (according to the analysis here) would not significantly degrade CV-IVFC sensitivity.

The relationship between false alarm rate (FAR) and bright pixels was more complex, since FAR was found to correlate with the number of “cell clusters” relating to both temporal and spatial distribution of bright targets. In practice, this suggests that relatively high background autofluorescence can be tolerated by the algorithm without
significant increase in FAR provided that bright pixels are physically separated in the image. As noted above, sensitivity and FAR can be traded-off by adjusting the “threshold” parameter in Step 1. In CV-IVFC, false alarms can be easily discounted by a human operator reviewing the original image sequence. Because the algorithm reports the time of detection in the image sequence, this is significantly more time efficient than manually counting cells with long (30 min or more) data acquisitions. Therefore, in practice higher FAR is generally preferable to lower sensitivity in CV-IVFC.

We also note that the results shown here pertain specifically to our existing CV-IVFC instrument and algorithms and do not represent a technical limit of the technique. Improvement of the CV-IVFC tracking algorithm is the subject of ongoing work in our lab; we are currently pursuing a number of alternative strategies, for example, by jointly solving the “detect and connect” problem, as opposed to our current two-step process. Likewise, we have noted that stationary but distributed bright regions (such as sebaceous glands) appearing in multiple images frequently trigger false-positives in the algorithm. As such, we are studying methods to automatically detect such rejoins and reject them as cell candidates. Therefore, we expect that better CV-IVFC performance may be achieved in the future.
Chapter 5 – Frequency Modulated DFFC Instrument

As described in section 1.6.2 of this dissertation, our lab previously developed two diffuse fluorescence flow cytometry (DFFC) prototypes that allowed detection and enumeration of rare circulating cells by sampling relatively large volumes of circulating blood. The first DFFC prototype, built by Zettergren et al., illuminated a sample (phantom, mouse tail or hindleg) with a 642 nm laser and collected diffuse fluorescence light with a ring containing 6 optical fibers connected to PMTs. The output signal of the system was a trace which contained “spikes” indicating a fluorescent cell moved through the field of view.

For this prototype, motion artifacts were a hindrance, especially in the leg when compared to the tail even when tightly securing the anesthetized mouse. The artifacts resulted in spikes in the signal with similar amplitude and temporal characteristics to spikes from cells that could not be removed with data post-processing. The second DFFC prototype, built by Pestana et al., used an open optics system (instead of fiber optic light collection) to improve collection efficiency. It also allowed detection of light at two wavelengths, so that the background signal (and associated motion artifacts) could be removed from the fluorescent signal. The sensitivity was improved on the order of 1 cell/mL of circulating peripheral blood or better.

The candidate developed a number of further improvements on this design as part of this dissertation, which are described in the following chapter. First, the single arm
open optics system based on the work done by Pestana et. al. was expanded to four
detector arms arranged in a ring around the sample. Second, two excitation lasers were
modulated at well separated frequencies in the kHz range, and the output of the PMTs
were analyzed at each frequency with custom built filter and demodulation and circuits.
The goal of this design was to improve both detection sensitivity and tomographic
localization accuracy.

5.1 Methods and Materials

5.1.1 Detection System

A schematic diagram and photograph of the frequency modulated (FM)-DFFC
system is shown in figs. 32 (a) and (b), respectively. Samples (tissue-mimicking optical
phantoms) were placed in the center of system field of view. Two 637 nm diode lasers
(CUBE 637-25C, Coherent, Santa Clara, CA) were placed on opposite sides of the
sample. The output of each laser passed through a 640 nm with 10 nm bandpass ‘clean-
up’ filter (640/10, Edmund Optics, Barrington, NJ) and illuminated the sample with a 1
mm spot size and 5 mW average intensity. The lasers were controlled by two separate
analog output channels of a multi-functional data acquisition card (DAQ NI USB-6212,
National Instruments, Austin, TX). A custom algorithm was written in LabView
(National Instruments, Austin, TX) to control the modulation (amplitude and frequency) of the two lasers.

Each of the four detector arms was positioned 60 degrees from the axis of the lasers’ illumination. Fluorescent light was collected with a 12.5 mm diameter plano-convex lens (Edmund Optics, Barrington, NJ) followed by an 700 nm with a 30 nm bandpass emission filter (700/30, Edmund Optics, Barrington, NJ) to remove any laser light leakage and background autofluorescence. After filtering, light was focused onto a single anode PMT (H6780-20, Hamamatsu Photonics, Bridgewater, NJ). The emission filters were chosen to work well with commonly used fluorophores such as Alexafluor 680, Cyanine 5.5, and Vibrant-DiD.
Figure 32- Schematic diagram (a) and photograph (b) of the FM-DFFC system. D, detector arm; L, laser; f, frequency; DAQ, data acquisition card.
The output of each PMT was passed to a circuit mounted on a printed circuit board with two separate channels made of three distinct blocks as shown in the flow chart in figure 33. Each channel separated the signal according to the two modulation frequencies of the lasers. In the first block, the signal was filtered and amplified by dual stage, fourth order multiple feedback bandpass filter at specific frequencies (2.3k Hz or 6.1k Hz). The signal then passed through an active full-wave rectifier to create an envelope of the signal specific to its frequency channel. Finally the signal was transmitted through a low pass filter with a cutoff frequency of 100Hz corresponding to the known frequency range (0.1-10Hz) of the fluorescent spikes established in our previous work [62-65]. Hence, the fluorescence signal generated by both lasers could be detected simultaneously, eliminating the need for the “off/on” illumination scheme used in our previous work [66].
Figure 33- Flow chart of demodulation circuit on the printed circuit board.

The printed circuit board (PCB) was manufactured by ExpressPCB using a designed we generated with schematic tools (Figure 34) and PCB layout CAD tools (Figure 35). The boards were 3.8 by 2.5 inches with 4 layers (MiniBoardPro).
Figure 34 - Demodulation circuit schematic for one PMT output.
Figure 35- Top silkscreen layer of the demodulation circuit schematic for one PMT output.
5.2 Results

Figure 36 shows the details of each stage of the demodulation circuit. The frequency sweep of the filters (fig 37a) shows the magnitude of the signal passed at each frequency. The bandpass filters with center frequencies, 2,320 Hz and 6,165 Hz, were built to minimize leakage of signal between the two channels. Both bandpass filters have a gain of 7 while suppressing the opposite channel signal. The full wave rectifier clearly reversed negative AC signals (fig 37b) and is built to do the same for cell spikes which appear as negatives voltages from the PMT. The low pass filter passes the known frequency range of fluorescent cell spikes (0.1-10Hz) has unity gain (fig 37c). Figure 37 is a photograph of the assembled printed circuit board.
Figure 36. Characteristics of each stage of the demodulation circuit. (a) Bandpass filter frequency sweep magnitude of both channels. (b) Example of a 1V sinusoidal wave (blue trace) and the signal after rectification (yellow trace). (c) Low pass filter frequency sweep.
Figure 37. Photograph of the assembled printed circuit board.

5.3 Discussion and Future Work

The FM-DFFC prototype has been built, and the circuit has been debugged and optimized for demodulation of two frequencies.

The next step is to test the FM-DFFC prototype with optical phantoms and fluorescent microspheres suspended in PBS as we have done previously. Counting will be done using a threshold and peak detection algorithm or by direct visualization with our existing LabView software. The eight signals from four detector arms will then be analyzed to do tomographic reconstruction of the field of view. This will allow for the localization of the microsphere in the phantom which could be used to eliminate the double count of a microsphere that traveled in one direction in a tube and then come back through the phantom if the tube was folded as show in our previous DFFC system [64]. This mimics an event in vivo when a cell may be double counted due to artery/vein pairs and knowledge of the location could remove this double count event.
Chapter 6 – Near-Infrared Fluorescence Imaging of Nanoparticle Functionalized Spacers

As a separate area of research, we developed an imaging system to characterize nanoparticle diffusion in biological tissue. By using near infrared imaging, we observed fluorescence diffusion of nanoparticles both in phantoms in vitro and in mice in vivo. The imaging system described in this chapter is motivated by our collaborators novel therapeutic spacers and localized drug delivery. Sridhar et. al. have recently developed and reported novel implantable smart nanoparticle (NP) coated brachytherapy spacers which allow controlled, long-term release of chemotherapeutic drugs into tumors [84]. The spacers are coated with therapeutic nanoparticles (NPs) that encapsulate chemotherapy-drug and fluorescent imaging dye which are released steadily over time [78, 80]. Controlled temporal and spatial release of NPs is critical for therapeutic efficacy, but the diffusion kinetics in biological tissue is poorly understood. The goal of the project is to optimize these spacers’ diffusion characteristics for local drug delivery inside the body.

6.1 Motivation for Functionalized Brachytherapy Spacers with Nanoparticles

6.1.1 Goal for Radiation Treatment for Prostate Cancer

Prostate cancer is the most frequently diagnosed cancer and the second leading cause of cancer death in men [102]. Treatment for prostate cancer depends on the stage of
cancer given by levels of prostate specific antigen (PSA) or biopsy grade (Gleason score) [103]. While during the early stage of prostate cancer either radiation therapy (RT) or external beam RT (XRT) may be used. As the disease progresses the RT involving brachytherapy (BRT) is often combined with XRT [104-106]. Increased radiation leads to higher levels of toxicity in the anterior rectal wall and the urethra. While these higher levels assist in managing the cancer, the exposure of the radiation in the two areas mentioned are not acceptable. A set of studies by P. Nguyen et. al. determined that the high levels of radiation lead to toxicity levels where radiation therapy is no longer recommended, but after application of magnetic resonance imaging (MRI)-guided salvage brachytherapy, PSA was controllable 4 years after application in 70% of the patients [107, 108]. During the brachytherapy inert radio-opaque fiducials (XRT) or biocompatible spacers (BRT) are used to control the location and accuracy of the radiation by being implanted into the prostate. Spacers are useful in directing the therapy, but the spacers themselves do not currently have any therapeutic benefits [109-111]. While chemotherapy is used routinely in the treatment of prostate cancer, systemic toxicity due to intravenous delivery is a major problem [112, 113].

6.1.2 Motivation for Development of Nanoparticle Functionalized Spacers

Localized delivery of a chemotherapy agent would eliminate some systemic toxicity while allowing for increased dosage at the tumor site. As noted, spacers are
frequently used as radiation therapy guides and therefore offer the opportunity for in-situ delivery of drugs. By functionalizing the surface of the spacers with nanoparticle encapsulated drugs, the size, shape and delivery procedure for spacers would be unaltered but would carry the added benefit of localized drug delivery. This is shown schematically in fig. 38.

Figure 38- a) Illustration of drug release from a PLGA spacer. b) Photographs of the functionalized spacers and those currently used by clinicians.

Conceptually, spacers would become drug reservoirs, capable of sustained drug release over periods appropriate to radiation therapy. Implantation of the spacers improves the therapeutic ratio of the brachytherapy procedure by delivering radiosensitizer to the prostate without systemic toxicity. Prior work by Sridhar et. al., evaluated the slow-release polymers that release the radio-sensitizer docetaxel from brachytherapy spacers [114-116].
6.1.3 Nanoparticle Diffusion and Motivation for NIR Imaging

The use of NPs on the spacer allows for additional control of the distribution of chemotherapeutic agent. By controlling the speed with which the drug is released, a sustained local delivery of high dose would be ideal in long term treatments. For preclinical research, NPs allow for the addition of imaging agents for observation of distribution of the drug over time and efficacy of the treatment. A new fabrication approach was developed by Sridhar et. al. to produce a biocompatible matrix composed of PLGA polymer as the backbone of the spacers which further contains embedded silica NPs in the matrix as drug/fluorophore depot. These NP loaded spacers – termed “INCeRT spacers” - are morphologically identical to the routinely used brachytherapy spacers but with an additional therapeutic and diagnostic value. In this chapter, NPs were also loaded with a NIR fluorophore (Cyanine 7.5) so that they could be imaged and drug diffusion quantified \textit{in vivo}.

We developed and validated a novel near-infrared (NIR) fluorescence imaging system for measurement of NP diffusion for functionalized spacers in bulk tissue \textit{in vivo}. The goal of this instrument is to allow characterization and optimization of NP functionalization, for example with respect to size and surface chemistry. We chose to use near-infrared (NIR) fluorescent dyes, since NIR is minimally attenuated through biological tissue [3]. We used this imaging platform to study the kinetics of NP diffusion...
first in phantoms *in vitro* and then in mice *in vivo* [74]. It is anticipated that optimization of NP diffusion in time and space will yield new advance cancer treatments [114, 116].

6.2 Methods and Materials

6.2.1 Fluorescence Imager

A schematic diagram and photograph of the fluorescence imaging system used in these experiments is shown in figures 39a and b, respectively. Samples (mice or agar phantoms) were placed on a platform and trans-illuminated with the output of a tunable Titanium:Sapphire laser (Mai Tai, XF-1, Spectra-Physics, Inc., Santa Clara, CA). We chose to use a near-infrared fluorophore and laser combination because attenuation and autofluorescence of biological tissue is well known to be significantly reduced versus blue or green wavelength ranges [1]. The output wavelength of the laser was set to 740 nm (which matched the absorption spectra of the AlexaFluor750 and Cy7.5 dyes). The output of the beam was filtered with a 730 nm “clean up” filter with 30 nm bandpass (z730/30 nm, Chroma Technology, Rockingham, VT) to remove any out-of-band near infrared (NIR) laser output. The 1 mm diameter beam was expanded to approximately 150 cm in diameter using a lens pair in simple telescope configuration (f = 50 mm and 200 mm, Edmund Optics, Barrington, NJ) followed by a third expanding plano-concave lens (f = -25 mm, Edmund). The power at the sample was adjusted using a set of neutral
density filters and was approximately 15 mW (equivalent to 0.015 mW/cm$^2$) for these experiments. A ground glass diffuser plate was placed under the samples to create a relatively homogeneous illumination profile and remove any speckle or interference pattern from the illumination light.

Samples were imaged with a high-sensitivity electron-multiplied charge coupled device camera (EMCCD, iXon$^\text{EM}+855$ Andor Technology, Belfast, Northern Ireland) fitted with a 35 mm lens (NT54-689, Edmund). The imaging field of view was 105 mm x 105 mm at a working distance of 47 cm. Each image was 1004 x 1006 pixels. Camera exposure time and image acquisition was controlled using a personal computer running Andor control software. Although the camera could be operated with intensifier gain, this was turned off for the experiments described below. The camera was fitted with either a 740 nm filter (to record “intrinsic” images at the laser wavelength), with a 40 nm bandpass (et740/40m, Chroma) or a 780 nm longpass filter (et780lp, Chroma) to collect fluorescent light from the sample. The filters were mounted in a five-position filter wheel (LCFW5, Thorlabs, Newton, NJ). White light images of the sample were acquired by moving the filter wheel to an open position so that all wavelengths were detected by the camera. Mice were top-illuminated with a white light source (Fiber Optic Illuminator Model 190, Dolan-Jenner Industries, Lawrence, MA), whereas clear agar phantoms were back-illuminated with a LED ring (Digi-Slave L-Ring 3200, Edmund).
Figure 39-(a) Schematic and (b) photograph of the fluorescence imager used to acquire image sequences for this work. M, mirror; LP, long pass filter; BP, band pass filter; WL, white light.

### 6.2.2 Agar Phantom Preparation and Imaging

Agar phantoms were placed on the imaging platform and trans-illuminated as described above. The movement of free AlexaFluor (AF) 750 dye (Molecular Probes, Life Technologies, Carlsbad, CA), 30 nm nanoparticles, and 200 nm nanoparticles were tested in separate experiments to observe the effect of particle size on diffusion. Free dye and nanoparticles were injected directly into the clear phantom with an insulin syringe. The phantoms were made in 250 mm diameter by 25 mm deep cell culture dishes (Corning Inc., Corning, NY). The phantom material was made with 1.5 grams of Agarose (Mr=0.10, Acros Organics, Geel, Belgium) and 250 mL of distilled water resulting in 0.6% agar gel. The mixture was boiled for 10 minutes until the powdered
agar was dissolved and then poured into the cell culture dish to cool and harden [117, 118]. For experiments involving free dye, AF-750 was injected into the agar phantom and images were acquired at 15 minute intervals for a total of 3 hours (N=3). In this case, it was possible to continuously make measurements without removal of the phantom from the stage. Exposure times were 1 ms for intrinsic images (i.e. at the wavelength of the laser) and were between 0.5 and 4s for fluorescence images, depending on the acquisition time point (later times required longer exposures due to dye diffusion and dilution). For experiments involving nanoparticles (30 nm and 200 nm diameter), the phantoms were imaged daily for 15 days after the injection of the nanoparticles (N=3). To prevent drying of the agar, these phantoms were refrigerated between experiments. A wire marker was placed in the upper left hand corner to allow image co-registration between imaging sessions. For nanoparticle formulations, fluorescence images required between 0.5 and 30 s exposures (as above, longer exposures were required for later time points). White light images were taken of the phantoms by back-illumination with the LED ring during each imaging session.

6.2.3 In Vivo Experiments

The in vivo experiments were conducted in agreement with all relevant guidelines and regulations set by Northeastern University, and with approved institutional protocols by the American Association of Laboratory Animal Care. Six-to-seven week old male
Athymic nude mice (Hsd:Athymic Nude-Foxn1nu) were procured from Charles River lab. All spacers were implanted in the mice using clinical brachytherapy applicator needle.

Two nanoparticle or free-dye coated spacers were implanted in the left and right rear flanks of nude mice (N = 4 spacers per case). Each mouse was imaged once per day for 15 days following implantation. It is important to note that the mice were imaged in trans-illumination mode (as opposed to reflection mode). As we discuss, trans-illumination imaging (combined with image normalization) is significantly more quantitatively accurate when imaging in deep tissue with significant optical inhomogeneities. During imaging, mice were placed on a custom mask to avoid saturation of the camera by direct laser illumination. Three sets of images were acquired during each imaging session as follows: First, white light images were acquired with a 1 s exposure time. Second, intrinsic images (at the wavelength of the laser) were acquired with the 740 nm filter in front of the camera and 5 ms exposure times. This “intrinsic” image was used for normalization of the fluorescence images to improve quantitative accuracy [119]. Third, fluorescence images were acquired with the 780 nm filter in front of the camera and exposure times was between 7.5 and 100 s, depending on the imaging time point.
6.2.4 Image Processing and Data Analysis

We first performed basic pre-processing and normalization as follows. First, a dark field image was acquired (by capping the lens) and then subtracted from each image. All images were normalized to exposure time and laser intensity (which was measured on each day) so that the units were in photon counts/s/mW. Next, fluorescence images were normalized by pixel-by-pixel division by the corresponding intrinsic image. Other authors have shown previously that this normalization operation improves the accuracy of fluorescence images – particularly when imaged in trans-illumination geometry – since it accounts for the laser illumination profile and minor differences in illumination intensity. Normalization also improves quantitative accuracy in the presence of tissue optical property heterogeneities and improves imaging depth [119].

6.2.4.1 Phantom Data

Each of the data sets for agar phantoms injected with either free AF750 dye, 30 nm, or 200 nm nanoparticles was analyzed by first finding the center of the injection site using a custom written MATLAB (MathWorks, Natick, MA) routine. Images were then averaged radially from this center point for each time point. These intensity curves were then fit to a simple one dimensional, point source diffusion equation:

\[
I(x,t) = \frac{A_0}{\sqrt{4\pi Dt}} e^{\left(-\frac{x^2}{4D t}\right)}
\]
where \( I(x,t) \) was the intensity at each time and position, \( A_0 \) was the total amount of diffusing substance, \( D \) was the diffusion coefficient, \( t \) was the time following injection, and \( x \) was the radial position [120]. Equation 7 was fit to the averaged experimental data for each injection type to yield the diffusion coefficient \( D \). The complete spatial and temporal profiles were used in each fit for each case.

6.2.4.2 In Vivo Data

The diffusion process \textit{in vivo} is significantly more complex than in agar phantoms - for example, due to active transport of nanoparticles away from the site of injection, tissue boundaries and inhomogeneities, light diffusion – which prevented application of simple diffusion fitting as above. Therefore, diffusion of nanoparticles and AF750 dye from the implanted spaces was quantified using three additional metrics: i) diffusion area, ii) diffusion profile, and iii) spacer intensity. For the first (diffusion area), we considered the maximum fluorescent intensity for each time point and then computed the fluorescence area for pixels exceeding 50\% of this value on each day (i.e. the “full width at half maximum area”). Pixel numbers were converted to area (in mm\(^2\)) using the calibrated image pixel size of 0.011 mm\(^2\). This area was calculated for each spacer and for each time point (N=4 for each). Second, we computed intensity (diffusion) profiles for the spacers at each time point. Since spacer orientation differed between mice, we first applied a thresholding routine to automatically identify the long and short axes of the
cylindrically shaped spacer in the *in vivo* fluorescence images. Next, the fluorescence profiles were computed by averaging 11 lines orthogonal to the long axis of each spacer. Third, the mean fluorescence intensity of each spacer was computed as a function of time.

### 6.3 Results

#### 6.3.1 Phantom Results

Example fluorescence images measured from agar phantoms injected with AlexaFluor-750, 30 nm nanoparticles and 200 nm particles are shown in figures 40a-d, 42e-h and 42i-l, respectively. As is evident from these image sequences, the free dye diffused significantly more rapidly than the nanoparticles (i.e. on the order of minutes versus days) presumably due to their larger size. As described above, normalized corrected fluorescent profiles were computed and are shown in figures 41a-c for the three sets of injections. By inspection of these figures, diffusion of the nanoparticles was small but significant over the 15 day period, whereas the diffusion of the free dye occurred rapidly over the span of a few hours. In all cases, the measured fluorescence intensity steadily decreased over time.
Figure 40-(a-d) Fluorescent image sequence of an example AlexaFluor 750 dye phantom diffusion at 1 minute, 15 minutes, 30 minutes, and 60 minutes respectively. (e-h) Fluorescent image sequence of one 30 nm nanoparticle phantom diffusion for 1 minute, 6 days, 11 days, and 15 days. (i-j) Fluorescent image sequence of one 200 nm nanoparticle phantom diffusion for 1 minute, 6 days, 11 days, and 15 days. All images are normalized to the maximum at minute 1.

This is shown explicitly in figure 41d, where the maximum intensity is shown as a function of measurement time (intensity data for the free dye is shown in figure 41d inset). In combination, figures 41a-d indicate the anticipated size-dependant diffusion behavior, i.e. that the larger nanoparticles diffused less than either the smaller nanoparticles or the free dye. Likewise, the 1-dimensional diffusion equation (equation
7) was then fit to the data from figures 41a-c. The resulting diffusion coefficients (D) for each of the three sets of phantoms are shown in figure 41e. As shown, the diffusion coefficient for the free dye was 24.92 mm$^2$/day, 0.02 mm$^2$/day for 30 nm NPs, and 0.01 mm$^2$/day for 200 nm NPs. This agrees well with visual inspection of the diffusion behavior in figures 41a-d and the anticipated size-dependency.

**Figure 41**- Diffusion profile curves of the averaged phantom data (N=3) normalized to the intensity at x=0 per time point for (a) free AF750 dye, (b) 30 nm nanoparticles, and (c) 200 nm nanoparticles. (d) Average maximum fluorescent intensity normalized to initial time point per phantom type showing the continual decrease in maximum fluorescence over time. Inset time scale of free AF750 dye diffusion is on the order of minutes compared to days in part (d). (e) Diffusion coefficients found by fitting the diffusion profile curves to Eq. 1 on a logarithmic scale.
6.3.2 *In Vivo* Results

Spacers coated with either free dye (Cy7.5), 30 nm or 200 nm nanoparticles were implanted in the rear flanks of nude mice (N = 4 for each) and then imaged daily for 15 days. Example representative image sets obtained for implanted spacers at a single time point *in vivo* is shown in figure 42. A white light image of the mouse is shown in figure 42a, with the position of the implanted spacers indicated by the red arrows. The normalized fluorescence image is shown in figure 42b. For anatomical reference, an overlay of the normalized fluorescence image over the white light image of the animal is shown in figure 42c. After the animals were euthanized, we also removed the skin layer and the spacers to image the residual fluorophores and nanoparticles without light scatter due to the skin tissue. As shown in figure 42d (in this case for Cy7.5 coated spacer), the residual fluorescence was still clearly visible and demonstrates the continuous diffusion from the spacer over time.
Figure 42. *In vivo* example image sequence of Cy 7.5 spacers. (a) White light image, (b) normalized fluorescence image, (c) normalized fluorescent image overlaid on white light image at minute 1, and (d) Overlaid image on day 15 after dissection of spacer from the mouse.

Complete time-series for each of the 3 spacer types (representative images) are shown in figure 43. As is indicated, all of the spacers decreased in intensity over time due to continuous diffusion of the fluorescence from the spacers, and more decrease was observed for spacers coated with nanoparticles than for free dye.
Figure 43- \textit{In vivo} example image sequence of Cy 7.5 spacers in row 1, 30 nm NP spacers in row 2, and 200 nm NP spacers in row 3. Each row represents the changes over the 15 days of a specific spacer. The images in each row are normalized to minute 1.

Likewise, the fluorescence area appeared to increase over time due to this diffusion process. This is shown explicitly in figure 44. The diffusion profiles for the free dye, 30 nm and 200 nm nanoparticles as a function of distance from the spacer over time are shown in figures 44a-c, respectively. A small amount of diffusion (~1 mm) beyond the profile measured on day 1 is evident in all cases (we note that the diffusion profile observed on day 1 is principally due to light diffusion in scattering biological tissue, as opposed to fluorophores or nanoparticle diffusion from the spacer). The normalized fluorescence intensity for each case is shown in figure 44d, indicating continuous diffusion of the fluorophores and nanoparticles over time. Likewise, the full width at half maximum fluorescence area is shown in figure 44e. As shown, the area of diffusion reached its maximum on day 4 for the 30 nm nanoparticles and day 6 for the 200 nm particles, indicating a size-dependence of the process (i.e. diffusion was slower
with the larger nanoparticles. The fluorescence area decreased after this maxima, presumably due to active transport of the nanoparticles away from the site of the spacer, for example by blood vessels.

![Diagram of fluorescence profile curves](image)

Figure 44- Diffusion profile curves of the averaged *in vivo* data (N=8) normalized to the intensity at x=0 per time point for (a) Cy7.5 dye spacers, (b) 30 nm nanoparticle spacers, and (c) 200 nm nanoparticle spacers. (d) Average maximum spacer intensity normalized to initial time point showing the continual decrease in maximum fluorescence of all the spacers over time. (e) Diffusion results represented by the full width half max area of fluorescence per day.
6.4 Discussion and Conclusions

In phantom testing, the size-dependency of the diffusion process was clearly observed and quantified by fitting to the experimental data to the diffusion equation. Larger diffusion was observed for smaller particles and free dye. Size-dependant free dye and NP diffusion was also consistently observed \textit{in vivo}. However, the diffusion process \textit{in vivo} is significantly more complex than in phantoms due to, e.g. active transport of NPs away from the site of injection, tissue boundaries, and light diffusion. Development of more detailed models for NP diffusion \textit{in vivo} is the subject of ongoing work. Nevertheless, our fluorescence imaging system allowed robust visualization and quantification of NP diffusion from implanted brachytherapy spacers \textit{in vivo}. We were able to consistently observe size-dependent diffusion properties of NPs from the spacers. We next plan to use our fluorescence imaging methodology to optimize NP diffusion in space and time, for example with respect to NP size and surface coating.
Chapter 7 – Conclusions and Future Work

7.1 Computer Vision – In Vivo Flow Cytometry

7.1.1 Summary of Results

CV-IVFC was designed to detect, enumerate, and track rare circulating cells in small animals in vivo. The main advantage of this system over previous fluorescence microscopy systems was due to the number of artery-vein pairs sampled in a 5 x 5 mm$^2$ area which corresponded to a larger circulating blood volume. CV-IVFC was first tested in phantoms in vitro and in mice in vivo. The overall sensitivity was estimated to be approximately 20 cells/mL. The automation of cell detection eliminated the need for a human operator to tediously count cells. In contrast to microscopy-IVFC, CV-IVFC also has the ability to image circulating cells which allowed for observation of cell behavior, e.g. measurement of their speed of travel or the observation of cells docking to the blood vessel walls. Another advantage of CV-IVFC versus other IVFC instruments is that it generates video files that give the user an opportunity to review cell tracks and manually eliminate false alarm counts easily by eye.

In order to determine the performance of the CV-IVFC in low contrast situations, we next fabricated and optimized a phantom model that replicated a range of imaging and contrast characteristics that are observed in vivo when there is increased autofluorescence
or less brightly labeled cells. With the low contrast phantoms, we tested three microsphere types with different intensities. To determine the effect of contrast on system performance, a total of three different phantom batches were tested, and the relationship between the contrast and sensitivity studied. The contrast data was a poor predictor for sensitivity, particularly at low contrast levels where a wide range of sensitivities were seen at a single contrast level. We then analyzed the number of bright pixels per frame above the average microsphere intensity, i.e. the number of pixels where the noise on the pixel was comparable to microsphere intensity. Our analysis indicated that this directly correlated with sensitivity. A linear fit to the bright pixel versus sensitivity data implied that CV-IVFC would retain 80% detection sensitivity for the case when the contrast is 38 times lower versus our in vivo studies and 50% of microspheres would still be detected for the case with 159 times lower contrast. The relationship between FAR and contrast was more complex. It was determined that there was a direct correlation between the number of bright pixel clusters (related to bright pixels near each other in multiple frames) and suggests that FAR would not be significantly affected by a high background autofluorescence provided the bright pixels are well separated temporally and spatially. The sensitivity and FAR have a trade-off by adjusting the threshold set in Step 1 of the algorithm, the higher the threshold the lower the resulting sensitivity and FAR will be.
7.1.2 Future Work

Although CV-IVFC performed well in the models studied here, CV-IVFC can be further optimized to improve system performance. For example, the optimization of collection optics could yield performance improvements. Use of a higher powered objective would improve image contrast and increase the size of circulating cells, but this will result in a smaller field of view, smaller depth of field, and less blood volume interrogated at a time.

The computer vision algorithm could be further optimized. One approach would be to combine the two step detection process and solve the detection problem in a single step. Another simple approach would be to analyze specific regions of the image sequence to minimize false alarm candidates. For example, sebaceous glands that appear as stationary bright regions in multiple images could be automatically detected and removed from the analysis. Likewise, the area of analysis could be reduced to only the blood vessels where cells are most likely to appear. However, this would eliminate the capillary bed where approximately 5% of the total cells were observed, so that this would result in some loss of sensitivity. Both of these solutions would add a pre-processing step to the algorithm but would reduce the computational load of the trajectory merging step.

Additional algorithms could be implemented to the cell tracking algorithm to detect and characterize adhesion, rolling and extravasation of immune cells in response to inflammation along blood vessel walls. For example, it would be possible to study
leukocyte homing in response to a perturbation of the tissue (inflammation). The number of cells recruited and the distribution of recruitment times could be measured [121-124]. Visualization of the adhesion cascade of leukocytes to the inflammation location and transmigration through the endothelium would be beneficial in the study of the disruption of this behavior following drug intervention such as anti-inflammatory agents. In order to see this behavior, a higher-powered objective is necessary but again there will be a smaller field of view and smaller depth of field.

Performance of the CV-IVFC under low contrast conditions has been tested \textit{in vitro}, but the system should also be tested with a variety fluorescently labeled cells \textit{in vivo}. Fluorescent labeling schemes of interest include red fluorescent proteins and receptor-targeted nanoparticles. Green fluorescent proteins are used in many biological models, and CV-IVFC could be modified to work in this wavelength region. This would require changing the excitation laser along with corresponding excitation and emission filters. While our \textit{in vitro} models suggest this is feasible, this should be directly verified experimentally.

\section*{7.2 Frequency Modulated – Diffuse Fluorescence Flow Cytometry}

FM-DFFC uses two modulated lasers and four detector arms, yielding eight simultaneous measurement channels after demodulation of the signals. The FM-DFFC has many ongoing technical improvements. For hardware, the printed circuit board can
redesigned for additional layout optimizations and bandpass filter improvements. The frequencies of modulation can be adjusted to fit any special user requirements. A power board can be laid out on a printed circuit board and optimized to ensure user control over PMT gains. Additionally, adding more sensors will improve the accuracy of cell location and sensitivity.

Future work for this system includes testing and validation with phantoms \textit{in vitro} and in mice \textit{in vivo}. In addition to detection, the capability of FM-DFFC to localize fluorescent targets should be verified both in phantoms and in mice. The tomographic localization will help in eliminating double counts of cells seen both in arteries and veins (where blood flows in opposite directions) and provide a more accurate count of cells.

\section*{7.3 Fluorescence Imaging of Nanoparticle Diffusion}

\subsection*{7.3.1 Summary of Results}

The fluorescence imaging system allowed robust visualization and quantification of NP diffusion \textit{in vitro} and implanted brachytherapy spacers \textit{in vivo}. Results showed consistent size-dependent diffusion properties of NPs from spacers. First, larger diffusion for small particles (i.e. free dye) was observed. Secondly, larger NPs took longer to reach a peak area of diffusion meaning that they could provide longer treatment but more spacers may be needed in the bulk tissue to treat the entire area of concern.
Finally, fluorescence area decreased after these maxima due to active transport of the NPs away from the site of the spacer. This transport may provide an idea for optimal NP size to prevent the removal of fluorescent dye (drug) from the area of interest.

### 7.3.2 NP Imaging Future Work

Improvements to the system include a more systematic approach to mouse positioning on the imaging platform. This would remove any shifts in body position due to different limb positions which were corrected with adhesive in this study and improve image co-registration between imaging sessions. When illuminated with appropriate wavelengths for the fluorophore of interest, other dye could be detected, e.g. Alexa Fluor 647 which has excitation around 635 nm and emission around 660 nm. NIR fluorescent probes are optimal compared to shorter wavelengths in whole mouse imaging due to the light absorption and scattering characteristics of tissue. The imager was used in a wide-field macroscopic setting, but the fluorescent light collection optics and the camera could be moved closer for a smaller field of view. This would provide higher image contrast, but the imaging depth would decrease.

The current approach was a simple one dimensional diffusion equation which did not account for all the variables found in vivo, such as tissue type, boundaries between tissues, blood flow, and removal of NPs and dye (or drug) by macrophages. Future work with this system would be to develop more detailed models for NP diffusion in vivo (e.g.
models that would include active transport from the site from blood and lymphatic vessels). Additionally, more NP testing and optimization can be performed, for example, to quantify diffusion characteristics with respect to NP size and surface coating.

7.4 Future Work

In conclusion, all three systems were able to track and monitor targets of interest with near infrared fluorescence imaging. A possible future application that would incorporate both our novel in vivo flow cytometry technology and the widefield fluorescence imaging system would be to monitor cancer metastatic progression and new treatment strategies. The integrated system, for example, would allow study the growth, metastatic spread and response of PC3 prostate tumors to therapeutic nanoparticles. Mice would be implanted either sub-cutaneously or orthotopically with $10^6$ PC3 cells suspended in matrigel. Nanoparticle-coated fiducials will be implanted in the PC3 tumors as above, and the diffusion of the drug will be imaged and quantified. We will also add a second excitation laser (660 nm) and appropriate optical filters to the setup to allow imaging of Cy5.5 fluorophores; this will allow detection of apoptosis (using an Annexin-V-Cy5.5 conjugate) in the tumors in response to the drug bearing fiducials. In combination, the two techniques will allow us to quantify the effect of the therapy on the tumors, both in terms of chemotherapy induced apoptosis, tumor growth and number of circulating metastatic cells.
While cancer may have been the motivation behind the individual systems, the applications can be expanded to other diseases or cells of interest. The *in vivo* flow cytometry systems can be used to track any fluorescently labeled circulating cell of interest, i.e. red blood cells affected by sickle cell anemia and T-Lymphocytes in HIV animal models. Finally, the nanoparticle spacers can be used in other applications where there is a need for long term drug release, i.e. birth control and macular edema patients.
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