Understanding Neurodegeneration with Multi-Scale Images
- An Integrated Neural Image Analysis System

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Abstract

Neurodegenerative disorders are a heterogeneous group of genetic disorders characterized by loss of neuronal structures and functions. This is a family of most prevalent and least understood diseases. For example, to date, the Alzheimer's disease remains incurable and current treatment options only temporarily ameliorate the symptoms and do not stop disease progression. In neurodegenerative diseases, a pronounced loss of neuronal projections and synapses has been observed in the brains of patients as well as in murine models. The morphological changes in neuronal structures are heavily involved in neural functions such as information transmission, learning, memory and attention. These changes significantly precede any other pathological features and provide an early indicator of disease progression. To study the mechanisms of neurodegenerative diseases and develop therapies, images taken from different scales are employed to illustrate structural deterioration or changes to provide powerful information for disease mechanism research and drug development. As most of neurodegenerative diseases involve dysfunction of synapse, imaging synaptic structures is of particular interests. In this work, we propose a system approach designed for large-scale mechanism studies and drug development by analyzing multi-scale neuron images of synaptic structures, including dendritic spines, synaptic vesicles and neurites. These three structures are the most important components in synapse functions including synaptic information transmission and storage. We propose curvilinear structure detector based dendritic spine detection strategy and employ a novel level set method to segment the spines. A pipeline based on multi-scale variance stabilizing transform followed by region based adaptive thresholding is proposed to accurately segment and quantify synaptic vesicles in an automated manner. To illustrate how the system can be used in large-scale screenings, an example of screening more than one thousand small molecules to identify inhibitors of neurite loss induced by Aβ peptide is provided using proposed neurite image processing module. The screening results are further analyzed as potential treatment for Alzheimer's. With the proposed system, scientists and biologists could employ it to large-scale study on responses of a population of neural cells under different chemical, genetics, or radiation perturbations without manual labeling and quantification to promote the neurodegenerative disease research and treatment development.
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To my family...
I. INTRODUCTION

A. An overview

Some of the most prevalent and least understood diseases are those that affect nervous system function. Neurodegenerative disorders are a heterogeneous group of genetic disorders characterized by loss of neuronal structures and functions. Neurodegenerative processes result many neurodegenerative diseases including Alzheimer’s disease, Parkinson’s disease and Huntington’s disease. Neurodegeneration can be observed in many different neuronal circuit levels, ranging from molecular to systemic. Morphological changes, even loss of neuronal structures are common among these diseases, such as dendritic spine density reduction and neurite outgrowth fail. In Alzheimer’s disease, for example, as well as other neurodegenerative diseases, a pronounced loss of neuronal projections and synapses has been observed in the brains of patients, as well as in murine models. These changes significantly precede any other pathological features and provide an early indicator of the disease progression. Parallels between different neurodegenerative disorders including atypical protein assemblies are identified as well [1]. In addition, many similarities among these diseases on a sub-cellular level appear and relate these diseases to one another. These parallels and similarities enable the possibilities that neurodegenerative diseases could be studied and addressed by similar methodologies. Although mechanism research and therapy development were conducted for decades, unfortunately, this disease category remained one of the least understood disease categories and no cure to slow or stop the disease progression.

To study the mechanisms of neurodegenerative diseases and develop therapies, thanks to the development of imaging techniques, images taken in different scales are employed to illustrate structure modifications to provide powerful information in both academia and industry. As illustrated by Figure 1, functional MRI (fMRI) can be used to investigate the involvement of brain regions in various cognitive and perceptual tasks. Neuropathology assay is a commonly used method based on standard histological techniques to detect off target toxicology effects leading to gross morphological changes at the tissue level. Specialty stains include neurons,
myelin fibers, glial cells, and others. However, these two imaging strategies do not provide information about the subtle morphological changes with their resolution scales. The morphological changes in neuronal structures are heavily involved in neural functions such as information transmission, learning, memory and attention [2, 3]. For example, the variations in dendritic branch morphology and spine density provide insightful information about the brain function and possible treatment to neurodegenerative disease, such as investigating structural plasticity during the course of Alzheimer’s disease.

As synaptic dysfunctions appear in most of neurodegenerative diseases, images of synaptic structures are of particular interests. These structures include dendritic spines, synaptic vesicles and neurites, which are most important synapse related structures, from post-synapse, pre-synapse, to synaptic information transmission. Therefore, with technology advances in imaging, subtle changes in neural structures can be traced under different conditions or in a time series to provide powerful information in mechanism research and drug development. Similar to the modern communication systems, these three structures covers three must components in neuron signal transmission: signal sender (synaptic vesicle in presynaptic structures), signal transmission cable (neurites) and receiver (dendritic spines). Therefore, these structures are not functionally related, but also structurally connected to each other.

The dendritic spines, neurites and synaptic vesicles are imaged at different scales to study the mechanisms of neurodegenerative diseases and develop therapies. Figure 1, from left to right, illustrates synaptic vesicle bouton image, dendritic spine image and neurite image, in the cellular level with different resolution scales. The resolution scale of dendrite spine imaging is 0.01-0.1 \( \mu m / pixel \). The analysis of dendritic spine morphology is an important endeavor of neurobiology research, since over 90% of excitatory synapses in the brain occur on dendritic spines and protrusion [4]. Dendritic spine morphological characteristics are closely related to neural functions such as learning, memory and attention. The dendritic spine assay is developed to study the post-synapse mechanism. The synaptic vesicle assay is developed to estimate the activity and function of live or cultured neurons in presynaptic imaging. The vesicle bouton imaging has the finest resolution among these three modalities, 0.001-0.1 \( \mu m / pixel \). Recent studies on neurodegradation diseases, such as Alzheimer’s disease and Rett syndrome, include evidence provided by analysis of synaptic vesicle activities [5-10]. The quantity and intensity of
vesicles in presynaptic imaging are the most significant features for estimating the activity and function of live or cultured neurons [9, 11, 12]. Among these three imaging scales, neurite imaging has the coarsest resolution at 1-5 μm/pixel. Since proper neuronal migration and establishment of circuitry are key processes for nervous system functioning, the neurite outgrowth imaging can be used to quantitative determine the factors that influence neurite formation and repulsion based on prior hypothesis. Therefore, these different imaging modalities under different scales include the information initialization, storage, receiving and transmission in the neural network, which, of no doubt, can be employed in neurodegenerative disease research with both structural and functional connections.

Figure 1 Multi-scale neuron images to study neurodegenerative diseases.

In the neural network, neurites develop toward other regions of the nervous system, thus neurite outgrowth length is a critical event in neuronal development, formation and remodeling of synapses and so on [13]. Neurites are comprised by axons and dendrites, where locate the synaptic terminals. In synapse structures, the presynaptic part is located on an axon while the post-synaptic sites are located on a dendrite. With the synaptic vesicle bouton image and dendritic spine image, quantification of the subtle synaptic alterations including both presynaptic
bouton function and postsynaptic dendritic spine morphology can be traced at the neurite terminals. These are the structure connections of the mentioned three scale neuron images.

What makes things better is that, with the advances in technology in the system biology and medicine, large-scale imaging is available to generate huge amount of image data, such as high content screening (HCS) of a 1,000-compound-library would typically generate more than 50,000 images, each containing at least 500 synapse structures. Techniques have been developed for analyzing pre- and post-synaptic activities at extremely high sensitivity. Among these techniques, optical microscopy imaging is increasingly used for its many advantages [11]. Compared to traditional electrophysiology, imaging methods allow the locations of neurons and synaptic connections to be identified, and hundreds or even thousands of neurons can be studied at a time so that a more comprehensive understanding of circuit function can be obtained. Optical microscope imaging is also less technically demanding and can be carried out with fewer manual interventions. With automated fluorescence microscopes now becoming affordable, regular labs can readily acquire more than 50,000 images daily, thus image acquisition is no longer a hurdle for neuroscience research [14]. By combining the HCS technique and advances in imaging neuron structures, especially the synapse structures with microscopy, statistically reliable and consistent large-scale experiments with images data might lead to new discovery and treatment for neurodegenerative diseases.

With all above imaging modalities and advances in large-scale imaging, there is a further step needs to be realized: how to extract information from images. Information can be acquired by image quantification and statistical analysis to infer biological conclusions. Morphological features, such as size, length, shape, as well as intensity are key elements to measure outcomes of a potential drug screening, a gene silencing study, a treatment experiment and other types of procedures [15]. With quantified features and statistics, how to draw a convincing conclusion is significant to answer the initial scientific question or to validate the original hypothesis. For example, given certain morphological signatures, whether a treatment is effective, or a knocked-out gene has a significant effect on the downstream pathway, answers to similar questions are usually the original purposes to develop the assay or similar types of experiments with those signatures. Therefore, image analysis is of great significance and necessity in the image-based studies for neurodegenerative disorders. For large amount data generated in large-scale studies,
automated method is valuable. Taking the HCS screening example mentioned earlier, even an expert would take years to manually process all the images reliably. Moreover, while prominent structures can plausibly be scored by human visual inspection, small changes in the proportions of various phenotypes, which may be informative, would likely be missed. In addition, visual inspection is susceptible to observer bias, and thus will not always lead to reproducible and comparable results. Therefore, to acquire information from image data, automated, fast and batch processing capable detection and quantification are necessary for a HCS system even for some small-scale experiment.

In this work, we will propose a system to process and analyze multi-scale neuronal images of synapse related structures to systematically study the neurodegenerative diseases with state-of-the-art imaging technologies. It closes the gap between informatics and wet lab experiments. By imaging these synaptic structures in different scales, the proposed system can be used in mechanism studies, early stage in vivo drug testing and pathway studies by tracking the morphological changes and neuron loss under different conditions. In addition, with the development of high content screening (HCS) applied in neurology studies, the proposed system is ideal for large-scale screening based studies for drug testing and development. By analyzing image data generated from HCS with neural structure detection and quantification, as well as statistical analysis, we could significantly improve the efficiency and consistency in large scale screening results analyses. This approach can be applied to any of these three image analysis combined as a systematical and powerful proof of study or individually addressing function of any structure of interest. This system includes novel image processing algorithms to detection different neural structures by addressing three structures individually. In addition, these algorithms are optimized for HCS processing, such as automated, fast, loose parameter settings and so on. We also propose different metrics to measure the neuron activities and screening results to provide quantifications. In Figure 2, we summarize the input and the key features of the system. With the proposed system, scientist and biologist could employ it to study responses of a population of neural cells under different chemical, genetics, or radiation perturbations without manual labeling and measuring for different purpose to speed up the neurodegeneration research.
Before we provide detailed descriptions of our approach in the next chapters, we briefly explain the algorithms developed in this study for different synaptic structures. In this thesis, we illustrate this system and provide image data in the context of studying Alzheimer's disease (AD) related neuronal dysfunction at the pre- and post-synaptic levels to evaluate its performance as an example for other neurodegenerative diseases, as well as some neurodevelopmental disorders. First, it is increasingly clear that dendritic spine abnormalities may underlie part of the pathophysiology of neuropsychiatric and neurodegenerative disorders [16]. Consistent with this hypothesis is the observation that neurons derived from the Tg2576 mouse model of AD exhibit significant degenerated dendritic changes, as compared to wild-type neurons [17]. Considering the in vivo image quality in one of our data source and high diversity of complex spine structures, we propose an automated algorithm based on curvilinear structure detection and a modified level set method to identify and quantify dendritic spines. This method works for both in vivo image that traces dynamics of spines and in vitro HCS image so that high-throughput studies for effective compounds discovery can be carried out on dendritic spine structures. Second, recent studies suggest that pre-synaptic dysfunction might be a converging early pathogenic event.

**Figure 2** System level description of the proposed system with its inputs and key functions.
before neurodegeneration in diseases such as AD [18]. Consistent with these findings, beta amyloid (Aβ) peptide was recently discovered to be a positive regulator of pre-synaptic release probability. It was hypothesized that the synapse activity enhancing role of Aβ might point to the primary pathological events that lead to compensatory synapse loss in AD [9]. HCS for compounds attenuating the pre-synaptic dysfunction caused by the Aβ peptide may lead to the discovery of novel therapeutic treatments. We propose a novel pipeline with multi-scale variance stabilizing transform and modified adaptive thresholding to achieve the fully automated image analysis so that pre-synaptic activities can be analyzed in a high-throughput manner. Third, to obtain statistical quantifications from neuronal images, we process neurite outgrowth images from nuclei and neurite channels separately. In the nuclei channel, nuclei are segmented using the marker-controlled watershed method. In the neurite channel, neurites are treated as 2D curvilinear structures, which allow the detection of the center points as well as the local directions. We screen the NINDS custom collection compound library II containing 1,040 known bioactive small molecule compounds, for reagents protecting Aβ1-40 induced degeneration of neuronal processes. The algorithm accurately measured a reduction of neurite length in response to Aβ1-40 peptide treatment and allowed us to assess the rescuing effect of each compound. As we mentioned, all algorithms for different structures in various scales require minimal human intervention and are optimized for the HCS large-scale studies.

In the following sections of this chapter, we will provide background knowledge in the biology, neurology and system development. Information such as introduction to neurodegenerative disease, how these structures are imaged and detailed reason why this system is valuable and unique will be provided in the remaining of this chapter.

B. Background knowledge in biology

In this section, to better explain why the multi-scale neural image analysis system is necessary and illustrate what problem we are trying to solve, we will provide basic biology backgrounds and stories of related topics, such as brief introduction to neurodegenerative disease, Alzheimer's disease (AD) and beta amyloid (Aβ). In addition, a closer look at functions and structures of dendritic spine, synaptic vesicle and neurite will be provided. Most of the
introductions are very general to prepare readers for the rest of this dissertation. Technical details are referred to references.

1) Neurodegenerative disease

Neurodegenerative diseases are defined as hereditary and sporadic conditions that are characterized by progressive nervous system dysfunction. Neurodegenerative diseases include Alzheimer's disease, Huntington's disease, Parkinson's disease and so on. These disorders are often associated with loss of structure or function of neurons, including death of neurons of the nervous system. They slowly cause all bodily function to cease working, making the concerned patient in a vegetable state. Unluckily, the cause of neurodegradation remains not fully discovered, one reason is the complexity of involvement of different levels of neuronal circuitry ranging from molecular to systemic. Therefore, neurodegenerative disease is one of the most dangerous and incurable diseases known today.

Alzheimer’s disease (AD) is a typical neurodegenerative disease suffered by seniors. It is an irreversible, progressive brain disease that slowly destroys memory and thinking skills, and eventually even the ability to carry out the simplest tasks. In most people with Alzheimer’s, symptoms first appear after age 60. Estimates vary, but experts suggest that as many as 5.1 million Americans may have Alzheimer’s disease [19]. Alzheimer’s disease is the most common cause of dementia among older people. Dementia ranges in severity from the mildest stage, when it is just beginning to affect a person’s functioning, to the most severe stage, when the person must depend completely on others for basic activities of daily living.

Although how the Alzheimer’s disease process begins still remains unknown, it seems likely that damage to the brain starts a decade or more before symptoms become evident. People do not show any sign of symptoms but toxic changes are taking place in the brain during the preclinical stage of Alzheimer’s disease. The cause and progression of Alzheimer's disease are not well understood. Strong links with amyloid plaques and tau tangles throughout the brain are indicated by several independent research [20]. Hypotheses based on tangles and plaques are dominated in the pathology studies as well as drug discovers. Before long, the damage spreads to a nearby structure in the brain called the hippocampus, which is essential in forming memories. As more neurons die, affected brain regions begin to shrink. Over time, neurons lose their ability
to function and communicate, and die eventually. Currently no treatments to delay or halt the progression of the disease are, as of yet, available.

Figure 3 Pathology features of Alzheimer's disease. (a) brain comparison; (b) illustration of AD pathology in developing tangles and amyloid plaques.

Cerebral plaques laden with β-amyloid peptide (Aβ) and dystrophic neurites in neocortical terminal fields as well as prominent neurofibrillary tangles in medial temporal-lobe structures are important pathological features of Alzheimer's disease [21]. Loss of neurons and white matter, congophilic (amyloid) angiopathy, inflammation, and oxidative damage are also present. Among this, the "amyloid hypothesis" is the most widely accepted mechanism by the community[21]. This hypothesis states that an imbalance between production and clearance, and aggregation of peptides, causes Aβ to accumulate, and this excess may be the initiating factor in Alzheimer's disease [21, 22]. As a natural product of metabolism, Aβ is neurotoxic to synapses. Alzheimer's disease may be primarily a disorder of synaptic failure [6]. In mild Alzheimer’s disease, there is a reduction of about 25% in the presynaptic vesicle protein synaptophysin [21]. With advancing disease, synapses are disproportionately lost relative to neurons, and this loss is the best correlate with dementia [21].

With the development of Alzheimer’s disease, neuropil in vulnerable brain regions undergoes extensive degeneration, including neurite morphological changes and dramatic synapse and dendritic spine loss [22]. Dendritic spines, small protrusions from dendrites, form
the postsynaptic element of the vast majority of cortical synapses and serve to compartmentalize postsynaptic signals [23]. Most excitatory synapses in the brain are located in dendritic spines which are lost in Alzheimer’s disease [24]. The loss of synaptic structures is fundamental to the collapse of brain function because synapse loss is a strong correlate of cognitive decline in AD [22]. Synapse loss appears particularly relevant because it closely correlates with degree of cognitive impairment. In addition, Changes in spine morphology and density are postulated to be structural correlates of plasticity involved in higher cognitive functions such as learning and memory[25, 26]. From a biochemical and genetic perspective, accumulation of Aβ seems to be the driving force behind disease progression; however, the relationship between Aβ and synapse loss remains unclear[22]. Synapse and spine loss have been observed in several strains of transgenic mice that develop amyloid pathology, with the most pronounced loss near dense-cored amyloid plaques[22]. Loss of synaptic connections and neuronal projections has been shown to be a common feature of many neurodegenerative diseases including Alzheimer’s disease.

2) Microscopy imaging

To visualize the structural change in neuron morphology, different imaging technologies are development. Microscopy is the technique using microscopes to observe and imaging samples and objects that cannot be seen with the unaided eye. Two types of microscopes are widely used in neurological studies including AD, both of which are fluorescent microscopes and objects are neuron cells and tissues from mouse models. The fluorescence microscope is based on the phenomenon that certain material emits energy detectable as visible light when irradiated with the light of a specific wavelength. All fluorescence microscopy methods share this principle. A sample is illuminated with light of a wavelength that causes fluorescence in the sample. First, the microscope has a filter that only lets through radiation with the desired wavelength that matches the fluorescing material. To become visible, the emitted light is separated from the much brighter excitation light in a second filter. The fluorescing areas can be observed in the microscope and shine out against a dark background with high contrast. Figure 4 illustrate two types of microcopies and their differences in imaging principles.
The first one is confocal microscopy, which is an optical imaging technique that increases both optical resolution and contrast of a micrograph by employing point illumination and a spatial pinhole to eliminate out-of-focus light in specimens that are thicker than the focal plane [27]. 3D structures can be reconstructed from image obtained by confocal microscope. Confocal microscope employs point illumination and a pinhole in an optically conjugate plane in front of the detector to remove out-of-focus signal. The resolution of image acquired by confocal microscope is much higher than that of wide field microscopes because it detects only light produced by fluorescence very close to the focal plane. However, as much of the light from sample fluorescence is blocked at the pinhole, this increased resolution is at the cost of decreased signal intensity. Therefore, long exposures are often required. In our studies, neurite image data are generated by confocal microscopy.

Figure 4 Confocal (a) and multi-photon microscopy (b) and their imaging principles (c).
The second common configure is multi-photon laser scanning microscopy. In our studies, both dendritic spine image and synaptic vesicle image data are generated by tow-photon microscopy. Two-photon microscopy allows imaging of living tissue up to a very high depth. It employs red-shifted excitation light which also excite fluorescent dyes. Two photons of the infrared light are absorbed for each excitation to minimize scattering in the tissue. Meanwhile, the background signal is significantly suppressed due to the multi-photon absorption. These configurations advance an increased penetration depth of the microscope. Two-photon excitation can be a superior alternative to confocal microscopy due to its deeper tissue penetration, efficient light detection and reduced phototoxicity[28].

3) Neuron morphology

**Figure 5** An illustration of neuron structures [29].
The morphological shapes of nerve cells are extraordinarily complex. Unlike most other cells, neurons have long and branching processes: axons and dendrites with spines [30]. Dendrites conduct signals from postsynaptic terminals to integration site, which is often the cell body. Axons conduct signals from the cell body to presynaptic terminals. The functions have been reveals more than one hundred years ago [30]. An illustration of neuron structures, including dendritic spines, synaptic vesicles and neurites, is provided in Figure 5.

**Dendritic spines** are bulbous membrane protrusions that form the postsynaptic specializations of the vast majority of excitatory synapses in the central nervous system (CNS) [31]. Most excitatory synaptic transmission happens at dendritic spines. The spine structure and density are important features of synaptic function [22, 31, 32]. An important feature of dendritic spines is that their volume and density can be dynamically regulated. Stimuli that induce long-term potentiation (LTP) and long-term depression in hippocampal slices lead to rapid changes in spine volume by activation of NMDA receptors [31, 33-35]. In addition, it is increasingly clear that dendritic spine abnormalities may underlie part of the pathophysiology of neuropsychiatric and neurodegenerative disorders [16]. Prominent changes were observed in AD on the dendritic spine structures [36, 37], where most glutamatergic synapses form and the establishment and remodeling of connectivity within neuronal circuits occur [22, 38]. Spine alteration is thought to be one of the earliest events in AD and was observed on embryonic neurons from transgenic APP (amyloid precursor protein) mutant animals [17]. Development of techniques that can prevent or rescue these alterations may lead to therapeutic strategies to intervene with the earliest pathological event in AD and, therefore, achieve efficient treatment of the disease.

A **neurite** refers to any projections, including axon and dendrite, from the cell body of a neuron. These neurites develop toward other regions of the nervous system or other structures on which the neurons will form synapses, such as glands, muscle, etc. Neurons eventually become assembled into functional networks. Since the proper functioning of the nervous system depends on the formation of proper connections, neurite outgrowth length is a critical event in neuronal development, formation and remodeling of synapses, response to injury, and regeneration [13]. Changes in the pattern of neurite outgrowth have been implicated in neurodegenerative disorders as well as traumatic injuries. Consequently, the neurite length can be used in drug discovery and
pathway analysis as an important quantitative measurement of drug effectiveness or significant component in a signaling pathway. In AD, neurite degeneration is observed in the areas responsible for higher cognitive functions, which are the primary areas affected by this devastating disease. It significantly precedes neuronal cell death suggesting that neurite damage and loss might be the primary cause of the decline in cognitive function [39].

**Synaptic vesicle**, located in the pre-synaptic axon terminals of nerve cells, is a small membrane-bound structure that contains various neurotransmitters and releases them by exocytosis when an action potential reaches the terminal [40]. They play essential roles in nerve impulse propagation when they release neurotransmitters at synapses [40]. The quantity and intensity of vesicles are of most significance features to estimate the activity and function of live or cultured neurons [9, 11, 12]. Recent studies suggest that pre-synaptic dysfunction might be a converging early pathogenic event before neurodegeneration in diseases such as AD [18]. Presenilins, the major cause of familial AD, has been shown to have a role in the regulation of pre-synaptic neurotransmitter release [41]. Subjects with mild cognitive impairment display a paradoxical elevation in glutamatergic pre-synaptic bouton density [42]. More recently, it was shown that endogeneous Aβ can enhance the pre-synaptic vesicle releasing probability, and it is hypothesized that the synapse activity enhancing role of Aβ might point to the primary pathological events that lead to compensatory synapse loss in AD [9].

4) **High content screening**

With the development of imaging technology, large-scale studies are employed to enable the power of the screening technique and statistical analysis in mechanism studies and drug discovery. High content screening (HCS), one of the most commonly used large-scale screening strategy, allows functional analysis of targets and pathway modulation in cells by drug compounds in a high-throughput manner. It has emerged as a promising solution to improve the quality of decision making in drug development. Cell-based assays are assays prepared in multi-well formats, such as 96-well and 384-well plates, for high-throughput screening to study responses of a population of cells under different chemical, genetics, or radiation perturbations. They are widely used for the development of new drugs starting from primary screening to in vitro toxicology. Extracting quality information in bioassay development and screening is
enabled by a powerful combination of multi-dye fluorescence imaging, flexible analysis algorithms, and full system automation. HCS is a powerful tool for disease diagnosis and prognosis, drug target validation, and compound lead selection. However, tools required for processing and analyzing HCS data are rather immature. Different types of screened objects require different automated processing procedures. Current optical microscopy techniques, coupled with a large arsenal of fluorescent and other labeling methods, generate tremendous numbers of digital images ready for quantification. However, the analytical tools have not kept pace with these developments. While existing tools, such as NIH Image (available as Scion Image or Image/J), MetaMorph, UTHSCSA ImageTool, QED Image, and In Cell Developer, perform reasonably satisfactorily to process standard images, they are extremely limited in their scope and capability for high content cell image analysis, especially for complex shapes or multi-spectral correlations of neuronal cells. Therefore, although the synaptic alterations and neurite outgrowth can be well resolved by microscopic imaging, high-content screening (HCS) studies for discovering novel synaptic alteration inhibition compounds or identifying reagents that may prevent neurite losses or stimulate neurite regeneration have been prevented or hampered by the lack of bioinformatics tools capable of automatically quantifying the subtle change on the delicate synapse.

C. Desired properties and quantifications needed by the system

Starting with brief introduction to neurodegenerative disease and Alzheimer's disease, we explained the microscopy image acquisition, synapse related structures and HCS system. This information prepare us with basic idea of what kind of problem we are facing and what resources are available to us. However, there is still a missing part. To capture statistical trend and make powerful and reproducible conclusions, the large-scale studies in neurodegenerative disease should be performed and analyzed unbiasedly, efficiently and consistently. Due to the nature of this type of study, automated methods would be valuable to keep consistent benchmark, save biologists’ efforts and meet the requirements of larger scale analytics. In this section, we will define the desired features of the proposed multi-scale neuron image analysis system and important measures of the synaptic structures designed for large-scale studies.
1) **Automated and batch processing**

The high throughput manner recruits robotics, data processing and control software, liquid handling devices, and sensitive detectors to allow researchers to quickly conduct millions of chemical, genetic or pharmacological tests. Once the assays are prepared in larger scale studies, the advances in microscopy or other types of imaging techniques allow fully automated imaging and data storage. However, these large-scale screenings still suffer in lack of fully automated processing tools which can extract information and statistics without human intervention with reliable results. For example, with more detailed in Chapter V, we screened the 1,040 compound known bioactives NINDS custom collection library II in primary mouse cortical neurons in the presence of Aβ1-40 and intended to identify inhibitors of Aβ-induced neurite loss. It is impossible for human manually labeling and measure for image data generated from 1040 compounds and couple of duplications for each compound. An automated neurite image quantification method is must for processing thousands of image data generated. It takes even experts years to manually label and quantify all the neurites on thousands of 2048-by-2048 images. There are some semi-automated dendrite detection software available which still cannot meet the requirement in such a large-scale study because they all need user to manual assign the starting and ending point of each single neurite. This example illustrate that in a large-scale study, the automated pipeline is very valuable for this type of screening.

Besides capability of automated processing, the large-scale data also require batch processing ability to process the large amount data generated under the same or very similar system/parameter settings. The batch processing enables automated processing for files of the same structure or type in the same folder. In our applications, the data are different scale of neuron image data including dendritic spine image data, neurite image data and synaptic vesicle image data. All these categories require different processing pipelines as well as parameter settings. Therefore, in the system design for large-scale studies, the parameters are preferably loosely set to be robust to process data with a certain range of variance of features. In addition, adaptive strategy is desired for large-scale studies as well for the same reason that certain features, such as width of neurite, length of spines may vary in a large data set. To summary, we
conclude that automated and batch processing capable methods are necessary conditions for large-scale screening studies.

2) *Information in spine morphology*

![Figure 6](image)

**Figure 6** An illustration of neuron structure morphology. (a) dendritic spines; (b) synaptic vesicles; (c) nuclei channel of neurite assay; (d) neurite channel of neurite assay.

A dendritic spine is a small protrusion from dendrites that usually receives input from a single synapse of an axon, as illustrated by Figure 7 (a) [38]. The dendrites of a single neuron can contain hundreds to thousands of spines [13]. Dendritic spines provide a memory storage site
for synaptic strength and synaptic transmission of electrical signals to the neuron's cell body, as well as serve to increase the number of possible contacts between neurons [38]. They form the postsynaptic element of the vast majority of cortical synapses and serve to compartmentalize postsynaptic signals [22, 43]. They contain neurotransmitter receptors, organelles, and signaling systems essential for synaptic function and plasticity [43]. Spine formation, plasticity, and maintenance depend on synaptic activity and can be modulated by sensory experience.

Dendritic spines consist of a head (volume 0.001–1 μm$^3$) connected to the neuron by a thin (diameter<0.1 μm$^3$) spine neck [43]. Spine morphology and density determine the strength, stability and function of excitatory synaptic connections that subserve the neuronal networks underlying cognitive function [25, 38, 43]. Spines vary in multiple shapes and sizes, which result a diversity of function. Several classes, including thin, stubby, mushroom and branch, has been distinguished based on morphological features [43]. Smaller spines are less stable and more motile and as a result, more plastic than large spines [44]. Recent data indicate that the morphology of spines is associated with numbers of docked presynaptic vesicles [45], numbers of postsynaptic receptors [46], and hence with synaptic strength. Therefore, learning and memory, which are function modulated by postsynaptic mechanisms, are related to spine morphology [22, 47]. In addition, diffusional coupling between dendrite and spine is influenced by the spine length and diameter [48]. Spine density and shape regulate the degree of anomalous diffusion of chemical signals within the dendrite [38].

3) Information in synaptic vesicle morphology

Recent studies on neuronal and synaptic activity related neurodegradation diseases, such as Alzheimer’s disease and Rett syndrome conclude with evidences provided by analysis of synaptic vesicle activity results [5-10]. Synaptic vesicles store various neurotransmitters. They play essential roles in nerve impulse propagation when they release neurotransmitters at synapses [40]. The quantity and intensity of vesicles are of most significance features to estimate the activity and function of live or cultured neurons [9, 11, 12]. All presynaptic functions, directly or indirectly, involve synaptic vesicles [40].
Under a microcopy, a vesicle can be visualized as a bubble or a punctum of liquid with or without neurotransmitters, as illustrated in Figure 8 (b). It cannot be labeled by fluorescent proteins directly. Upon stimulation, exocytosis causes vesicular membrane to fuse with cellular membrane, and dye binds to exocytosing membrane. Stained membrane is then endocytosed, and after washing out excess dye from the superfusate, endocytosed vesicles can be destained by a second round of stimulation. By imaging the membrane staining and destaining process, a set of presynaptic activities such as endocytosis, recycle time, vesicle-pool dynamics, and exocytosis can be studied [49]. More recently, it was also shown that the method could be used to detect early synaptic deficit in neurodegeneration condition [9].

A set of image containing before and after destaining processing could be used to measure the neuron activity base on the statistics, such as density, intensity, average size of synaptic vesicle boutons. For large scale data generated by high-throughput screening and high content screening in drug development or other biological applications, automated analysis method would be valuable [50]. As an image based method, information can be acquired by image quantification and statistical analysis. Therefore, we sought to develop programs for the automated analysis of FM dye neuron images. Success of the program will enable large-scale screening for the effectors on pre-synaptic activities, and greatly accelerate the neuronal mechanism study and drug screening.

4) Information in dendrite outgrowth

Proper function of the nervous system depends on communication through neurons via axons and dendrites. The formation of these specialized cell processes first begins with neurite outgrowth. Loss of synaptic connections and neuronal projections has been shown to be common features of many neurodegenerative diseases including AD as well. Measurement of neurite outgrowth is a convenient method to gauge neurotoxicity in screens for drug safety as well as a drug discovery tool for diseases of neurodegeneration. Thus under a high content screening circumstance, detection of dendrite and summarize statistics, such average dendrite length per neuron cell, could be acquired as key measurement to evaluate the screening results. Example images are shown in Figure 9 (c) and (d).
Neurite loss are early events in AD and during Aβ induced neurodegeneration of cultured neurons, this suggests that the in vitro system could be utilized to screen for compounds able to prevent neurite damage or allow neurite regeneration in AD which could represent the first step towards a novel approach to AD prevention and treatment. Although some controversy remains, most researchers agree that the primary cause of AD is the abnormal accumulation of the amyloid β (Aβ) peptide, the primary component of the amyloid plaques [51, 52]. Aβ is a 40-42 amino acid hydrophobic peptide derived through proteolytic cleavage from the amyloid precursor protein (APP) [39, 53] Aβ induced neurodegeneration can be relatively easily modeled in vitro using either primary rodent cortical or hippocampal neurons or Aβ sensitive neuronal cell lines. Treatment with aggregated synthetic Aβ 1-40 or Aβ 1-42 has been shown to lead to altered neuronal function, loss of synaptic connections, loss of neurites and eventually to neuronal cell death. This in vitro system has been used extensively to decipher the early molecular events occurring in response to Aβ treatment [54]. Many of these events have been later confirmed in vivo in AD mouse models and in patient tissue samples, demonstrating that this system represents a useful model for study of AD associated neuronal damage and death.

D. Summary

In this chapter, we provided a system level review of the biological and scientific questions, including the backgrounds, problem definition, and proposed system to address these questions. To understand the complicate mechanisms of neurodegenerative disease and develop effective drugs, large-scale studies using different image scales addressing different portions of neuron structures have become dominate in recent. However, the absence of analytics that could automatically, accurately and properly catch the subtle but functionally significant changes of neuron structures results difficulties to extract information from the massive amount of data. Therefore, to solve this issue and enable large-scale study to boost its scientific value, we will study the neurodegenerative diseases with high content screening and develop a system level solution to help understand the disease and develop effective drugs.

The remaining of this thesis is organized as follows: The current neuroscience research on neurodegenerative disease with large-scale studies and existing analytic methods and related
works will be introduced in Chapter II. In Chapter III, we will propose an automated pipeline to analyze dendritic spine image, which is able to process both in vitro and in vivo data. We introduce the proposed synaptic vesicle assay, data analysis and scoring procedures in detail in Chapter IV. In Chapter V, we provide the methodology to process neurite image data and more importantly, we present a screening of more than 1000 compounds in drug development as an example to illustrate how the system works. In Chapter III to Chapter V, we provides data collection steps, image processing and quantification algorithms, important result and scientific issue and algorithm related discussions. We conclude our work in Chapter VI.
II. CURRENT NEUROSCIENCE AND INFORMATICS RESEARCH WITH DENDRITIC SPINES, SYNAPTIC VESICLES AND NEURITE

This is a literature review chapter. We will first review current approaches in neuroscience research addressing neurodegenerative diseases with dendritic spine imaging, synaptic vesicle imaging and neurite outgrowth assay. The reviews on the biological research not only introduce biological background and stories but also present the needs for powerful automated and robust methods to benefit scientific research. Then we will provide a brief literature review on current approaches processing similar types of data used in biological researches and show the limitations of these approaches in the HCS studies with neurodegenerative disorders.

A. Neurodegenerative disease research using neuron morphology

In this section, we will provide examples of current neurodegenerative disease research and drug development to demonstrate the capability of image-based method in these types of researches. Examples are emphasized by different neuron structures to illustrate various functions of these structures in the neuron network and neurodegenerative disease research.

1) Spine morphology used in validation of the Aβ hypothesis and drug development

In previous studies, the literature suggested that during the course of Alzheimer’s disease (AD), neuropil in vulnerable brain regions undergoes extensive degeneration, including changes in neurite morphology and dramatic synapse and dendritic spine loss [32]. Since synapses are strongly related to the degree of cognitive impairment, synapse loss appears particularly important to understand the cause of AD [13, 55]. Although the possible cause of synaptic loss in AD remains uncertain, which could be secondary to neuronal loss [56], neurofibrillary tangles [57] or the postulated synaptotoxic effect of amyloid-β(Aβ) [58], our collaborators develop mouse model to validate these hypotheses and propose their own biological reasoning [22, 55].
Mouse models that overexpress amyloid precursor protein (APP) have been developed in which the development of plaques extraordinarily similar to those observed in AD patient. Moreover, these models do not recapitulate neurofibrillary pathology or widespread neuronal loss [22, 55]. These phenomena allow interpretation of results in the context of amyloid-related pathologies.

Studies of these transgenic mice demonstrate dendritic spine loss occurs in APP transgenic models [22, 32, 59, 60]. Since spines form the postsynaptic element of the vast majority of cortical synapses and are the proposed sites of synaptic plasticity underlying learning and memory, this observation is very important. Disrupted cortical synaptic integration, which is considered correlated with plaque formation, has been observed in a study using in vivo electrophysiology in the Tg2576 mouse model of AD [61]. The amyloid hypothesis holds that disrupted amyloid processing leads to neuron dysfunction and death in AD.

In previous work [62], the expression levels of three presynaptic markers, synaptophysin (general synaptic marker), synaptotagmin (marker of synaptic activity), and growth-associated protein 43 (GAP43; marker of synaptic sprouting) in the brains have been analyzed with frozen tissue from the frontal cortex. They found that compared with control subjects, mild (early) AD cases had a 25% loss of synaptophysin immunoreactivity. Levels of synaptotagmin and GAP43 were unchanged in mild AD, but cases in late stage had a progressive decrement in these synaptic proteins.

In another study [63], authors presented the hypothesis that synapse loss and possibly neuritic dystrophy in the senile plaques are the result of a process of regenerative failure akin to that which occurs following axotomy in the adult CNS due to trauma. They proposed that the dystrophic neurites in AD are undergoing abortive regeneration rather than aberrant sprouting, which is another hypothesis that has been put forth to explain neuritic dystrophy is that it is caused by aberrant sprouting.

To test whether accumulation of amyloidin plaques in the mouse brain perturbs synaptic connections, our collaborator developed a method using green fluorescent protein (GFP) injection into cortical neurons to observe fine structure of processes with in vivo three-dimensional (3D) multi-photon microscopy. Observing plaques and neurons in the living brain and postmortem analysis of immunostaining, questions of whether dense plaques cause local
disruptions of dendrites, can answer by tracking morphological changes in axons, and dendritic spines. In addition, neurons in control animals and those in Tg2576 cortex proximal to and distal from plaques are compared to examine the effects of amyloid deposition on cortical microarchitecture. Astriking focal synaptotoxic effect of plaques and importantly an overall loss of dendritic spines even quite far from plaque shave been observed through the experiment. To the extent that dendrite morphology and dendritic spines reflect fundamental structures necessary for the integration of signals in neocortical neurons, these changes likely contribute to the breakdown in electrophysiological integrity and behavioral abnormalities documented previously in Tg2576 mice [22, 55, 61].

2) Synaptic vesicles employed to quantify neuron activities

As we mentioned in the introduction chapter, synaptic vesicles store various neurotransmitters that are released at the synapse. As all other vesicles, whose membrane is similar to that of plasma membrane, synaptic vesicles can fuse with the plasma membrane to release their contents. The contents inside vesicles are usually different from the cytosolic environment because they are separated from the cytosol. Therefore, vesicles are a basic tool used by the cell for organizing cellular substances. Vesicles are involved in metabolism, transport, buoyancy control and enzyme storage. The activity dependent dye FM 1-43 serves as a marker of synaptic vesicle turnover upon stimulation, which can be further quantified to indicate presynaptic activity. This implantation is based on the mechanism of vesicle recycling through endo-exocytosis process, which release the content inside the vesicle and recycle the microenvironment content from outside.

There are recent studies of neurodegradation diseases, such as Alzheimer’s disease and Rett syndrome, focus on evidences provided by analysis of synaptic vesicle activity analysis results [5-10]. In [9], the authors examined the acute effects of endogenously released Aβ peptides on synaptic transfer at single presynaptic terminals and synaptic connections in rodent hippocampal cultures and slices. The production and subsequent release of Aβ positively correlate with the level of neuronal and synaptic activity [9]. More specifically, it has been suggested in [64] that synaptic vesicle recycling through coupled endo-exocytosis is the primary mechanism that mediates activity-dependent Aβ production and release. On the other hand, the strongest
structural correlation of cognitive decline in AD patients is synapse loss [65]. The down regulation of synapse density has been observed in studies on mouse models of AD which indicate that soluble Aβ oligomers might be the cause. To answer the question that how endogenously released Aβ regulates synaptic transfer in normal, non-transgenice hippocampal circuits on a fast timescale, the authors in [9] employed the activity-dependent dye FM1-43 as a marker of synaptic vesicles to assess the acute effects of changes in endogenous extracellular Aβ on synaptic transmission during different patterns of neuronal activity in rat hippocampal cultures and in acute hippocampal slices. The functional properties of synapses were evaluated by estimating the release probability and spatial distribution of active presynaptic terminals[9]. The amount of releasable fluorescence at each bouton and density of FM puncta per image have quantified to evaluate the total presynaptic strength. With these configurations, they found that both elevation and reduction in Aβ levels attenuated short-term synaptic facilitation during bursts in excitatory synaptic connections. These observations suggest that endogenous Aβ peptides have a important role in activity-dependent regulation of synaptic vesicle release and might point to the primary pathological events that lead to compensatory synapse loss in Alzheimer’s disease [9].

Another study [66] shows that DNA methyltransferase (DNMT) inhibition in hippocampal neurons results in activity-dependent demethylation of genomic DNA and a parallel decrease in the frequency of miniature EPSCs (mEPSCs), which in turn impacts neuronal excitability and network activity by using FM 1-43 dye based synaptic vesicle imaging strategy. The results demonstrated an intimate relationship between DNA methylation in neurons and the function of the transcriptional repressor MeCP2 (methyl-CpG-binding protein 2) whose mutation causes the Rett syndrome [67]. Rett syndrome is a neurodevelopmental disorder of the grey matter of the brain that affects almost exclusively females. By identifying the different behavior of total recycling synaptic vesicles from control and compound treated synapses measured by total fluorescence changes, the data of [66] indicated a role for DNA methylation in the control of synaptic function, which shares a common pathway with the methyl-binding protein MeCP2.

3) Neurite outgrowth used in inhibitors identification and traumatic spinal cord injury research
High-throughput image-based screening can be defined as the process of collecting large number of screening images under different drug treatments via automated microscopy. High content screening (HCS) extends high-throughput image-based screening by processing images to extract features and converting extracted feature data to readout information that can be used to answer in-depth biological questions.

Amyloid-beta peptide oligomers, has emerged as a principle trigger of progressive loss of neuronal function and cell death during Alzheimer’s disease [68]. At the cellular level, this disease is characterized by degeneration of neuronal projections, loss of dendritic spine density and neuronal loss [69]. The development of cognitive impairment strongly correlates with the accumulation of soluble Aβ oligomers in AD brains [70]. Significant effort has been made to understand the mechanisms of cell death triggered by Aβ. However, there is evidence that cognitive decline correlates better with synaptic changes than with the eventual neuronal death. For example, no significant neuronal death was observed at the onset of cognitive decline in the Tg2576APPSw mice [71]. Furthermore, loss or dystrophy of neurites is a common feature of several neurodegenerative processes such as Parkinson's disease, dementia with Lewy bodies and Amyotrophic lateral sclerosis [72]. Therefore, development of an assay, which would allow identification of new classes of molecules capable of protecting neurites, will be very useful for many areas of neurodegeneration. An automated pipeline to process HCS data would be valuable which could be used in reliably screening and characterizing small molecules that are protective against Aβ-induced neurite loss. To the best of our knowledge, the system approach that is specifically designed for identification of drugs that prevent neurite loss caused by Aβ in primary neurons does not exist.

Regeneration of neuronal function following traumatic spinal cord injury requires axonal regrowth and re-establishment of synaptic termini. However, myelin, covering axons in adult neurons, provides inhibitory stimuli for axonal outgrowth, precluding efficient neuronal regeneration. Specific signaling events involved in myelin-induced suppression of axonal outgrowth have been extensively studied in recent years. At least three myelin components have been found to contribute to the inhibition: Nogo-A, myelin associated glycoprotein and oligodendrocyte myelin glycoprotein [73, 74]. At least two signaling receptor complexes, which may contribute to the axon regeneration suppression, have also been described and characterized [75].
Studies utilizing peptide disrupting Nogo-A/Nogo-66 (Nogo Receptor) or soluble Nogo-66 clearly demonstrated that disrupting these signaling events provides a feasible strategy for functional recovery [76-78]. However, therapeutically feasible small molecule inhibitors targeting these pathways have not yet been described.

B. Current solutions in neural image analyses

The difficulty of interpreting large image datasets from all types of neuron assay based compound screening varies with the readout. The acquisition of high-content images for neuron assay based compound screening is relatively straightforward, and image-based screens focusing on subcellular structures, such as the nucleus, can be analyzed by off-the-shelf software. However, analyzing more complex structures, such as centerlines of neurites and attaching nucleus in neurite images, neuronal spines in in vivo images and synaptic vesicles images are of great challenges to be fully automated. Manually inspecting, segmentation and quantification complex neuron structures are time consuming and daunting tasks. For example, neuron assay based screen of ten thousand molecules or compounds generates more than 23,040,000 images (10,000 compounds × 384 wells/plate × 6 image fields/well), and it will take even an expert months, if not years, to process all the images reliably. Moreover, while prominent structures could plausibly be scored by human visual inspection, small changes in the proportions of various phenotypes, which may be informative and crucial, are likely to be missed, especially when more than one phenotype is scored. In addition, human manual quantification is easily biased and inconsistent, and bias are as difficult as quantification to be removed. Moreover, human manual processing will not always lead to reproducible and comparative results. Therefore, visual inspection and manual quantification of these large-scale image data is laborious, time consuming, and prone to errors. Fully automated analysis of neurons combined with high resolution fluorescence imaging will allow consistent and efficient scoring of phenotypical changes.

Current optical microscopy techniques, coupled with a large collection of fluorescent and other labeling methods, generate tremendous numbers of digital images ready for quantization. However, the analytical tools have not kept pace with these developments. While existing tools,
such as NIH Image (available as Scion Image or ImageJ), MetaMorph, UTHSCSA ImageTool, QED Image, and In Cell Developer, are able to process common image data, however, they are extremely limited in their scope and capability for high content cell image analysis, especially for complex shapes, different scale of structures and images with noise and inhomogeneity issues.

In the following of this part, we will give some typical examples of existing methods on HCS large-scale image processing and statistical analysis.

1) **Current approaches for spine detection and matching**

Spine morphology has been extensively studies through years. Recent publications indicate that the morphological characteristics of neuronal structure viewed from in vitro microscopy are closely related to neural functions such as learning, memory and attention [22, 38]. Dendritic spines could be followed over time to identify whether they are stable, eliminated, or if new spines form providing readout of structural plasticity that could be used to understand the pathophysiology of neurodegenerative diseases and to investigate therapies, such as antibody treatment. In addition, scientists have been studying the biochemical pathways by examining the morphological and statistical changes of the dendritic spines at the intracellular level. As an example, [79] and [80] show that the TSC pathway regulates soma size, the density and size of dendritic spines, and the properties of excitatory synapses in hippocampal pyramidal neurons. Moreover, current imaging methods allow tracking of morphological changes of the spines before and after treatment or under different condition in a large screen manner, for example, HCS. In vivo imaging enables directly observation of the effectiveness of a treatment [26]. RNAi (RNA interference) has been applied in pathway identification by tracking the dynamics of synapse structure [81].

To track the dynamics of spine morphology with a large amount of data, an automated neuroinformatics tool would be valuable to save biologists time and labor to manually label and quantify the statistics. Although algorithms have been proposed recently for this topic [26, 82-85], including NeuronIQ developed by our group, the problem still remains imperfectly unsolved. The image data used currently in such applications are generated by the 2-photon laser scanning microscopy to image very small subcellular compartments within brain slices or in vivo, making it possible to study biochemical signaling that occurs at the intracellular level. Pyramidal neurons
in organotypic hippocampal brain slice cultures transected with are imaged in z direction with a certain distances [81]. In some applications, time-lapse image data are generated at a predefined interval to track the dynamics of morphological changes. We will discuss the reasons as well as challenges in developing a fully automated tool for neuronal spine segmentation and tracking as follows.

There are algorithms addressing spine detection and segmentation, which can be classified as following classes. The first category is from the in vitro image data analysis in [86] and [87]. Their pipeline started with thresholding-based segmentation to create binary images. Then a morphological thinning operator was applied on binary images to acquire central lines of dendritic structures and a line structure filtering was applied to get the backbone, detached spines and branch points. This method is very straightforward and fast. However, the boundaries acquired by global thresholding failed to accurately depict the actual shape of the neuronal structures. In addition, it failed in in vivo neuronal image due to it over-simplifies the segmentation of spines, which is a very difficult and challenging task.

The second one is based on adaptive thresholding with local information of each pixel [82]. This method detected detached and attached spines with different segmentation strategies. This automated included an adaptive thresholding method, which can yield better segment results than the prevalent global thresholding method. It also introduced an efficient backbone extraction method, a SNR-based, detached spine component detection method, and an attached spine component detection method based on the estimation of local dendrite morphology. It was able to accurately segment spines with limited number of false positives from images with good quality and high SNR containing a small number of neuron cells. However, if the image is contaminated by noise, for example, for in vivo image data that have more noise and blurred boundaries, this method suffered both false positives and negatives.

The third algorithm category is line detection based segmentation strategy used in [85]. This strategy is much more robust to noise and can be used in in vivo neuron image processing. Due to the nature of in vivo imaging which illustrates the live environment, the in vivo image data are often corrupted by noise and blurs. This strategy treated every object as a line structure, including both dendrites and spines. Attached spines were considered as branches of the dendrite backbone. The detection of boundaries of the neuronal structures is based on the strength values
of the line points and their neighbor points. Although the curvilinear structure detector is able to identify most spine and dendrite structures, the boundary detection strategy fails to locate accurate edges in an in vivo image data corrupted by noise.

There is a common issue shared by these three categories of spine detection methods, which is the reason why the automated detection remains unsolved through years of development. The first one is number of parameters. Although in the algorithm design, although in [82] and [85] equipped procedures to reduce the number of parameters or enable the loose parameter setting to allow the algorithm can be widely employed for various data sets generated under different conditions and from different labs, however, we still need to custom parameter settings for different image data to achieve optimal results. Though this issue can be solved manually by changing parameters by experienced engineers, it still takes time and makes automated algorithms not smart enough for different applications.

Matching spines for different time points is another task which assists in estimating whether the treatment is effective or not. Dynamics of dendritic spines can be followed over time to provide readout of structural plasticity that could be used to understand the pathophysiology and to investigate therapies, such as antibody treatment. Morphological changes of spines as well as spine formation and elimination can be observed from the time series dendritic spine image data. These information would be useful to study the pathology, signaling pathways and in drug screening.

In [88], there was an assumption of constant number of spines at different times. However, in our studies, we do not have any prior knowledge on the number of spines. Therefore, this assumption will not stand in our application. Moreover, the literature on cell and other structure tracking methods usually employed Kalman filtering and mean-shift methods [89], both of which required a long time sequence. In our case, there are only two or three time points available. The tracking methods requiring long sequence of time points are not appropriate.

2) Challenges and existing methods to detect and quantify synaptic vesicles

The dynamic changes of synaptic vesicles before and after the electric stimulation provide a convincing quantitative criterion to determine the neuron activity. It can be used in any large
scale studies of neuron activity as measurements. Based on the developed high throughput or high content imaging strategy to visualize neurons and synaptic vesicles, an automated imaging quantification pipeline to characterize the neuron activity using vesicle dynamics in a large scale will be very valuable. The dynamics for evaluating neuron activity is measured by detected and quantified vesicles.

To our knowledge, there are no literatures directly addressing the synaptic vesicle detection and segmentation in a large scale data set manner. However, there are several popular algorithms for spots or similar shape objects detection. In cell segmentation, the common strategy is the marker controlled watershed algorithm [90, 91]. But cell images are usually collected from multi-channels, and the mutual information is rich, in which nuclei channel images can be used to detect and segment cytoplasm channel images [90]. However, for the synaptic assay, we only have a single channel’s information and markers are not easy to obtain. Besides, the size of synaptic vesicles is much smaller than that of cells. So it is impractical to follow the cell segmentation pipelines to process vesicle images. Another strategy, usually applied to address inhomogeneous images, LBF level set algorithm [92-94] is a popular algorithm in medical image processing. However, most vesicles are located in structures with bright background such as presynaptic region in axon terminals as well as axons. The LBF level set method fails to accurately identify the boundaries of such spots and misses the weak spots with low intensity as well. In turn, it segments the background structure containing several spots as a single region. Moreover, the standard thresholding [95] based methods are not able to solve the inhomogeneity issue as well. As a denoising and enhancement method, MSVST (a Multi-Scale Variance Stabilizing Transform) [96] is reported as a very effective method for this type of applications. However, this method individually cannot achieve high detection accuracy for our specific application in vesicle detection.

We will discuss several challenges in the detection and quantification of vesicles for this application as specified below. The first one is noise corruption. Medical images are often noisy due to the physical mechanisms [96]. These types of noise are comprised by Gaussian white noise and Poisson noise. Gaussian random noise can be easily removed by filtering. On the other hand, the Poisson noise is introduced during the imaging process. Thus, it is a result of the random nature of photon emission and needs extra effort to eliminate. Imaging systems using
photon-counting strategy are dominated by Poisson noise. These imaging modalities include PET, SPECT, and fluorescent confocal microscopy imaging. These imaging systems that operate in low-light conditions or in low-dose radiation conditions are often affected by photon noise which can be modeled as a Poisson process [96]. Poisson noise can be reduced if we increase the intensity of light or extend the exposure time. However, photobleaching will occur, when the fluorescent molecules are permanently destroyed and fail to send fluorescent signal. Therefore, this aberration introduced in the imaging process needs to be corrected in image analysis.

Besides the random noise, another source of aberration is blurring and out-of-focus issue for some regions of interest. The objects of interest in this study are spots, or fluorescent puncta, which are relatively small compared with other structures. The spots do not have clear boundaries, and in turn, the intensity of objects gradually fades from center to periphery. The blurring is caused by the diffraction phenomenon and imperfection of the optical system. Moreover, since cultured neurons do not ideally lie on the same surface, some regions of interest may be out of focus. These intrinsic blurring and artificial blurring hinder accurate detection and segmentation of vesicle spots.

The first two issues can be solved by MSVST to a large extent [96]. Apart from the noise and the blurring, which are global aberrations of images, there are some undesired structures in the image preventing accurate segmentation of vesicles. These structures include inactive vesicles and vesicle-like structures introduced by imperfect staining. In addition, some parts of membranes of the neuron axon are stained by the FM dye due to the staining imperfection. These structures should be addressed in the prepossessing or the post processing procedures.

Another issue is image intensity inhomogeneity. Vesicle spots across the image have different intensity distribution as well as their background. For example, some dark spots might be recognized as background if we set the global intensity threshold too high. On the other hand, we observe that intensities of some vesicle background regions are even higher than that of some vesicles with low intensity. In addition, these high intensity spots usually cluster in a group with bright background. These bright backgrounds are typically presynaptic regions in axon terminals. Moreover, there are spots lined up in axons, which have lower intensity than the axon terminal regions but still higher than the axon background. Besides these high intensity spots, weak spots or vesicles, referred as spots with low intensity, are easily missed in conventional detection
methods. The weak spots possess neither obviously high intensity nor dramatic intensity gradient. If we set the intensity threshold low enough to detect these weak spots, it is hard to obtain the accurate boundary of each vesicle. Moreover, false positives will dominate the results. In addition, in this specific application, there is another category of weak vesicles that are considered as relative weak vesicles. This spot type is usually located in bright background regions. The intensity change between before and after stimulation is not as obvious as other vesicles, which is the reason we name it as relatively weak vesicle. As discussed above, the inhomogeneity issue should be addressed and local information should be employed to accurately detect and segment vesicles for this synaptic vesicle assay.

Last but not least, the size of vesicle is very small, thus some available algorithms designed for large size spots detection and related topic of much larger objects do not succeed in this application. Compared with cells, which have similar morphological signatures as vesicle spots, objects in this study are much smaller. Therefore, even if the pipeline for cell image processing looks promising, it fails in some steps, such as the current methods [97] to solve the overlapped vesicles and the detection of region centers. Although some segmentation strategies are able to identify part of spots for this application, the numbers of both false positives and false negatives are not acceptable.

To summarize, accurate detection of neuron vesicles is impeded by difficulties in noise removal, imperfection in imaging, inhomogeneity, as well as the nature of the objects.

3) Current approaches to identify and quantify neurite

Loss of synaptic connections and neuronal projections has been shown to be a common feature of many neurodegenerative diseases including Alzheimer’s disease [6, 57, 60]. In AD, neurite degeneration is observed in the areas responsible for higher cognitive functions, which are the primary areas affected by this devastating disease. It significantly precedes neuronal cell death suggesting that neurite damage and loss might be the primary cause of the decline in cognitive function [6]. Neurite loss is also observed following traumatic brain and spinal cord injuries. Chemical compounds targeting neurite loss prevention or neurite outgrowth stimulation could represent a promising novel treatment in AD and following spinal cord trauma. Loss of neuronal projections in AD can be modeled in vitro in primary mouse cortical neurons treated
with the amyloid β peptide, the primary cause of neurodegeneration in AD [51, 98], neurite loss precedes induction of neuronal death in this disease model. It can be easily assessed visually either in live neurons through bright field microscopy or by immunostaining following fixation. Given recent advances in automated microscopy, this technique could be adapted for use in high throughput screening to identify compounds specifically suppressing amyloid-induced neurite damage and loss. However, without automated analytic tools to assess neurite outgrowth, it has been impossible to conduct such high throughput neuron assay screening.

In addition, neurite outgrowth has been extensively used different scenarios other than AD. In [99], authors employed neurite outgrowth image to find that that PGRN levels are reduced in the cerebrospinal fluid from FTLD patients carrying a PGRN mutation and conclude that PGRN/GRN is a neurotrophic factor with activities that may be involved in the development of the nervous system and in neurodegeneration. Another example, in [100], neurite outgrowth image was used to demonstrate that in autosomal dominant Parkinson’s disease, mutations in the leucine-rich repeat kinase 2 (LRRK2) gene are frequent and LRRK2 homologs in non-vertebrate systems play an important role in chemotaxis, cell polarity, and neurite arborization.

The direct exploratory tracing algorithms automatically detect the initial points and extract the centerlines of the line structures in a tracing-based iterative way. Although these algorithms are computationally inexpensive, the tracing performance is inadequate for images with poor quality, discontinued line structures, complex branching patterns. Moreover, the algorithm requires semi-automated selection of the parameters, such as the length of the templates and the maximum expected width of the vessels, which may limit its possible applications to certain types of images. The tracing performance is mainly determined by the selection of the parameters and the design of the stop conditions. The other approach [101] is referred to as line-pixel detection algorithms in which local geometric properties of the lines are modeled and examined for each image pixel, followed by linking the successive line pixels that are most likely to represent the centerlines of the neurites. Although computationally expensive, these algorithms yield highly precise extraction of the line structures. Cohen et al. [102] presented a method for automatic 3-D tracing based on skeletonization and graph extraction. Koh et al. [86] and Weaver et al. [103] described a 3-D structure approach utilizing modified medial axis to locate dendritic branch centerlines. Recently, Meijering et al. [104] suggested a 2-D
semiautomatic neurite tracing method that employed local principal ridge direction to guide the live-wire algorithm to track centerlines. Their method has been validated carefully, but its need of frequent user interaction precludes it from being used in large datasets.

C. Summary

In this chapter, we presented different scenarios where high content screening strategy is applied to various neuron structures, including dendritic spines, synaptic vesicles and dendrites to study neurodegenerative disease, especially for AD. Through these interesting biological stories, we could conclude that the lack of information analytics and automated system would significantly impair the capability of the powerful screening system and even fail the research to answer the scientific questions. Then for each structure component, we reviewed current approaches to process different types of microscopy neuron images. As some of them are not fully automated and need intense human intervention, which fail to satisfy the HCS information extract standard, most of them were not able to achieve high accuracy results that are not designed specifically for neurodegenerative disease screening. Therefore, in the following chapters, we will provide novel strategy, exclusively designed components of the multi-scale image analysis system for studying neurodegenerative disease and related drug development.
III. DENDRITIC SPINE DETECTION AND TRACKING

Dendritic spines are small protrusions from dendrites. They serve as receivers on the postsynaptic end to collect electrical or chemical signals which then are transmitted by dendrites and axons. In addition, they also function as information/memory storage sites. Scientists discovered that dendritic spines are highly related to neural functions such as learning, memory and information transmission. Therefore, the density of spines on dendrite as well as morphological features are heavily employed in current image based research on neuron network, neurodegenerative diseases and drug development. As one of the most important parts in our multi-scale studies, automated detection and quantification of dendritic spines could provide solid support to research tracing the dendritic spines.

In this chapter, we will start with a brief introduction to our data, then introduce the spine detection and tracking methods in detail, illustrate the detection results with comparison to manual segmentation by biologist as well as some statistics.

A. Materials and methods

In this section, we first describe how we acquire and pre-process our neuron images. Then we present each step for spine detection and tracking of these images in detail. Figure 10 displays a flow chart of our approach. We start with curvilinear structure detection to get the essential information about points and lines. Then spine boundaries are detected by the proposed aLBF level set model. We extract shape information of the spines, which finally allows us to match spines at different time points and to model the dynamics of the dendritic structures. We also briefly describe a 3D level set method of spine segmentation as a comparison with our proposed spine extraction method at the end of this section.

1) Spine detection

a) Dendritic spine image data acquisition
Figure 10 The pipeline of the proposed spine detection and tracking strategy.

The image data were collected at the MassGeneral Institute for Neurodegenerative Disease, Massachusetts General Hospital by Drs Tara Spires-Jones and Bradley Hyman. Anesthetized mice with a cranial window were positioned on a multi-photon microscope. Surgical details are provided in Spires *et al.* (2005). Green fluorescent dextrans were injected into primary somatosensory cortex. Emitted light in the ranges of 380 to 480 nm, 500 to 540 nm, and 560 to 650 nm was collected by three photomultiplier tubes. To avoid photo damage, lowest laser power
that could detect all spines was used for acquisition. Z-stack images were collected with an inter-slice interval of 0.5 \( \mu m \) and each stack contained 15-20 images. The dendrites were reimaged under the same conditions one hour later. Dendrites were analyzed in green channel images. The \( xy \) resolution of the image data was 0.06 \( \mu m/\)pixel. The image sizes are 512 by 512.

b) Preprocessing

In vivo images exhibit noise, blurs and sharp variations in intensity. Image pre-processing is therefore necessary before further analysis. Our pre-processing involves the following steps: We first deblur the 3D image data, project the 3D stack of each time point into 2D, and then register 2D images of different time points in the same data set.

Deblurring: Because of the relative movement between different frames in the same stack and corruption by noise, the projection of the original image is blurred. We deblur 3D images by deconvolving with a Gibson’s point spread function (PSF,[105]) to correct artifacts introduced by breathing and heartbeat. In our data, main structures appear at the middle of the stacks. Gibson’s PSF considers the non-stationarity of the PSF and asymmetry focal plane. We use this property of Gibson’s PSF to obtain optimum deblurring results at the middle of the stack.

Projection: After deblurring, we use maximum intensity projection (MIP) to project 3D images into 2D, which collects the maximum intensity along \( z \)-stack.

Registration: Figure 11(a) displays an overlapped image of two time points prior to registration. Red pixels represent one time point and green pixels represent the other. The yellow pixels in Figure 11(b) stand for the overlap of two time points. We observe considerable movement and small deformation between two time points, therefore image registration is necessary. We apply the Iterative Closest Point (ICP) algorithm [106] to align the skeletons. ICP finds the closest point on a geometric entity, which is the foreground object (dendritic structures in our case) in an image to a given point. The ICP algorithm always converges monotonically to the nearest local minimum of a mean-square distance metric, and the rate of convergence is fast [106]. Here, we select three key points marked by different signs in Figure 11(a) on the dendritic structure at each time point as the control points to guide the registration. Figure 11(b) shows an
overlapping view of both time points with ICP registration applied. Since no severe deformations
are observed, we find it sufficient to consider only rigid transformations with the ICP algorithm.

![Figure 11 Illustration of registration. (a) Before registration. The two colors represent two time
points. Control points are paired with different marks; (b) After registration. Yellow denotes the
overlap of green and red.](image)

c) **Backbone and Spine Detection**

In our algorithm, it is important to first identify the following key structures: backbone, medial axis and branch points. A backbone is the centerline of the dendrite structure. A medial axis is the connected centerline of spines and dendrite structures. A branch point is a pixel, which has no less than three branches. In the following, we present our approach to backbone and spine detection: curvilinear structure detection, followed by line linking and branch point detection.

(1) **CURVILINEAR STRUCTURE DETECTION**

A curvilinear structure is a line or a curve with some width, such as dendritic structures in the neuron image and roads in aerial images [101, 107, 108]. A desired curvilinear structure detector should be able to robustly distinguish lines with a certain range of width and high curvature. In our problem, we would like to precisely detect the central lines of the neuron structures. However, in Steger (1998), there was a primary restriction on line width because the
method were designed to find narrow and long structures with small variation of widths along the line direction. We adopt the strategy described in [107] and [108] which allows an adaptive width. The steps are as follows:

(i) **Local point detection, direction estimation and linking:** To detect the lines, we first apply a Gaussian derivative kernel, which essentially smoothes (de-noises) the image before obtaining its gradient. The variance of the Gaussian kernel should be large enough to detect wide lines. However, the thin structures or curves are blurred out. By employing the Hessian matrix calculated for each pixel, we vary the variance of the Gaussian kernel to find the largest eigenvalue and its corresponding eigenvector to determine local direction of the line. The magnitude of the second derivative of the image is considered as the strength map. The points with strengths larger than a given threshold are identified as line points. This method overcomes the drawback of Steger's method of a fixed line width. More importantly, our method works well for the weak lines whose strengths are relatively small. To link the detected line points, the user specifies two line strength thresholds for the starting and end points for a data set. The linking starts at the points with strength values higher than the upper threshold and ends at points lower than the lower threshold. Figure 12 demonstrates the line direction detection result. Figure 12(a) illustrates the relationship between the line direction marked by the red arrow and its normal direction presented by the green line. Figure 12(b) displays a magnified view of the spine. The blue points are the detected center line points of the curvilinear structures and the cyan arrows denote normal directions.

![Figure 12](image.png)

**Figure 12** (a) Illustration of line direction and normal direction; (b) Illustration of line detection algorithm. The cyan arrows denote the normal direction. Red dots are the rough estimates of the boundary.
(ii) **Backbone linking:** As mentioned above, we classify structures into two classes: backbone and spines. The backbone structures are detected lines with lengths larger than a user specified threshold. It is possible that backbones are disconnected due to noise and inhomogeneity. Therefore connection is necessary. We search for the points which fit the predefined criteria for boundary points described in [108] on the strength map. The boundaries are rough estimates of the line segment positions marked as red dots in Figure 12(b). We link two backbone structures if their estimated boundaries overlap in the eight-neighborhood area and linking radius is smaller than the linking threshold. Here we employ the Bresenham line linking algorithm [109] to connect the backbone segments.

![Figure 13(a) Original spine and backbone detection results of R10XB time point one without post processing. Cyan: backbone; Red: spines; (b) Line linking results of (a).](image)

Figure 13(a) shows the line detection results of the MIP image of the R10XB data sets at time point 1 without any post-processing. The blue lines denote the detected backbone while the red ones indicate the spines. Ideally, according to our previous definition, the backbone should be a smooth line without many trivial branches. However, due to attached spines to the dendrite and the poor quality of the image, as shown in Figure 13(b), some spines are connected to the backbone as branches. We could extract the backbone without branches by line filtering as mentioned in[82]. However, this is unnecessary because our objective is to extract the medial axis of all the dendritic structure, including backbones and spines, then, more significantly, to
locate the backbone branch point. We will describe in the next part that, instead of chopping off the branch, we link detached spines to the backbone.

(2) LINKING DETACHED SPINES TO THE BACKBONE

Medial axis is a linked line structure of detached spines and backbones. Figure 13(b) is the linked results of Figure 13 (a), which displays the medial axis of the entire dendritic structure. In the part, we will explain in detail how we link spine segments to the backbone.

Figure 14 Some possible situations in line linking: (a) Two branches with obvious orientation differences; (b) Both branches are smoothly connected with the main structure; (c) Illustration of branch point selection on the backbone; our algorithm will choose the yellow marked point B. Purple arrow is the direction of \(v_p\) and orange arrows are the direction of \(v_b\).

We first describe how to identify a candidate end-point on the spine segment to link. For simple cases where the spine segment has no branch, the closest end-point to the backbone is chosen as the candidate for connecting. There are some special cases that are more complicated. The first is that the central lines of the detached spines have more than one branch. In this case, the end-point along the direction of the main structure would be chosen, because usually spines do not curve sharply. Figure 14(a) illustrates this circumstance where branch 1 follows the true direction. In Figure 14, the cyan lines represent the backbone and the red line segments indicate spines, while the yellow dots are end-points. Another case is that there are two possible branches that are following the ‘right’ direction. As shown in Figure 14(b), branch 3 and branch 4 are both smooth extensions of the line before branching. We choose the branch with the smallest distance from the branch end points along its direction to the backbone. As illustrated in Figure 14(b),
branch 3 would be our choice since line section 33" is shorter than 44", which indicates smaller
distance. We formulate the previously described problem as follows:

Equation 1

\[
\text{end point } i = \begin{cases} 
\arg \max_{i=1,2}(V_{bp} \cdot V_{p_i}) & \text{if } |V_{bp} \cdot V_{p_i} - V_{bp} \cdot V_{p_j}| \geq C \\
\arg \min_{i=1,2}[\text{distance}(p_i,b)] & \text{if } |V_{bp} \cdot V_{p_i} - V_{bp} \cdot V_{p_j}| \leq C 
\end{cases}
\]

where \( p_i \) represents the end-points on the proximal-end-of-spine branch to the backbone, \( V_{bp} \) and
\( V_{p_i} \) denote the direction vectors at the spine branch-point and end-point respectively. \( C \) is a
threshold, which determines whether a distance condition is required for identifying the end
point. \( b \) represents the backbone. The model can be easily extended to instances with more than
two branches.

Secondly, we describe the approach to connect spines to their corresponding backbone. With pre-computed products, we remove spine structures which are away from the backbone based on a distance threshold. After that, the candidate end-point are connected to the backbone according to the end-points’ direction with direction vector \( V_{p_i} \). We examine each point on the backbone to calculate the distance and the direction vector from the backbone point to spine end-point with direction vector \( V_{p_i} \). By taking the inner product, we find the largest magnitude of inner products and define the corresponding point at the backbone as a branch point for the spine. Then we use the Bresenham line linking algorithm to connect two points to get the medial axis of the neuron structure. In the case that there are multiple backbone segments, we avoid cyclic structures by choosing the candidate point that has the smallest distance to the spine end-point. We formulate the problem of locating the branch point \( j \) on the backbone for a given spine end-point \( i \) as follows:

Equation 2

\[
\arg \max_{j=3,2,...}(|V_{p_j} \cdot V_{b_j}|)
\]
where $V_{ep}$ denotes the direction vectors of a given end-point on the spine and $V_{jb}$ represents the direction vector of a candidate branch-point on the backbone.

Here, we would like to add one more comment on the direction of the end-points. As shown in Figure 12(b), where cyan arrows present the normal direction, the end-point $p$ is oriented perpendicularly to the other line-points in the same line structure. In this instance, we use the averaged direction vector of neighbors of the end-point. In Figure 12(b), the average of the direction vectors of two points in the yellow circle would be utilized as the end-point direction vector. We summarize the above description as follows:

Equation 3

$$V_{ep} = \begin{cases} V_{ep} & \text{if } V_{ep} \cdot V_n < 0.7 \\ V_n & \text{if } V_{ep} \cdot V_n > 0.7 \end{cases}$$

where $V_{ep}$ stands for the direction vector of the end-point and $V_n$ is the average of the direction vector of the neighboring points mentioned above. The threshold, which is a measure of similarity of the endpoints’ directions, is determined by experiments on several data sets.

(3) BRANCH POINT DETECTION

In this section, we describe the method for detecting branch points on the central lines of neuron structures. There are two types of branch points on the backbone. One is generated by attached spines and the other appears after connecting detached spines to the backbone. Different strategies are adopted to detect two categories as follows.

A template of n-by-n matrix of 1s is applied to the backbone to identify branch points introduced by attached spines. Points with the largest magnitude of a template image on the backbone are considered as branch points. If two or more points have the same value, the neighboring information is used to choose the point with a larger value to its left or right as the branch point. One or two pixel uncertainty of the branch points is acceptable because: 1) the probabilistic model for tracking in the next section is robust to 10-20 pixel variance; 2) the
registration inaccuracy is larger than 1 pixel; 3) the neighboring attribute for tracking, such as curvature and the direction of the branch point would not fluctuate much in a small neighborhood.

Another kind of branch point is generated by linking detached spines to the backbone. As described in the previous part of detached spine linking, we locate the branch point on the backbone of its matching spine end point by finding the maximal inner product between two vectors: \( V_p \) and \( V_b \). The process is illustrated in Figure 14(c). The pink arrow is the direction of the spine end point and the orange ones are the other kind described in the above paragraph. We choose point B at the backbone as the branch point of its corresponding spine.

The central line of the dendritic structures, the branch points of spines and backbones are now available for subsequent processing. Figure 15 and Figure 16 respectively illustrate line detection results of two images, which we will discuss thoroughly in the results section. We can easily measure the spine length on the medial axis by removing the backbone [82].

d) Boundary detection with an aLBF level set model

In order to obtain shape information of the spines, we need to accurately locate the boundaries of the dendritic structures. The boundaries can provide information such as area and perimeter which are more explicit to biologists than the medial axis.

The previous extracted medial axis provides a good approximate of dendritic structure and can be used as an initialization for most active contour methods, such as edge based models. The energy function of the piecewise constant (PC) model [110] is

Equation 4

\[
E(c_1, c_2, C) = \mu \text{Length}(C) + \nu \text{Area}(\text{in}(C)) \\
+ \lambda_1 \int_{\text{in}(C)} |I(x) - c_1|^2 \, dx + \lambda_2 \int_{\text{out}(C)} |I(x) - c_2|^2 \, dx
\]

where \( I(x) \) is the image domain. \( \mu \geq 0, \nu \geq 0, \lambda_1 \geq 0, \lambda_2 \geq 0 \) are fixed parameters. \( \text{in}(C) \) and \( \text{out}(C) \) stand for regions inside and outside of the contour \( C \). \( c_1 \) and \( c_2 \) are average intensities in \( \text{in}(C) \).
Figure 15 Line structure detection results of data set R10XB, time point one; (a) Original image; (b) The line detection result without post processing; (c) Medial axis derived by line linking. Branch A and B are false positive detected branches; (d) Manual labeling result.
Figure 16 Line structure detection results of data set R10XB, time point two; (a) Original image; (b) The line detection result without post processing; (c) Medial axis derived by line linking; (d) Manual labeling result.

and out(C). Li et al. (2007) [111] modified the model (4) to solve inhomogeneity by defining a local binary fitting (LBF) energy function for each point given by

Equation 5

\[
\varepsilon_1(C, f_1(x), f_2(x)) = \lambda_1 \int_{\text{in}(C)} K_\sigma(x - y) |I(y) - f_1(x)|^2 dx dy
\]

\[
+ \lambda_2 \int_{\text{out}(C)} K_\sigma(x - y) |I(y) - f_2(x)|^2 dx dy
\]

where \(I(x), \lambda_1\) and \(\lambda_2\) are the same parameters as those in the PC model. \(K_\sigma(x)\) is a Gaussian kernel with variance \(\sigma\), whose value is decreased as \(|x|\) increases. Its expression is given by
Equation 6. \( f_1(x) \) and \( f_2(x) \) are the average intensity inside and outside the contour \( C \) and with the effect of the local kernel \( K_\sigma(x) \), they are actually the average intensities near point \( x \). The local Gaussian kernel is defined as:

Equation 6

\[
K_\sigma(t) = \frac{1}{(2\pi)^{\frac{D}{2}} \sigma} \exp(-\frac{x^2}{2\sigma^2})
\]

The LBF model outperforms the PC model in LBF’s strength in processing inhomogeneous images. However, we need to set \( \lambda_1 \) and \( \lambda_2 \) large to detect all the dendritic structures, including weak spines with low intensities. However, large \( \lambda_1 \) and \( \lambda_2 \) can also detect low intensity noise and irrelevant structures. On the other hand, small \( \lambda_1 \) and \( \lambda_2 \) will constrain the zero level set to high intensity regions, which tends to miss some important structures. We solve this problem by adaptively changing \( \sigma \) of the Gaussian kernel while setting \( \lambda_1 \) and \( \lambda_2 \) large enough to detect all the structures. To do this, we first define the following function to calculate \( \sigma \) for each pixel.

Equation 7

\[
\sigma_x = \begin{cases} 
\frac{d_x}{1 + g_x} \sigma_s & \text{if point}(x) \not\in \{\text{Medial axis}\} \\
\sigma_c & \text{if point}(x) \in \{\text{Medial axis}\}
\end{cases}
\]

Where \( x \) represents pixel, \( \sigma_x \) is the variance of the Gaussian kernel at each pixel, \( d_x \) is the distance between pixel and the medial axis, \( g_x \) is the magnitude of the gradient at each pixel. \( \sigma_s \) is the starting variance value of the Gaussian kernel on each pixel except for the medial axis points whose variance is \( \sigma_c \). We named our modified LBF model as adaptive LBF (aLBF) model. The meaning of Equation 7 is that when the pixel is far away from the medial axis, the image is smoothed to avoid irrelevant structures and noise. At the same time, since gradients indicate edges, the variance should be small enough to be sensitive to these edges. For points on the medial axis, which is the initialization of the level set formulation, we also employ a small \( \sigma \) to make sure that the weak boundaries are detected. To save calculation cost, if \( \sigma_x \) is larger than
a given threshold $\sigma_{\text{max}}$, we set it to $\sigma_{\text{max}}$. In this formulation, the LBF energy for each pixel in Equation 7 becomes:

Equation 8

$$\varepsilon_i(C, f_1(x), f_2(x)) = \lambda_1 \int_{\text{int}(C)} K_{\sigma_y}(x-y) |I(y) - f_1(x)|^2 dx dy$$

$$+ \lambda_2 \int_{\text{out}(C)} K_{\sigma_y}(x-y) |I(y) - f_2(x)|^2 dx dy$$

Figure 17 shows a comparison between LBF and aLBF segmentation results and details will be provided in the results section. With segmented images, we can calculate signal to noise ratio (SNR) which is an important measurement of system robustness. SNR is defined as

$$\text{SNR} = 10 \log_{10} \left( \frac{P_s}{P_n} \right)$$

where $P_s$ is the power spectrum of the signal while $P_n$ is the counterpart of noise. Here the signal is segmented dendritic structures and noise is the background.

Figure 17 Comparison of (a) LBF level set model and (b) aLBF level set model. The yellow circled regions are weak spines.

2) Extraction of morphological information of spines and branch points

The dendritic structure information obtained so far includes medial axes, branch points and boundaries of the dendritic structure. With this information, we can quantify some morphological
characteristics, such as spine area and attributes of branch points which include curvatures, positions and branch directions.

When the medial axis image is overlapped with the boundary image, which is illustrated in Figure 18(a), the medial axis passes through the spine region detected by the aLBF method. We calculate the spine area in the following manner: 1) remove aLBF detected regions without intersections with the medial axis and remove the isolated regions where other regions intersect with the same branch. The result is shown in Figure 18(b); 2) extract regions that are detached from the main structures. These are the detached spines, whose areas are calculated as areas of the corresponding detached spines; 3) calculate attached spine areas. Since branch points located on the backbone are available, we follow the attached spine detection method described in Cheng et al. (2007) [82] to identify the attached spines. The results are shown in Figure 18 (d). The red regions are the attached spines that are detected by the automated method.
Now the detached spines and attached spines are successfully quantified in spine areas. By representing each branch point as spines, we build an attribute vector $V$ which will be used for spine tracking. For each branch point, six attributes are defined: the Euclidean positions $x_i$ and $y_i$, the curvature $\kappa_i$, the normalized spine area $\bar{a}_i$, and the direction information $o_i$ and $\omega_i$. We represent each set as:

$V = \{v_1 \cdots v_m \mid v_i \equiv [x_i, y_i, \kappa_i, \bar{a}_i, o_i, \omega_i] \in \mathbb{R}^6 \}$

where $m$ is the total number of branch points at each time point.

Next, we describe how to calculate the attribute vector of the $i$-th branch point. To simplify the notation, we drop the ‘$i$’ in $v_i \equiv [x_i, y_i, \kappa_i, \bar{a}_i, o_i, \omega_i]$ temporarily. The curvature of the $i$-th branch point is given by

$$\kappa(t) = \frac{\dot{x}_\sigma(t) \ddot{y}_\sigma(t) - \dot{y}_\sigma(t) \ddot{x}_\sigma(t)}{[(\dot{x}_\sigma(t))^2 + (\dot{y}_\sigma(t))^2]^{3/2}}$$

where the image domain is parameterized by $t$ and $\dot{x}_\sigma(t) = x(t) * \hat{g}_\sigma(t)$, $\dot{y}_\sigma(t) = x(t) * \hat{g}_\sigma(t)$ where $g_\sigma(t) = \frac{1}{(2\pi)^{n/2}} \exp(-\frac{t^2}{2\sigma^2})$ is the Gaussian kernel with variance $\sigma$, which is different from the $\sigma$ in
the aLBF level set part. \( \dot{y}_\alpha(t) \) and \( \ddot{y}_\alpha(t) \) follow similar formulations as \( x(t) \). Here the Gaussian kernel helps smooth the image and makes the algorithm numerically computable.

We normalize the spine area as:

\[
\bar{a}_i = \frac{(a_i - a_{\text{min}})}{(a_{\text{max}} - a_{\text{min}})}
\]

where \( a_i \) is the area of each spine and \( a_{\text{min}} \) and \( a_{\text{max}} \) are the largest and smallest area respectively.

For different time steps, the backbone shape will not change dramatically, hence the curvatures of the corresponding branch points will not fluctuate significantly and neither does the area of the spine region. \( o_i \) is the tangent value which points from the branch point to a point several pixels away. We compute \( o_i \) as follows:

\[
o_i = \frac{(y_{bp} - y_{ap})}{(x_{bp} - x_{ap})}
\]

where \( x_{bp} \) and \( x_{ap} \) denote the x-axis Euclidean position of the branch point and the point several pixels away respectively and \( y_{bp} \) and \( y_{ap} \) are the corresponding y-axis Euclidean positions. We usually consider positions five pixels away, which is equal to the line length lower threshold. Another reason is that usually the central line of the spine does not have a sharp curvature at the beginning of the structure. However, when two branches point to opposite directions, we cannot differentiate these directions only with \( o_i \). Therefore we add the left or right pointed term \( \omega_i \) to remove this confusion. The left or right pointed term represents the branch’s relative location to the skeleton. We denote the left-pointed branch as -1 and the right-pointed as 1. The term is determined using a technique similar to the one we used to detect the branch point.

3) **Spine tracking based on maximum likelihood estimation (MLE)**

We represent the dendrite structure that we have extracted at each time point as a dendrite graph \( G_i(V) \) of a dendritic structure comprised of the branch points and their attributes. We, then, match two dendrite graphs \( G_1 \) and \( G_2 \) of time points T1 and T2 by establishing the graph homomorphism [112] between two graphs under with maximum likelihood estimation (MLE).
With dendrite graph, we translate the tracking problem to a graph homomorphism problem by representing each image as a graph with each branch point as a vertex. A graph homomorphism is a mapping \( f : V_1 \rightarrow V_2 \) from the dendrite graph \( G_1 \) to \( G_2 \). Most of the existing methods assume constant number of branch points. However, in our problem, there exist spine formation and elimination due to the evolution of the neuron and the occurrence of spurious branch points. We employ MLE-based techniques to find the best homomorphism between the two graphs, which can solve spine evolution. Let \((o, e)\) be a pair of vertices at two time points. \(v_o\) is a point in data set \(V_1\) of the first image and \(v_e\) is a point in the evolved data set. The objective is to find a mapping which maximizes the posterior \( p((o,e) | v_e = f(v_o)) \). According to Bayes’ law, we get

\[
p((o,e) | v_e = f(v_o)) = \frac{p(v_e = f(v_o) | (o,e))p((o,e))}{p(v_e = f(v_o))}
\]

where \( p(v_e = f(v_o) | (o,e)) \) is the likelihood, \( p(v_e = f(v_o)) \) is the evidence and \( p((o,e)) \) is a uniform distributed prior. Therefore, our problem reduces to finding the best \( e \) that maximizes the likelihood, which can be expressed as follows:

\[
\arg \max_e p(v_e = f(v_o) | (o,e))
\]

We model this problem as a 6-dimensional Gaussian distribution with a diagonal covariance matrix because the six attributes are uncorrelated with each other. In our experiments, we assume the diagonal terms of the covariance matrix are \([10 \ 10 \ 0.02 \ 20 \ 0.05 \ 0.001]\). 10 is the variance of the Euclidean position of the branch points, which allows ten-pixel movement due to imperfect registration and noise to guarantee the robustness. The typical curvature of the branch points in our problem is in the range of \([0.01, 0.20]\). Hence, we assume the variance of the curvature is 0.02. 20 is the variance for the area. As mentioned above, the mistake that matches a left-pointed branch to a right-pointed one is considered unacceptable, so we assumed a small variance for the left or right pointed term. We also set the variances for the tangent value as 0.05 and the left or right pointed term as 0.001. All these parameters are optimally determined by experiments. The Gaussian distribution \( N(0, \Sigma) \) is formed as
\[ p(v_e = f(v_o) \mid (o,e)) = \frac{1}{(2\pi)^{d/2}} \exp(-\frac{1}{2}(v_e - v_o)^T \Sigma^{-1}(v_e - v_o)) \]

We developed a dynamic programming (DP, [113]) solution to find the best e that maximizes the likelihood probability \( p(v_e = f(v_o) \mid (o,e)) \) for all the branch points on the two dendritic sections. We build the DP algorithm for our problem as follows,

Let \( d(i, j) \) be the probability of \( p(v_j = f(v_i) \mid (i,j)) \). \( d(i, j) \) serves as the decision in the DP. \( S(i, j) \) is the score for the best correspondences of the spines 1 to i in \( V_1 \) with spines 1 to j in \( V_2 \). The score is considered as a state in DP.

The equation of state is

\[
S(i, j) = \begin{cases} 
S(i-1, j-1) + d(i, j) & S(i-1, j-1) + d(i, j) \geq \max[S(i-1, j), S(i, j-1)] \\
S(i-1, j) & S(i-1, j) > S(i, j-1) \\
S(i, j-1) & \text{Otherwise}
\end{cases}
\]

Equation 9 expresses how we update state when a new decision arrives. The termination condition is to find \( S(n,m) \), where \( n \) and \( m \) are the total numbers of branch points in the two data sets.

4) Spine segmentation with 3D level set approach

3D image based strategies segment dendritic structure without image projection. We employ a LoG (Laplacian of Gaussian) based level set approach (Zhou et al., 2008) to segment neuron dendrites, attached spines as well as detached spines. We will compare 3D level set segmentation results with our proposed projected image based strategy to demonstrate the capability of our method in the Discussion section.

The preprocessing starts with a 3D median filter with a kernel size of 3 by 3 by 3 to remove shot noise introduced by imaging device. Then a top-hat operator is used to correct uneven illumination degradation and enhance the images. Considering \( C \) as a closed contour and \( \phi \) as a
signed distance function, which is negative for points inside C, positive outside C and zero at C, we define the energy function as:

\[ F(\phi) = \lambda E(\phi) + \alpha L(\phi) + \beta V(\phi) \]

where \( \lambda, \alpha \) and \( \beta \) are weighting constants for three energy terms respectively defined as:

\[ E(\phi) = \int_{\Gamma(\phi=0)} \nabla (G_{\sigma_1} * I) \vec{n} d\Gamma \]
\[ L(\phi) = \iiint_{\Omega} \delta(\phi) |\nabla \phi| dxdydz \]
\[ V(\phi) = \iiint_{\Omega} g(\nabla I) H(-\phi) dxdydz \]
\[ g(\nabla I) = \frac{1}{1 + (1+|\nabla (G_{\sigma_2} * I)|^p)} \]

Let \( \Omega \) denote the image domain and I be an image, \( E(\phi) \) is LoG energy while \( L(\phi) \) and \( V(\phi) \) denote the area of zero level set surface and volume inside zero level set surface respectively. \( G_{\sigma_1} \) is Gaussian kernel with standard deviation \( \sigma_1 \) and \( \vec{n} \) denotes the outer normal direction of zero level set. \( \delta \) is Dirac function, \( H \) is the Heaviside function and \( g \) is a positive non-increasing function defined as: \( g(\nabla I) = \frac{1}{1 + (1+|\nabla (G_{\sigma_2} * I)|^p)} \), in which \( G_{\sigma_2} \) is a Gaussian kernel with standard deviation \( \sigma_2 \). To minimize \( F(\phi) \), the Euler-Lagrange equation for \( \phi \) can be written as:

Equation 10

\[ \frac{\partial F}{\partial \phi} = -\delta(\phi) \left[ \lambda \Delta G_{\sigma_1} * I + \alpha \nabla \left( \frac{\nabla \phi}{|\nabla \phi|} \right) + \beta g(\nabla I) \right] \]

The first term of Equation 10 aligns the curve close to the zero crossings along the edge. The second term keeps the surface of the zero level set smooth all along the evolution. The third term is a balloon force, which acts to speed up the evolution where the image gradient is small or slow down the evolution where the gradient is large.

**B. Experimental results and validation**

1) Results for dendritic structure detection
Figure 15 and Figure 16 show the dendritic structure detection results. Figure 15 (a) and Figure 16(a) are raw images without processing. Figure 15(b) and Figure 16(b) illustrate the line detection results without post-processing. The cyan lines denote the backbone and the attached spine structures, while the red lines represent the detached spines. There are some line segments far away from the main dendritic structures and they are considered as noise that can be easily filtered. Figure 15(c) and Figure 16(c) show the dendritic structures after linking detached spines to the backbone. The manual spine detection results are displayed in Figure 15(d) and Figure 16(d).

In Figure 15, by comparing (c) and (d), we could tell that using our algorithm, we can detect all the spines marked manually by the biologist. The manual detection is performed in 3D without knowing the automated detection results. An expert observes several neighboring slices before marking each spine. The expert can zoom in every region during the observation. In addition to the manually marked spines, our method detected spines marked by capital letters, which are considered as false positives. The expert cannot confirm whether spine A is a positive one because it is overlapped with some uncorrelated structures even in the 3D domain. For spine B, we noticed that the expert first marked it and then deleted it based on the 3D information.

Figure 16 presents the dendritic structure detection result for the same data set at time point 2. From the result shown in (b), we could tell that we successfully detected the central line of this spine (Spine A in Figure 16(c)). By comparing (c) with (d), we can see that our method finds all of the spines except the one manually marked as number 6. Note that spine number 6 is parallel to the backbone. The error appeared in the line linking process because of the parallelism. Another false positive happened at spine B which was ignored by the expert since it was too small.

2) Results for dendritic structure boundary extraction

Figure 17 shows the results of the standard LBF model and proposed modified aLBF model using the same parameter settings. The red contours in both images depict boundaries. Here, we set $\lambda_1$ and $\lambda_2$ to the smallest value that can identify the weak spines A and B marked by yellow circles in Figure 17(a). These two spines have relatively lower average intensities than the other
spines. In Figure 17(b), with the same $\lambda_1$ and $\lambda_2$ setting, our method can identify spine A and B more accurately. The accuracy is important for spine tracking. We can see that in Figure 17(a) there are more irrelevant structures and noises detected at the northwestern part than that at the same position in Figure 17(b), as well as in some other locations. Furthermore, the boundaries identified by aLBF were tighter and more precise than its counterparts in LBF. This desired property is an outcome of our adaptive setting for $\sigma$. Therefore, the aLBF model outperforms the LBF level set method in detecting weak spines.

![The Sigma matrix](image)

(a) (b)

**Figure 19** The value of sigma. (a) 3-D view; (b) 2-D view.

Figure 19 displays the value of $\sigma$ in the whole image domain. As mentioned above, we set the $\sigma$ as $\sigma_{\text{max}}$ if it is larger than $\sigma_{\text{max}}$. From Figure 19(a), a 3-D view of $\sigma$, we can tell that when the pixel is far away from the main structures, $\sigma$ is set to $\sigma_{\text{max}}$. The value of $\sigma$ near the medial axis varies according to Equation 7. In Figure 19, the red color denotes the large value while the blue one represents small.

3) **Results for spine tracking**

In this section, we present the spine tracking results with our MLE based method. Figure 20 shows the tracking result of two time points in data set R10XB. As the cyan two-way arrows illustrate, we find 14 pairs of the matching spines at two time points. Their branch points are circled by green rings. Yellow squares in the left image, which present the first time point, denote the spines for which we cannot find their evolved versions. They are considered as
eliminated spines or false detected spines of our method. On the other hand, in the right image, the pink triangles at the second time point highlight the spine branch points without matching spines with its previous time point, which are newly formed ones. By comparing with the manual results, we notice that we successfully match 14 of the 17 spine pairs and locate one disappeared spine at the first time point and one newly grown spine at the second time point.

**Figure 20** Tracking results for the two time points in data set R10XB. Cyan arrows denote the matching relationship. Green circles denote branch points that can find its matching point. Yellow squares represent the eliminated spines or noise while purple triangles are the newly formulated spines.

4) **Validation**

To evaluate our proposed algorithm for dendritic backbone and spine detection, we compare our results with manual labeling by visual inspection done by an expert. The protocol of derivation of manual results is described in early part of this section. The validation process is designed as follows: we randomly select 8 neuron images from a total of 14 images available to us. 8 images are 4 pairs of two time point data sets. Each pair of images was taken from different regions or different dendrites of mouse brain. For each image, we randomly select one dendrite region to lighten the burden of the manual process. We manually label spines in each selected
dendrite and use the results as the ground truth. Our proposed algorithm is then applied to each
dendrite and results are compared with the manual results.

The manual method detected 107 spines while the automated method detected 115 spines. Among these spines, a total of 103 spines were detected by both methods; four additional spines were detected only by the manual method and they were false negatives that we fail to identify using the automatic method; 12 spines were detected only by the automated method and they were found to be false positives. As mentioned above, some of the false positives are introduced by noise and some are too small for the expert to mark. Table 1 quantitatively summarizes the results.

Table 1 Comparison of spine detection results between manual and the proposed automatic method.

<table>
<thead>
<tr>
<th>Image name</th>
<th>Spine number (Manual)</th>
<th>Spine number (Our method)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total number</td>
<td>False positive</td>
</tr>
<tr>
<td>R10XAT1</td>
<td>6</td>
<td>7</td>
</tr>
<tr>
<td>R10XAT2</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>R10XBT1</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td>R10XBT2</td>
<td>17</td>
<td>18</td>
</tr>
<tr>
<td>R10XDT1</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>R10XDT2</td>
<td>10</td>
<td>12</td>
</tr>
<tr>
<td>L10XBT1</td>
<td>20</td>
<td>21</td>
</tr>
<tr>
<td>L10XBT2</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>107</td>
<td>115</td>
</tr>
</tbody>
</table>
Figure 21 shows manual spine labeling results for two time points in data set R10XB. The manual labeling of the spines strictly follows the shape of the spines until the edge becomes too weak. Figure 22 illustrates the spine length comparison between the manual and automatic methods. From Figure 22(c) we notice that in most cases, the spine lengths of the automatic method are shorter than the manual measurement. However, a small part of the automatic results are far larger than the manual ones because of noise and uncorrelated structures linked to the spines. From Figure(a) and (b), respectively the CDF (Cumulative Distribution Function) comparison and the quantile-quantile plot, we conclude that the manual and automatic results follow the same uniform distribution. The spine areas calculated from the automated and manual method are consistent. For attached spines, results are similar in both methods. For detached spines, manual results are larger than automated ones because our neurologist included the area between detached spines and dendrites. The comparison of R10XB time point 2 is illustrated by Figure 23. KS (Kolmogorov-Smirnov) test confirms that automated and manual area measurements are drawn from the same distribution.

Figure 21 Manually labeled results for two time points in data set R10XB.
Figure 22 (a) CDF of spine length distribution; (b) Quantile-quantile plot of spine length; (c) Spine length comparison between manual and automatic results.
To validate the tracking results, we compare our results with manual results. We use the same data sets as those used in the spine detection validation part. The results are listed in Table 2. From Table 2 we observe that the total number of matched spines is 51 and our method identifies 43 of them. Compared with the manual results of 2 eliminations and 3 formations, our results are 15 and 14 respectively, much larger than the manual ones. The errors mainly introduced by the false positives in the spine detection as well as imperfect registration results. The errors in elimination are induced by false negatives in the first time point or by wrong matching results with time point 2. The errors in formation are caused by false positive detection of spines and also due to errors in tracking. Dynamic programming is a forward optimization technique, assuming that the solution previous to the current decision is optimal and it would not resort to the former decision to update current state. Therefore, although it is a global optimization technique, matching errors may occasionally occur during the optimization process. A possible solution to improve the tracking performance is to incorporate more time points and integrate all information to eliminate false positives with “future” time point information. The false negatives can be corrected if the same spine appears in the previous time and the subsequent time but not in the current time point. Once we derive the accurate spine detection result with multiple time points instead of only two, dynamic programming will be able to find the global optimal solution.

Figure 23 Area comparison between automated and manual method on data R10XB, time point 2.
Table 2 Comparison of tracking results between the manual and the proposed automated method.

<table>
<thead>
<tr>
<th>Data sets names</th>
<th>Spines number (Manual)</th>
<th>Spine number (Our method)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Matched</td>
<td>Elimination</td>
</tr>
<tr>
<td>R10XA</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>R10XB</td>
<td>16</td>
<td>1</td>
</tr>
<tr>
<td>R10XD</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>L10XA</td>
<td>19</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>51</td>
<td>2</td>
</tr>
</tbody>
</table>

5) Parameter selection

Table 3 lists typical parameter values used in this work. The standard deviation for the Gaussian kernel is determined automatically in the curvilinear structure detection part. Other parameters for the curvilinear structure detection, such as the upper threshold, the minimum line length, and the linking distance, can be fixed loosely using a large range and do not affect the final results significantly. This conclusion is derived by a large number of test experiments. The parameters for the backbone and spine detection include the weakness threshold and the minimum backbone length. These two parameters may vary for different images, but can be fixed for a set of images from the same biological experiment, or images with similar intensity distributions. In addition to the detection part, the parameter settings for the aLBF level set are also listed in Table 3. The other unmentioned parameters are the same as the original LBF model in Li et al. (2007). The average running time is only in minutes for processing a 512*512*10 neuron image with a Pentium IV2.8G Hz processor for the selected parameters.
Table 3 Parameter settings used in our method.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Typical Value</th>
<th>Description</th>
<th>Parameter</th>
<th>Typical Value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Upper/lower threshold</td>
<td>3/1</td>
<td>Curvilinear structure detection</td>
<td>Lambda1 / Lambda2</td>
<td>3/3</td>
<td>aLBF</td>
</tr>
<tr>
<td>Linking distance/Line length</td>
<td>5/5</td>
<td>Curvilinear structure detection</td>
<td>Maximal value of sigma</td>
<td>20</td>
<td>aLBF</td>
</tr>
<tr>
<td>Minimum backbone length</td>
<td>80</td>
<td>Curvilinear structure detection</td>
<td>Starting sigma</td>
<td>10</td>
<td>aLBF</td>
</tr>
<tr>
<td>Vector inner product difference</td>
<td>0.1</td>
<td>Spine Linking</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

C. Discussion

3D based segmentation methods are employed in *in vitro* neuron spine detection. We also employ a 3D level set method to segment dendritic structures in *in vivo* data. A rotated version of our 3D segmentation result of R10XB time point 2 is shown in Figure 24(a). The uncorrelated structures far away from the dendrite were filtered. We could tell that the structure surfaces were smooth. However, this method failed in detecting the weak spines, which are marked by yellow circle in Figure 24(b). We marked the corresponding region in Figure 24(a) where we could not see the spine. In 3D space, signals of this spine in each slice are very weak and they are contaminated by noise. However, in MIP projection, the largest intensities of each column of voxels are recorded in the projection image, thus the weak signal can be detected in the 2D MIP
image. We then quantify the spines to get volumes (Zhou et al., 2008) and add this feature to the branch point feature vector. The variance of the Gaussian assumption for volumes in MLE model is set to 20. The tracking result is the same as previously reported results for data set R10XB and in other data sets.

![Figure 24](image)

Figure 24 (a) A Rotated view of LoG 3D level set segmentation of R10XB time point 2; (b) Image R10XB time point 2 overlapped with medial axis. The yellow circles indicate the corresponding position in the two images.

This result indicates that if \textit{in vivo} images are directly segmented and quantified in 3D, due to the low SNR, weak signals can be missed. Thus far, volume information does not improve the tracking result. Therefore, only 3D based methods may not be the optimal solution and in some case they are inferior to 2D based methods. Possibly a hybrid method including both 2D and 3D could improve spine detection as well as spine tracking. In addition, when 3D curvilinear structure detection is mature enough to detect lines with different widths in 3D space in a low SNR environment, we might generalize our whole pipeline to 3D.

Another issue is time series of images. The interval of our \textit{in vivo} image data is one hour. There are two time points in every data set. The details about these data sets are provided in Validation part in previous section. If we keep this interval, the algorithm will be able to process data of multiple time points two by two and a summarization of the time series is necessary to incorporate time series information together. The time series information helps to eliminate false
positives and false negatives. If the interval is larger, such as one day, the deformation and shifting make the problem much more difficult. A more complex non-rigid registration strategy should be necessary, as well as segmentation methods that are robust to changing and lower SNRs. Moreover, unlike in \textit{in vitro} imaging, it is very challenging to keep the object stable for days to record the same regions of interest using \textit{in vivo} imaging. Therefore, although time series images might be a potential improvement to derive better results, the difficulties of \textit{in vivo} imaging should be addressed first.

The last issue we discuss is the robustness of our system. Signal to noise ratio (SNR) is an important criterion to quantify the quality of an image as well as a measurement of system robustness. In order to find a SNR threshold which approximately indicates how bad an image our system is able to process robustly, we tested the SNR of our validation data sets and images contaminated by additive Gaussian noise.

![Box plot of SNRs of validation data set](image1)

**Figure 25** (a) Box plot of SNR of the validation data set; (b) SNR changes with additive white noise with different variance.

A box plot of SNRs of 8 images used as validation data is presented in Figure 25(a) with average SNR 24.2dB. We noticed that L10XBT1 had the worst SNR of 19.58dB. For image R10XBT1 and R10XDT2, the SNR are as low as 22.34dB and 21.92dB respectively, which was coincidental with the relatively large false detection error in spine detection. In addition, Gaussian additive noise with zero mean and different variance was added to R10XDT1, an image with relatively higher SNR (26.68) in our data set and results were illustrated by Figure
16(b). The SNR dramatically dropped when the variance increased from $1e^{-5}$ to $1e^{-3}$. When contaminated images were processed by our system, the false detection rate rises in accordance with the increase of Gaussian noise variance. We noticed that when SNR was larger than 10dB, the detection rate was higher than 75%. Therefore, we adopted 10dB as a SNR cut-off value for our system.

**D. Conclusion**

In this chapter, we have presented a novel strategy for spine detection and tracking for *in vivo* neuron images. There is little work in *in vivo* neuron image processing and quantification available in the literature according to our knowledge. The pipeline presented here will help neurologists automatically label and quantify small spines and find matching spine pairs at different time points. It overcomes the difficulties of poor image quality and extracts neuron dynamic changes from the real-time *in vivo* data. The algorithm detects the dendritic backbones and the detached spines from noisy and low contrast *in vivo* image data of live mice of an AD model. It is able to quantify the spine length and area, with the proposed adaptive local binary fitting (aLBF) level set method. The proposed aLBF model can accurately locate the boundary of dendritic structures using the central line of curvilinear structure as the initialization. Then, we present a maximum likelihood estimation based tracking algorithm to track the evolution of spines at different time points and employ dynamic programming to find the solution. Our tracking algorithm is able to detect spine elimination and formation. The algorithm is highly automated and fast. It requires minimum human interaction. With the results derived from the proposed pipeline, biologists can study the pathology of certain types of neuron diseases and sketch the spine dynamics without manually labeling and pairing spine structures.
IV. SYNAPTIC VESICLE DETECTION AND QUANTIFICATION

Synaptic vesicle dynamics play an important role in the study of neuronal and synaptic activities of neurodegradation diseases ranging from the epidemic Alzheimer's disease to the rare Rett syndrome. A high-throughput assay with a large population of neurons would be useful and efficient to characterize neuronal activity based on the dynamics of synaptic vesicles for the study of mechanisms or to discover drug candidates for neurodegenerative and neurodevelopmental disorders. However, the massive amounts of image data generated via high throughput screening require enormous manual processing time and effort, restricting the practical use of such an assay. This chapter presents an automated analytic pipeline to process and interpret the huge data set generated by such assays. This pipeline enables the automated detection, segmentation, quantification, and measurement of neuron activities based on the synaptic vesicle assay. To overcome challenges such as noisy background, inhomogeneity, and tiny object size, we first employ MSVST (Multi-Scale Variance Stabilizing Transform) to obtain a denoised and enhanced map of the original image data. Then, we propose an adaptive thresholding strategy to solve the inhomogeneity issue, based on the local information, and to accurately segment synaptic vesicles. We design algorithms to address the issue of tiny objects-of-interest overlapping. Several post-processing criteria are defined to filter false positives. A total of 152 features are extracted for each detected vesicle. A score is defined for each synaptic vesicle image to quantify the neuron activity. We also compare the unsupervised strategy with the supervised method. Our experiments on hippocampal neuron assays showed that the proposed system can automatically detect vesicles and quantify their dynamics for evaluating neuron activities. The availability of such an automated system will open opportunities for investigation of synaptic neuropathology and identification of candidate therapeutics for neurodegeneration.

This chapter is organized as follows. The Materials and Methods section describes our data and the workflow of our pipeline in detail. Here, we explain acquisition of images, image denoising and enhancement, adaptive thresholding to segment vesicles, post processing, feature extraction, scoring, and the supervised detection method. The experimental results and validation by comparing automated results with manual results are presented in the Experimental Results.
section. Algorithmic related issues and scientific generalization are discussed in the Discussion section. We summarize our work in the Conclusions section.

A. Method

As discussed in Chapter II, accurate detection of neuron vesicles is impeded by difficulties in image inhomogeneity, object size, noise removal, and artifacts introduced by imperfections in imaging. To address these issues, we developed an automated pipeline for vesicle detection and quantification to characterize synaptic activities using vesicle dynamics. Our pipeline employs a Multi-Scale Variance Stabilizing Transform (MSVST) to alleviate the Poisson noise and enhance the signals. Local information is then employed to solve the inhomogeneity issue. A new iterated procedure employing object size and local intensity in an adaptive thresholding manner is proposed to detect vesicles with high intensity background as well as weak vesicles. In addition, we define criteria especially for this application to filter false positives with morphological features. We also design novel algorithms to separate the overlapped tiny objects such as vesicles. We define a score to measure neuron activity using the density and intensity dynamics of vesicle boutons. This unsupervised method provides accurate results with automated, fast, and batch processing capabilities. In addition, we compare this method with a supervised method based on the popular SVM (support vector machine [114]) using most common image features for spot objects and feature selection strategies [15] and discuss the application of these two strategies. We will provide details of these algorithms in the following sections.

1) Image data acquisition

The resting buffer consisted of 25mM HEPES, 119mM NaCl, 2.5mM KCl, 2mM CaCl2, 2mM MgCl2, 30mM Glucose, 10μM 6-cyano-7-nitroquinocxaline-2,3-dione (CNQX), and 50μM D,L-2-amino-5-phosphonovaleric acid (D,L-AP5), pH 7.4. Buffer was balanced with sucrose to 315 mmol kg-1osM-1. Field stimulation was achieved through parallel platinum electrodes immersed in the perfusion chamber, delivering 70 V, 1 ms pulses at 10 Hz. Cells were washed with resting buffer twice before they were incubated with resting buffer containing 10 μM FM 1-
43. To load FM dye, cells were stimulated with 900 action potentials (APs). They were then washed extensively with resting buffer for 10 minutes. To destain, cells were continuously stimulated and fluorescence images were recorded for 3 minutes at 5 second intervals.

In this study, we made use of the first image before destaining and the last image after destaining. For imaging, cells were viewed via a 60× 1.4 NA objective on an inverted microscope. The microscope was equipped with a stage environment control box and the temperature was maintained at 37°C. In this application, each data set contained two images, the first was scanned after staining and washing, and the other was taken using the same hardware settings after the electric stimulation, which excited the neuron to release stained dye. If the stimulation forced a complete dye release, indicating that the neuron and vesicles were active, few bright spots were observed in the second image. Therefore, the differences between before and after stimulation can be employed as a measurement of activity.

2) Image preprocessing

Image preprocessing procedures are employed to correct artifacts to help achieve optimal results. Due to non-specific staining, some neuron structures other than vesicle boutons are stained and these structures are illuminated in both before and after destaining images. In addition, although biologists endeavor to keep identical conditions before and after stimulation, there are still slight differences, such as changes in locations of the same neuron structure and intensity of non-vesicle structures. If there is need for registration, we apply the Iterative Closest Point (ICP) algorithm [115] to align the two images. ICP finds the closest point on a geometric entity, which is the foreground object in an image, to a given point. The ICP algorithm converges monotonically to the nearest local minimum of a mean-square distance metric and the rate of convergence is fast [115]. For each data set, we manually select four reference points from the stained structures from the first time point image. Since no severe deformations are observed, we find it sufficient to consider only rigid transformation with the ICP algorithm. Note that the registration is an optional step in the pipeline, which can be enabled if necessary. After registration is the global background correction step. When the same neuron component has been aligned at the same or very close Euclidean location for two image slides, a consistent intensity
range is desired. To do this, linear image enhancement is applied to the second image to establish consistence with the first. For an image batch, we employ an averaged range to adjust the background.

Given a data set of registered and background corrected images, we can simply subtract the second image from the first to obtain the difference image. In this image, the staining artifacts are mostly removed. These structures include both inactive vesicles and non-vesicle structures. The following procedures, including MSVST enhancement and adaptive thresholding, will be performed on the difference image of each image set.

3) Image segmentation

One of the most challenging issues is overcoming image inhomogeneity to identify both weak vesicles and vesicles with high intensity. To address this issue, we implement the MSVST method to perform approximate gaussianization and stabilize the variance of a sequence of independent Poisson random variables filtered using a low-pass linear filter. This wavelet-based algorithm is fast and effectively controls the false discovery rate (FDR) [96]. This transform will provide an enhanced and denoised map of the difference image. Then we propose an adaptive thresholding-based iteration to directly address the inhomogeneity issue. The adaptive thresholding takes affect only on a limited number of vesicle regions and considers only local information. Thus, it is able to identify vesicles located in both bright and dark background regions. In addition, the proposed adaptive thresholding strategy addresses the size issue by varying the region size threshold to accurately acquire object boundaries. The parameters are loosely set to allow as small a number of false negatives as possible. The reason is that the loose thresholding will introduce some false positives; however, false positives can be eliminated in post processing but false negatives are very complicated to retrieve. To reduce the number of false positives, filters are defined to remove the fake vesicles including isolated spots, non-vesicle structures, and weak-vesicle-like structures.
We first briefly explain the MSVST method as an enhancement and denoising procedure. Assume \([Y_n]\) is a sequence of output RVs (random variable) of a finite impulse response (FIR) filter \(h\), and \([X_n]\) is independent RVs; Equation 11 stands and the goal is to stabilize the variance of \(Y_n\). In this case, the filter \(h\) functions as an averaging filter to increase the signal-to-noise ratio at the output.

Equation 11

\[ y_n = \sum_i h(i) x_{n-i} \]

Any form of transformations would not change the property of the variance of \([Y_n]\). The general form of VST (variance stabilization transform) is given as: \(T(Y) = b\sqrt{Y} + c\). We follow the definitions of \(b\) and \(c\) described by Zhang, Fadili et al. 2008 [96].

After the stabilization procedure, UWT (undecimated wavelet transform) is applied to enhance the signal, which is intensity in this case. A filter bank \((h, g)\) is employed by UWT, \(W = \{w_1, w_2, \ldots, w_J, a_J\}\), where \(w_J, 1 \leq j \leq J\) is the wavelet coefficient at scale \(j\), and \(a_J\) is the coefficient at the coarsest resolution. The update from one resolution to the next can be represented as: \(a_{j+1} = h[-k]^{(j)} \ast a_j\) and \(w_{j+1} = g[-k]^{(j)} \ast a_j\). The reconstruction is obtained as \(a_j = (\hat{h}^{(j)} \ast a_{j+1} + \tilde{g}^{(j)} \ast w_{j+1})\). The filter bank \((h, g, \hat{h}, \tilde{g})\) has to meet the reconstruction conditions defined by Zhang, Fadili et al. 2008 [96].

With the given definition and formulation, UWT denoising with MSVST involves the following three major steps: 1) transformation: to obtain UWT coefficients with MSVST; 2) detection: to identify significant wavelet coefficients by hypothesis testing; and 3) estimation: to iteratively reconstruct the final estimate with the identified wavelet coefficients. The detailed iterative reconstruction procedure is described in Box 1.
The MSVST process

The reconstructed map is an enhanced version of the original difference image. In this map, bright spots are more illuminated while weak spots and spots contaminated by noise have been strengthened. At the same time, the background noise has been reduced. Moreover, edges of vesicle boutons are more obvious than before the procedure. Figure 26 illustrates the effect of MSVST. Figure 26 (a) shows the original image while Figure 26 (b) is the MSVST transformed version of the difference image. Figure 26 (c) illustrates Figure 26 (b) in a 3D view with the value of MSVST map on the z-axis.

Box 1 The MSVST process

Given a filter bank \((h, g = \delta - h, \tilde{h} = \delta, \tilde{g} = \delta)\)

Initialization: \(a_0 = x\)

for \(j = 0\) to \(J - 1\)

do

Calculate the approximation coefficients \(a_{j+1} = h[-k]^{(j)} * a_j\)

Calculate \(w_{j+1} = T_j a_j - T_{j+1} a_{j+1}\) with VST as \(T_j a_j = \tau_1 \sqrt[1/2]{a_j + c_j}\)

Where \(\tau_k = \sum_i (h[i]^{(j)})^k\) and \(c_j = (7\tau_2 / 8\tau_1) - (\tau_4 / \tau_2)\)

Employ hypothesis testing-based denoiser \(\psi\) to \(w_{j+1}, \hat{w}_{j+1} = \psi w_{j+1}\)

end for

Reconstruct \(a_0\) as \(\hat{a}_0 = T_0^{-1}(T_j a_j + \sum_{j=1}^{J} \hat{w}_j)\)
Figure 26 An illustration of MSVST enhanced map. (a) Original Image; (b) MSVST enhanced map of the original image; (c) A 3D view of the enhanced map.

b) **Adaptive thresholding**

We propose an adaptive thresholding-based procedure on the MSVST enhanced map to address the inhomogeneity issue. The adaptive thresholding employs local information to identify vesicles of both high and low intensity. From Figure 26, we notice that if a global thresholding method is applied to such an inhomogeneous image, neither large thresholds nor small ones will accurately segment objects of interest. A large threshold will cause a false negative problem, which is unacceptable in most applications. On the other hand, a small threshold cannot accurately segment vesicles because it might derive loose boundaries. In our data, some vesicles cluster in a group with relatively high intensity background, such as dendrites.
and axon terminals. We name these vesicle groups HIC (high intensity clusters). Therefore, local information should be considered to solve this typical inhomogeneity issue.

**Box 2** The process of adaptive thresholding to detect vesicles in HIC.

We apply an initial intensity threshold to the enhanced difference image generated by the previous step. This threshold is low enough to allow very limited number of false negatives. Then, on the binarized image, we apply an area threshold $T_A$ to obtain regions larger than the given threshold for further processing. For each of these regions, we employ the MSVST-derived values and identify subregions with intensity larger than $T_i$. This $T_i$ will shrink or split the original region by selecting points with intensity larger than the threshold. If the shrunk or split subregions have smaller areas than the updated area threshold $T_A$, we save these updated regions as segmented spots and do not process them any further. For subregions with a larger area than the updated $T_A$, they will go through the next iteration and are filtered by increased threshold $T_i$. This iterative procedure is presented in Box 2 and illustrated by Figure 27.

The *StepLength* is the intensity increment. It linearly increases $T_i$ during the iteration until reaching the intensity upper bound. On the other hand, the update of the area threshold is not linear. $T_A$ approaches the lower bound in an inverse exponential manner. The lower bound of $T_A$
is usually a large portion of the average size of the spots; in our application, it is set to a value smaller than 75% of average spot size derived by experiments.

**Figure 27** A simulated example of the proposed process of adaptive thresholding. (a) Unprocessed image regions; (b) The effect of increasing $T_I$. The region splits into two subregions; (c) The effect of another iteration of updated $T_A$ and increased $T_I$. One subregion is saved and the other enters into the next iteration (d).

As illustrated in Figure 27, there is an HIC region and an isolated spot with low intensity and small area. The area of the isolated spot is smaller than the initial area threshold and is not processed by adaptive thresholding. Increased intensity threshold decreases the area of the HIC background, which is illustrated in Figure 27(b) and (c). Once spots fail to satisfy the area condition, which indicates identification of a vesicle from a relatively high intensity background, regions are saved as segmented spots. This process is illustrated in Figure 27(c) and (d).
c) Segment the overlapped vesicles

Figure 28 Segmentation of overlapped vesicles. Points A and B are central points for each overlapped spot and connected by a green line section. Points C and D are the crossing points and the black line section separates the two overlapped regions. Solid red line sections represent candidates to split the two regions while the red dashed-line sections illustrate the non-candidates, which fail to cross the green line section.

Overlapped objects are commonly found in cell assays. Accurate quantification and segmentation are required to measure neuron activity, and as such, the overlap issue must be addressed. There are two classes of algorithms solving this issue\cite{116, 117}. The first one relies on curvature to detect crossing points (points C and D marked by yellow squares in Figure 28), which typically have large curvature values on edges of overlapped spots. Once these points are detected, we can simply connect them with any line connection algorithm. However, in our case, the size of vesicles is too small (around 10 pixels) to accurately calculate the curvature of each point on the edge and to identify the crossing points. The second common strategy to solve this issue is based on the assumption that the shape of objects of interest can be approximated as round disks. The crossing points can then be identified using the radius and distance between centers of each object. However, in our application, the object size is too small to make any shape approximation or assumption. Therefore, this type of strategy also fails to accurately identify the crossing points. Here, we propose a method to solve this issue.

Our proposed strategy starts with distance mapping. The binary image derived by adaptive thresholding is iteratively eroded by a one-pixel radius disk template until all pixels are zeros. The number of iterations at which a non-zero pixel is eliminated is the distance from the pixel to the edge. Edge points always have distance as one and center points (points A and B in Figure 28)
usually have the largest values in the distance map. With the distance mapping we first locate the central points of each spot region. Overlapped vesicle regions may not contain the local maxima with maximal distance. In this case, we follow a strategy similar to the watershed algorithm on the distance map, which is illustrated by Figure 29. Since prior information about number of vesicles in an overlapped region is unknown, we first consider all the local maximum regions with the largest distance in the distance map as central regions for each candidate spot. Then we search along the downhill direction on the distance map until we identify another isolated local maximum. There are two typical cases illustrated in Figure 29 (a) and (b). Figure 29(a) demonstrates a case in which two overlapped regions have the same local maximum while Figure 29(b) illustrates a case in which one region has a larger local maximum than the other. To differentiate the cases in Figure 29(b) and (c), we also check neighbors of local maxima in the distance map. If a local maximum spreads over pixels, it is not considered as a center point. Since the radius of a circular vesicle or the minor axis of an ellipsoid vesicle usually varies from 2-5, the repetition of the decreasing on the distance map are performed twice in our experiments. For local maximal regions containing more than one pixel, we employ central points of these regions.

![Figure 29](illustration.png)

**Figure 29** An illustration of searching for the local maxima as the region central points.

On the distance map, points with a value of one are the edge points. An example of an overlapped region is provided in Figure 30. We use a template to detect all possible crossing points. In the distance map, we first search for 2’s (green squares in Figure 30(b)) with at least two 1’s (red squares in Figure 30(b)) within its 4-connection neighborhood, with 90 degree difference. These 1’s are candidate crossing points. This procedure reduces the amount of
candidate crossing points on the edge, which depends dramatically on the size and shape of the region. Then, for each pair of overlapped regions, centers are connected with a line section, illustrated by the green line in Figure 28. After that, we employ an exhaustive search on the candidate crossing points and save pairs whose connecting line section crosses the line section linking theregion centers. These sections are represented by the red solid lines in Figure 28; the dashed lines represent non-candidate pairs whose linking line sections fail to intersect with the line connecting region centers (green line). Finally, we use the pair with the smallest distance (white squares in Figure 30(b)) that crosses the center connection as the crossing point for the two overlapped regions. This line section is illustrated by the black line in Figure 28. In our data, spot regions are usually lined up without dramatic direction change so that the center connection line section is always within the overlapped region, indicating that two crossing points are located on opposite sides of the line. This strategy can be easily generalized to cases in which multiple regions are overlapped in a line.

![Figure 30](image.png)

**Figure 30** An example of identification of the crossing points of two overlapped regions. (a) An overlapped region example; (b) The distance map of Fig 7a. The candidates of crossing points are marked in red. The 2’s, according to which we select candidate crossing points, are marked green. Two white squares are the crossing points identified by our algorithm.
The purpose of post processing is to remove false positives from vesicle detection results. These false positives are comprised of three major classes: 1) isolated spots, 2) non-vesicle structures introduced by staining imperfection, and 3) weak-vesicle-like spots. We define these classes and provide detailed description of removal of these false positives in the following.

**a. Filtering isolated spots.** Vesicles usually cluster in neuronal structures such as dendrites and axon terminals, and seldom isolate with each other within a neighborhood. Isolated spots with high intensity are probably introduced by staining imperfection. Therefore, these spots should be reconsidered. We can simply define a neighborhood size and count the total number of spots in the neighborhood. If no other spots exist, the neighborhood filtering removes the isolated spot.

**b. Remove non-vesicle structures.** The non-vesicle structures are generally introduced by staining imperfections. The majority of these structures are dead spots. These objects are high in intensity in both before and after destaining slides when compared with active vesicles. Although we notice that decreases in average intensity are larger in part of these objects than in active vesicles, the changing ratio is lower than that of active vesicles. The numerical changing ratio is defined as the ratio between intensity decrease of a certain region from before to after stimulation and the intensity before stimulation. Usually the ratio of an active vesicle is 1.5 times the ratio of dead vesicles. By discarding spots with slight changes in average intensity, we remove the majority of the second false positive group. Other types of non-vesicle structures are neuronal structures such as line-structure dendrites and other irregularly shaped structures. These objects can be easily eliminated by shape descriptors such as major axis length, convexity, etc.

**c. Remove weak-vesicle-like structures.** There is another class of false positives that are difficult to distinguish from true active vesicles. This category is similar to weak vesicles in relatively low intensity as well as trivial intensity change compared to active vesicles. Unlike weak vesicles, these regions seldom contain a subregion with higher intensity than the region’s average intensity. Therefore, to distinguish this category, we first shrink these weak-vesicle-like regions by erosion according to region areas with 1 or 2 pixel radius disks. Then we calculate the change ratio defined previously for the shrunken region. For active vesicles, the intensity in the
center is higher; the intensity change ratio of the shrunken region between before and after stimulation is larger than that of an unmodified region. Based on this observation, we can discard regions with slight change to the ratio before and after shrinkage.

After the previous steps, including preprocessing, segmentation by adaptive thresholding on MSVST enhanced maps, and post processing to remove different categories of false positives, we now consider the result as our final detection result for vesicle image data.

e) Image quantification

Vesicle quantification extracts straightforward features of each vesicle bouton, including average intensity, area, perimeter, etc. On the other hand, there are certain image features that cannot be directly or spatially observed from an image. These features are extracted from the frequency transformed domain of the original image. In the following paragraphs, we will describe all extracted vesicle bouton features in both spatial and frequency domains.

The first feature category includes general information about vesicle shape, size, and intensity. This fourteen-feature set is comprised of maximal intensity, minimal intensity, average intensity, deviation of gray level, length of long axis, length of short axis, long axis/short axis, equivalent diameter, area, convex area, perimeter, eccentricity, orientation, and solidity.

The second feature category is Haralick co-occurrence texture feature [50], which can be extracted from each of the gray level spatial-dependence matrices. There are totally 13 co-occurrence texture features defined. They are angular second moment, contrast, correlation, sum of squares, inverse difference moment, sum average, sum variance, sum entropy, entropy, difference variance, difference entropy, information measures of correlation, and maximal correlation coefficient.

Another feature category is the wavelet coefficients. Gabor wavelets [118] are directly connected with Gabor filters because they can be designed for a number of scales and orientations. The Gabor filters can be considered dilation and rotation-tunable line detectors. In addition, the texture information can be characterized by statistical information, such as mean and variance in a certain patch. Here we adopt the strategy described in detail by Manjunath and Ma, 1996. With five scales and seven orientations, we have a seventy-element feature vector for
each input sample image: \([\mu_0, \sigma_{00}, \mu_1, \sigma_{01}, \ldots, \mu_6, \sigma_{46}]\). \(\mu_i\) is the mean and \(\sigma_{ij}\) is the standard deviation of the magnitude of the transform coefficients. \(i\) stands for the scale while \(j\) denotes the orientation.

The Zernike moment feature is a region-based shape descriptor that is used as a pattern feature in object recognition from shape. Zernike moments are derived from Zernike polynomials which present an orthogonal set [119]. The orthogonality provides the uniqueness of features extracted by the polynomials. In addition, the moments defined in polar coordination are invariant with respect to their magnitude under rotation. In our implementation, we first locate the centroid of each image. Second, we define the maximum radius \(R\) as the farthest pixel from the centroid. The images are then normalized with the center of mass and the maximum radius \(R\). The pixel \((x,y)\) in the original image is represent by \((\rho, \theta)\), which is the projected image from a Cartesian to a polar coordinate. With the notation \(I(x,y)\) and \(I(\rho, \theta)\) for intensities of original and projected images, respectively, the Zernike moment for each image is defined as:

Equation 12

\[
Z_{nm} = \frac{n+1}{\pi} \sum_{\rho} \sum_{\theta} V_{nm}^*(\rho, \theta) I(\rho, \theta)
\]

Where \(0 \leq m \leq n\), \((n-m)\) is even, \(\rho \leq 1\), \(V_{nm}^*\) is the complex conjugate of a Zernike polynomial \(V_{nm}\) of degree \(n\) and angular dependence \(m\), which is defined over the unit disk as \(V_{nm}(\rho, \theta) = R_{nm}(\rho) \exp(jm\theta)\). \(R_{nm}(\rho)\) is the Zernike polynomials given by

Equation 13

\[
R_{nm}(\rho) = \sum_{k=0}^{(n-m)/2} \frac{(-1)^k (n-k)!}{k!(\frac{n+m}{2}-k)!(\frac{n-m}{2}-k)!} \rho^{n-2k}
\]

The magnitude of \(Z_{nm}\) will be used as features. We choose \(n = 12\) so that we obtain 49 moment features in total. Details are provided by Boland et al, 1998 [120].

In addition to the features described above, we propose a feature category specially designed for this application. This category includes 1) the average intensity difference between before
and after stimulation of a certain region, 2) the ratio of 1) to the average intensity before stimulation, 3) the average intensity difference between before and after stimulation of a shrunken region, 4) the ratio of 3) to the average intensity before stimulation of the shrunken region, 5) the average intensity difference between before and after stimulation of an expanded region (following a similar manner to the shrinkage, but expanding the region instead), and 6) the ratio of 5) to the average intensity before stimulation of the expanded region. These features illustrate the intensity changes between the same regions in original, shrunken, and expanded cases, which are the most important features experts use in manual labeling. In total, we extract 152 features for each vesicle.

\[ f) \quad \text{Supervised method} \]

We briefly describe the supervised method to detect and segment vesicle images. Although the proposed post-processing criteria in the unsupervised strategy remove most predictable false positives, it is interesting to question whether there are better fitted models using more features to identify vesicles. Support vector machine (SVM) is widely employed in a variety of classification problems in bioinformatics. Its advantages in avoiding overfitting, managing large scale feature space, and incorporating diverse kernel methods make it a suitable solution to solve classification problems. In our study, we apply LIBSVM software [121] to classify vesicles and false positives. Five-fold cross validation (CV) is performed. In \( N \)-fold CV, the original data set is equally separated into \( N \) partitions. \( N-1 \) partitions are employed to train the classifier and one partition is left to test the performance of the classifier. This procedure is performed times for all \( N \) partitions and the accuracy is an average of all \( N \) tests. The training data is obtained from manual vesicle labeling by our experts. Details of manual labeling will be provided in the next section.

In the situation in which the dimension of the feature space is very large, the classifier easily tends to be overfitted. Feature selection is a solution to solve this problem and desirably, it also can increase the accuracy of the classification result and better interpret the model. SVM-RFE (Support Vector Machine-Recursive Feature Elimination [122]) is an efficient feature selection algorithm in a backward discarding manner. This procedure starts with all the features, then
removes one feature each time, and all the features are ranked in the end. The ranking principle for each step is the coefficients of the weight vector $w$ of a linear SVM. The procedure is illustrated in Figure 31.

**Figure 31** Procedure of SVM-RFE.

\[ tw = (w_i)^2 \]

\[ \text{Initialize with} \\
\text{Rank Index } R = [ ] \\
\text{Feature space } F \]

\[ \text{Train a linear SVM with} \\
\text{all training data in} \\
\text{feature space } F \]

\[ \text{Compute the weight} \\
\text{vector } w \text{ and ranking} \\
\text{score: } t_i = (w_i)^2 \]

\[ \text{Search for the smallest} \\
\text{ranking score feature } e \\
\text{and its index } j \]

\[ \text{Update} \\
\text{Rank Index: } R = [ j ] \\
\text{Feature space: } F = F - e \]

\[ \text{Are all features} \\
\text{ranked?} \]

\[ \text{Yes} \]

\[ \text{Output} \\
\text{Rank Index } R \\
\text{Feature space } F \]

\[ \text{No} \]

\( g) \quad Scoring \ system \)
To summarize neuron activity based on the synaptic vesicle assay, one straightforward idea is to employ the summarization of vesicle features, such as intensity and area; alternately, averaged features can be employed. However, the average intensity and area, defined as the summation divided by the total number of vesicles, suffer when a large image contains a limited number of vesicles with average vesicle quality. More specifically, this measurement cannot differentiate a decent synaptic vesicle image (with a large number of vesicles) from an image indicating poor neuron activity (with a similar image size but only a limited number of vesicles). In this case, the strategy based on averaged information fails to provide the obvious differences between the decent and the poor cases.

Equation 14

\[ \text{Score} = \frac{1}{\text{Area}} \sum_{i=1}^{n} (\bar{T}_{\text{Before},i} - \bar{T}_{\text{After},i}) \times k \]

Since intensity change of the same spot-region between before and after destaining is the most significant feature that allows the measurement of neuron activity, we employ this descriptor as the major factor to score the neuron activity. The definition of the score is provided by Equation 14. \( \bar{T}_i \) is the average intensity of region \( i \). Subscripts \( \text{Before} \) and \( \text{After} \) indicate the before and after stimulation cases. The \( \text{Area} \) is the size of the image data. \( k \) is a constant number, equal to 100 in our example to modify the score to around 1. Instead of averaging by the vesicle quantity, we utilize the size of the image to divide the total intensity of all detected vesicles. In this case, one image with a considerable number of vesicles has a higher score than the one with a limited number of vesicles of the same size. This is based on the assumption that neurons containing more vesicles releasing FM dye are more active or vigorous than those with a limited number of vesicles.

Since the fluorescence intensity of segmented puncta is the most important parameter for measuring the activity of pre-synaptic boutons, we propose a precise measurement strategy to accurately quantify this value. Subtraction images of the before and after destaining images allows global background correction; however, this method only measures the activity of fast recycling vesicles, which undergo exocytosis in the second round of stimulation (destaining). To measure the total vesicle activity, quantification of the image after FM dye uptake alone is
necessary. However, this is hampered by the inhomogeneous background as the pre-synaptic boutons reside on or near dendrites to which FM dyes nonspecifically bind. Global background correction is inappropriate in this case. To achieve accurate background correction, we propose the following method. First, the background neurite trace will be detected using curvilinear structure detector described in our previous work [94]. These results are illustrated in Figure 32(a). For puncta isolated from dendrites, a box with edge length equal to the major axis is drawn and the background intensity is calculated as the average inside the box other than the puncta area, as illustrated on the upper left in Figure 32(b). For the puncta intersecting with neurites, average background intensity is calculated as the inserted picture in Figure 32(b), in which a rectangular region is drawn along the direction of the background dendrite. The major axis length is then extended on both ends along the direction of central line by \( h \), which is usually 1/3 to 1/2 of the major axis length and can be further determined case by case. The background intensity is then calculated from the pixels inside the rectangle.

**Figure 32** Dendrite fluorescence background correction. Since synapses are projections onto dendrites, most pre-synaptic boutons are located on or near the dendrites and suffer from inhomogeneous fluorescence background. (a) Dendrites are tracked using the curvilinear structure detector and the centerlines are marked by green curves; b) an illustration of local background correction procedures.

**B. Experimental Results**

Figure 33 and Figure 34 illustrate results of the proposed automated vesicle detection pipeline. We compare these results with manual labeling and consider the manual labeling results as the ground truth. In both Figure 33 and Figure 34, panels a and b are original images before
and after stimulation, respectively. Panel d shows the manual labeling results on an enhanced version of the original image before stimulation. Panel e presents the unsupervised detection and segmentation results while panel c illustrates the supervised results. The vesicles segmented by the automated method are labeled by red contours in both panels c and e. Vesicle features are quantified for further use and can be integrated into the scoring method to offer a straightforward measurement for neuron activity. The segmentation statistics and scoring results of a data batch with four images are illustrated in Table 4 and Table 5.

**Figure 33** An example of detection and segmentation results of the proposed pipeline on Image 1. (a) Image before stimulation; (b) image after stimulation; (c) supervised detection results, detected vesicles are circled in red; (d) manual labeling results, positive examples are marked in red while negative examples are marked in green. The blue marked spots are positive examples labeled by one of our experts; (e) Results of the proposed method.
Figure 34 An example of detection and segmentation results of the proposed pipeline on Image 2. (a) Image before stimulation; (b) image after stimulation; (c) supervised detection results, detected vesicles are circled in red; (d) manual labeling results, positive examples are marked in red while negative examples are marked in green. The blue marked spots are positive examples labeled by one of our experts; (e) Results of the proposed method.

1) **Manual labeling for validation and training purposes**

The manual detection is performed without knowing automated detection decisions. Two biologists experienced in vesicle imaging marked images independently. They could zoom in every region during the observation and labeling process. In Figure 33(d) and Figure 34(d), the red and green circles are marked by both experts as positive and negative examples of vesicles, respectively. The negative vesicle examples serve only for the supervised training purposes. Blue circles indicate marking results in which the two experts disagree. We only considered the
inconsistency of positive examples. Manual labeling was performed on the enhanced version for better view; it is only for calculation of detection errors and it does not accurately segment vesicles due the size of objects.

Table 4  Statistics for 4 images in a batch.

<table>
<thead>
<tr>
<th></th>
<th>Manual (both)</th>
<th>Manual (one)</th>
<th>Automated</th>
<th>FP(both)</th>
<th>FP(one)</th>
<th>FN (miss)</th>
<th>FN (one)</th>
</tr>
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<tr>
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<td>6</td>
<td>119</td>
<td>10</td>
<td>4</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>Image3 (not shown)</td>
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<td>6</td>
<td>127</td>
<td>14</td>
<td>3</td>
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<td>109</td>
<td>11</td>
<td>3</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

2) Unsupervised method

From the enhanced and manually labeled image (panel d of both Figure 33and Figure 34), we notice that the dendrite structures, extremely bright staining failure spots, and other irregular and isolated bright spots were not marked by experts. As demonstrated in the previous section, most of these undesired structures remain in the images after destaining. It is impossible for these structures to be perfectly overlapped, even with registration and background correction procedures. Consequently, these imperfections result in errors that include both false positives and false negatives. The error statistics are listed in Table 4.

In Table 1, the results can be separated in two parts. As discussed above, vesicle boutons marked by both experts are labeled in red and inconsistent labels are marked in blue. We follow the same color configuration in the table. The false positives are comprised of two parts: 1) proposed pipeline-detected vesicles labeled by neither expert, noted as $FP(both)$ in the table, and 2) proposed pipeline-detected vesicle labeled by one of the experts, noted as $FP(one)$. The false negatives are categorized as 1) spots labeled by only one of the experts but not by the automated pipeline, noted as $FN(one)$; and 2) spots labeled by both experts but not by the automated pipeline, noted as $FN(miss)$. From both Figure 33(e) and Figure 34(e) as well as Table 5, we can conclude that the unsupervised pipeline is very effective and accurate at identifying vesicle
boutons, recognizing more than 98% of the spots labeled by both experts. If the false positives are considered as a detection error, the detection accuracy is still around 90%. We will discuss the source of false positives and negatives below.

3) **False positives**

Considering false positives labeled by the automated method but not by the experts, the overall proportion is about 10 percent. The results without post-processing are based on the adaptive thresholding results of the MSVST enhanced map calculated from the difference between before and after destain images. Therefore, due to the imperfection of registration, a major source of false positives (60% of total) is the movement of neuronal structures before and after the stimulation. It is impractical to achieve perfect registration because the movement of the releasing vesicle and other stained undesired structures is anisotropic. Thus, this type of false positive is very difficult to completely remove. On the other hand, as a thresholding-based method, the proposed strategy is sensitive enough to identify weak vesicles with low intensity. However, this sensitivity to weak spots will introduce false positives as well.

Although we performed background correction and local adaptive detection strategy, there were vesicles and false positives located in the regions with bright background. Consequently, another major source of false positives is due to the low threshold on the enhanced map and image inhomogeneity. This category is responsible for 30% of false positives. The principle of parameter setting is to reduce the amount of false negatives such that the detection of weak spots has higher priority than the elimination of false positives. It was also challenging for our experts to differentiate these false positives from true vesicles. We noticed that 50% of false positives labeled by only one of experts, but not both (marked blue in Figure 33(d) and Figure 34(d)) falls into this category. This indicates that even human manual labeling fails to achieve results without any false positives.

Lastly, dead spots are another type of false positives that are hard to completely eliminate. Although the intensity of a dead spot reduces largely after washing and stimulation, it is still brighter than the background. The automated pipeline identifies the dramatic intensity change but fails to differentiate some of these false positives from vesicles located in bright background areas, even with post-processing filters.
4) False negatives

False negatives are the spots labeled by the experts but not the automated method. Although false negatives are a limited proportion of detection failures, we prefer less false negatives over less false positives in our algorithm design. To achieve a balance, we accept the false negatives at a rate of two percent in our parameter settings. The majority of false negatives, as expected, are weak or relatively weak vesicles. Two procedures may cause false negatives. One is the parameter settings in MSVST enhancement and the other is post processing thresholding. Though thresholds have been set loose enough to include most vesicles, we allow a trivial portion of false negatives to avoid too many false positives.

5) Vesicle quantification

For detected vesicle candidates, the morphological and other features can be easily stored for future use, e.g., for supervised classification and scoring. Figure 35 shows statistics of several important features selected by biologists, including the average intensity of objects before and after stimulation, maximum intensity, object size, and the major axis length. These features are very straightforward and are applied in the unsupervised detection pipeline.

![Boxplots of vesicle features detected in Image 1-Image 4.](image)

**Figure 35** Boxplots of vesicle features detected in Image 1-Image 4. (a) Intensity related features; (b) vesicle area in pixel; c) major axis length of each region in pixel.
Table 5 Results of supervised detection. CV is the cross-validation accuracy with combined training data from Image1 to Image 4; w/ FS indicates the accuracy with SVM-RFE feature selection; the last two columns list the number of features employed in the classifier with and without feature selection.

<table>
<thead>
<tr>
<th></th>
<th>Manual</th>
<th>Automated</th>
<th>FP</th>
<th>FN</th>
<th>CV</th>
<th>w/ FS</th>
<th># w/o FS</th>
<th># w/FS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Image 1</td>
<td>160</td>
<td>188</td>
<td>38</td>
<td>10</td>
<td>82.1%</td>
<td>84.5%</td>
<td>152</td>
<td>87</td>
</tr>
<tr>
<td>Image 2</td>
<td>106</td>
<td>100</td>
<td>11</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Image 3</td>
<td>114</td>
<td>121</td>
<td>20</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Image 4</td>
<td>99</td>
<td>103</td>
<td>14</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

6) Supervised detection results

The SVM classifier is trained by manually marked vesicles from the adaptive thresholding result of the original image. This binary image contains all possible spots that could be a vesicle bouton. The training data include both positive and negative training samples from all four images mentioned above. The dubious vesicles labeled by only one expert are excluded from the training data and the resulting statistics. Our biologists randomly selected several regions (not shown in Figure 33 and Figure 34) that were not positive vesicle candidates to enlarge the size of the negative training set for balancing the positive and negative effects to achieve accurate classification results. We performed five-fold cross validation and grid search for optimal parameters for the classifier. Feature selection with SVM-RFE improves the classification accuracy by 2% and keeps 87 out of 152 features, nearly 57% of its original size. This feature subset is determined at the global maximum of classification accuracy during the SVM-RFE procedure. We applied the classifier to each image and present the supervised detection results in panel c of Figure 33 and Figure 34. The classification results are listed in Table 5. In addition, numbers of features in each feature category before and after feature selection are described in Table 6. From this table, we notice that most features in the general information group and features specially designed for this work group survive the feature selection. For features in the transformed domain, wavelet and moments features dominate the after-selection feature space in
numbers, which indicates these features are important to catch the characteristics of vesicle boutons.

**Table 6** Number of features of different category before and after feature selection (FS). The percentage is the ratio between the number of each class before and after feature selection.

<table>
<thead>
<tr>
<th></th>
<th>General information</th>
<th>Texture</th>
<th>Wavelet</th>
<th>Moments</th>
<th>Specially designed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong># (before FS)</strong></td>
<td>14</td>
<td>13</td>
<td>70</td>
<td>49</td>
<td>6</td>
</tr>
<tr>
<td><strong># (after FS)</strong></td>
<td>13</td>
<td>8</td>
<td>30</td>
<td>31</td>
<td>5</td>
</tr>
<tr>
<td><strong>Percentage</strong></td>
<td>92.9%</td>
<td>61.5%</td>
<td>42.9%</td>
<td>63.3%</td>
<td>83.3%</td>
</tr>
</tbody>
</table>

By comparing Table 4 and Table 5, we notice that numbers of both false positives and negatives of the supervised method are larger than those of unsupervised method proposed. From Table 5, shows that Image 1 and Image 3 suffer relatively large number of false negative issues. Aside from the false negatives, Figure 33(c) and Figure 34(c) show that spots with a bright background are the major source of false positives. The classifier fails to make the right decision for spots with high intensity in the destained image of each data set. It misses some weak spots as well. The overall performance of the supervised strategy is acceptable because it identifies most high confidence vesicles with obvious vesicle morphological features. However, for the weak and relatively weak vesicles, the classifier fails to produce result as solid as the unsupervised method.

7) **Comparison between supervised and unsupervised strategies**

Although we extract a comprehensive feature space that has been applied in the supervised detection, the supervised strategy fails to outperform the unsupervised pipeline that employs a small subset of the feature space. This might be because, in the proposed unsupervised pipeline, we have defined some hierarchical filtering conditions. For example, we first identify spots with average intensity higher than a given threshold and then check the intensity differences between before and after stimulation. In contrast, the SVM classifier lacks this ability to make such decisions.

For a given data set, the training process takes longer than the overall unsupervised detection processing time. However, if a data batch has a large number of images acquired under
the same conditions, once the classifier has been built, the processing time might be less than that of unsupervised method. Therefore, considering the large data size and our interest in high confidence vesicles, the supervised strategy is very suitable and efficient. In turn, if accuracy is of higher priority, the proposed unsupervised automated pipeline is able to achieve reliable results without human intervention.

8) Neuron activity measure

The activity of neurons is quantified by Equation 14. The scores of the four-image data are listed in Table 7. To better demonstrate the meanings of the scores, we employ several cropped patches from Images 1 and 2 to discuss different scenarios.

Table 7 Scores of different images and image patches cropped from Image 1 and 2.

<table>
<thead>
<tr>
<th></th>
<th>Image 1</th>
<th>Image 2</th>
<th>Image 3</th>
<th>Image 4</th>
<th>Patch 1</th>
<th>Patch 2</th>
<th>Patch 3</th>
<th>Patch 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Score</td>
<td>1.01</td>
<td>0.98</td>
<td>1.02</td>
<td>0.95</td>
<td>2.54</td>
<td>1.54</td>
<td>0.97</td>
<td>0.12</td>
</tr>
<tr>
<td>Source</td>
<td>Image 1</td>
<td>Image 2</td>
<td>Image 1</td>
<td>Image 2</td>
<td>High vesicle density region</td>
<td>High vesicle density region</td>
<td>Average vesicle density region with bright spots</td>
<td>Low density vesicle region</td>
</tr>
<tr>
<td>Remark</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 7 shows that there are no obvious differences between four images with similar vesicle density. Patches 1 and 2 are cropped from Images 1 and 2, representing cases with high density of vesicles. Patch 3 illustrates the image with an average number of vesicles. Patch 4 contains a limited number of vesicles. Patch 3 also includes several bright spots which are not vesicles but noise. All four cropped patches have the same image size. As expected, high vesicle density Patches 1 and 2 have higher scores. Due to the nature of the score, the higher the vesicle density, the more active are the neurons. This positive correlation relation holds between the score and the intensity difference between before and after stimulation.

In this work, we do not provide a score benchmark because different data batches have varying neuron density and intensity conditions. It is not appropriate to assign a global threshold.
to distinguish active and non-active neurons for data generated for different purposes. With scores provided by the pipeline, biologists can simply set a threshold for each case according to their research objectives.

9) Parameter settings

Since several parameters are employed in this work, we briefly discuss the parameter setting issue here. For MSVST enhancement, we followed the standard parameter configuration described by Zhang et al., 2008. To achieve balance between false positives and negatives, we set the threshold on the enhanced map at two. In adaptive thresholding, the lower boundary of candidate vesicle region size is set to five. Parameters used in post processing were discussed in the Methods section. All parameters are derived from a large number of experiments. Most of them are robust and can be fixed loosely within a range. The size of vesicles and the shrinkage or expansion of the radius will vary from case to case because vesicles can be imaged at different facilities and resolutions. Here, we use relative information as much as possible (such as the difference between an image pair and the ratio of changes instead of absolute values); this enables the proposed robust strategy to be applied to different data sets. The average running time to process a 512x512 pixel image is less than a minute on a Xeon 3.0G Hz processor workstation with 4GB RAM.

C. Discussion

To measure neuron activity, we consider the total intensity difference of vesicles divided by the area of the image data. In this scoring system, the number of vesicles, the sum of the average intensity of each vesicle, and the size of image data influence the activity score. Since we extracted various features from the vesicles, it might be possible to incorporate more features into the scoring system. For example, we can include the regression coefficients or soft classification coefficients into the score if the supervised method is employed. Other features, such as wavelet coefficients, are not appropriate for introduction to the scoring system because it is difficult to explain neuron activity with abstract numbers in a transformed domain. For the same reason, features in the moment group are difficult to directly connect to neuron activity with biological meaning.
Although the proposed unsupervised methods achieve desired results, there is still a possibility of improving the detection result by employing more sophisticated machine learning strategies. A potential choice is online SVM [123]. Rather than batch learning problems, online SVM updates the classifier with new samples and new labels. We can include high confidence data in each learning step and update the classifier to achieve better accuracy. In addition, some features can be generated and updated during the online learning processing. For example, as we noted earlier, vesicles tend to cluster in a neighborhood, as do dead spots. Therefore, we can count the number of both positive and negative training samples in the neighborhood region of a given spot. This information can be updated after each learning step and also can be used in the classifier to obtain the support vector. Although online SVM and other semi-supervised SVM may achieve better classification accuracy, these methods suffer lengthy processing time and frequent human intervention. In our future work, we will aim to achieve a balance between accuracy and time-consumption together with human effort.

The final issue is the robustness of the proposed system. PSNR (peak signal-to-noise ratio) is an important criterion for quantifying the image quality as well as a measurement of system robustness. In order to find the lower boundary of image quality, we calculate the PSNR of the original data and images contaminated by additive Gaussian noise. Figure 36 illustrates that, for Image 1, the PSNR decreases while the Gaussian noise variance level increases. The proposed system is able to identify about 70% of the spots detected in the added-noise-free version when the Gaussian noise variance increased to 1e-3. This corresponds to a cut-off value of PSNR at 33 dB.

D. Conclusions

Synaptic vesicles are involved in performing presynaptic functions. The synapse assay can be implemented in a high throughput manner via automated, multi-well plate microscopy to study neuron activity and to do drug screening in neurodegradation diseases. For the large amount of image data generated by large scale experiments, automated processing and statistical analysis of the synaptic vesicle image will provide significant statistical power and save
biologists from manually labeling and counting vesicles. However, to our knowledge, such quantization tools designed specifically for high throughput synaptic vesicle assays do not exist.

In this chapter, we presented a neural image quantization pipeline to analyze a synaptic vesicle neuron image stained by FM dye. We can detect active vesicles releasing the neurotransmitter, FM dye in this case, to measure the neuron activity. This pipeline is fully automated, time efficient, and ready for batch processing. We address the inhomogeneity issue by introducing local adaptive threshold on a MSVST enhanced map. This method is robust to detect both vesicles with high intensity background and weak vesicles with low intensity. In addition, we develop novel strategy to solve the overlapped spots issue for tiny objects such as synaptic vesicles. Several criteria have been proposed to remove various categories of undesired structures. When compared with a popular supervised method, the proposed pipeline provides better detection accuracy and time efficiency. With detection and segmentation results, we extract different types of image features to quantify each detection vesicle. These features include intensity, intensity change between before and after stimulation, perimeter, convexity, and other texture features, moment features, and wavelet coefficients. Finally, a quantified score is provided for each input image to measure the activity of the neuron in a whole image. This score allows the statistical quantification of the neuron activity, which is measured by differences between before and after stimulation. To summarize, we develop an automated, batch processing-ready, and cost-effective pipeline to measure the neuron activity based on the

Figure 36 PSNR of Image 1 contaminated by Gaussian noise.
synaptic vesicle image data used in neurodegenerative disease research, which will help neuroscientists and researchers analyze high-throughput imaging data in neuropathology studies and drug screening.
V. DENDRITE OUTGROWTH IMAGE ANALYSIS AND AN HCS CASE STUDY

In this chapter, we will illustrate how dendrite outgrowth assay could be employed in the large-scale screening in neurodegenerative disease drug development. We will first introduce the automated pipeline to obtain accurate quantification of neurite lengths in high-density neuronal cultures. It provides quantitative interpretation of automatic fluorescence microscopy images, in particular, for the labeling and measurement of neurites. This pipeline processes images from Nuclei and Neurite channels separately to obtain statistical quantifications from neuronal images. In the second part of this chapter, we will present an example high content screening designed to identify small molecule inhibitors of neurite loss induced by Aβ peptide. We employ proposed neurite detection and quantification methodology to quantify the loss of neuronal projections induced by Aβ peptide. The screening enables us to identify new classes of neurite protective small molecules, which may represent new leads for AD drug discovery. In this large scale screening, we identified thirty-six inhibitors of Aβ-induced neurite loss from the 1,040 compound National Institute of Neurological Disorders and Stroke (NINDS) custom collection of known bioactives and FDA approved drugs.

A. Neurite image analysis: detection and quantification

In this section, we will provide data acquisition details for neurite image data used in this work first. We imaged both neuron nuclei channel and neurite channel separately. We developed different methodologies to process those images in different channels to capture the morphological features of various neuron structures. Processed image are presented to illustrate the capability of the system. Details are provided as follows.

1) Neuronal staining and image acquisition

Briefly, C57BL/6 mouse embryo (E15) brains were dissected and trypsinized at room temperature (RT), followed by trituration. Neurons were resuspended in DMEM medium.
supplemented with 10%FBS, 100 units/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine, and seeded in 384-well plates at 1×104 cells/well. The following day medium was changed to neurobasal with B27, penicillin/streptomycin, and glutamine and incubated for 4 days. Aβ1-40 was dissolved in 0.1 M Tris-HCl buffer (pH 7.4) at 200 μM, incubated for 1-4 hr (37°C), diluted with neurobasal media at a final concentration of 5 μM. Where indicated, compounds were added prior to Aβ 1-40 treatment. Following treatment, neurons were incubated (37°C) for 3 days before fixation and staining.

Neurons were fixed with 4% paraformaldehyde for 30 min (RT). Cells were permeabilized with 10% normal donkey serum and 0.4% Triton X-100 for 30min (RT). Primary TUJ1 antibody, and secondary donkey anti-mouse Cy3- conjugated antibody were used to stain neurons; nuclei were stained with SytoxGreen. Neurons were imaged on automated CellWorx microscope (Applied Precision) at 10X magnification and 488 nm (SytoxGreen) and 550 nm (Cy3) wavelengths. In Figure 37, (a) and (b) illustrate differences between untreated and Aβ treated neurons, from which we could observe obvious differences in both neurite length and density. Nuclei image and neurite images are acquired separately using automated fluorescent microscopy with 384-well plates in Figure 37(c) and (d).
Figure 37 Image in the neurite channel from untreated (a) and Aβ treated (b) group; Raw image acquired by high throughput fluorescence microscope (2048×2048 pixels, 0.1μm/pixel), (c) Image in the nuclei channel, and (d) in the neurite channel.

2) Nuclei image processing

The nuclei channel image provides the number of neuron cells to acquire statistics of neurite outgrowth, thus the first step is to detect and segment all the nuclei. Nuclei are round objects that vary in intensity and size. Compared with spine detection, nuclei detection is straightforward and conventional watershed algorithm is able to solve the problem. Figure 38 shows the flowchart of the proposed nuclei detection and segmentation method.

Figure 38 Nuclei channel image processing

Firstly, to correct the degeneration of the images, a data-driven background correction algorithm [124] using the cubic B-Spline method to estimate the background iteratively is employed in the image preprocessing step. Then we employ the nuclei detection algorithm based on gradient vector field and watershed in [125]. In this algorithm, a Gaussian filter is used at first to remove noise, smooth the texture, and generate a unique local intensity maximum to locate nuclei inside each nucleus. Then, to spot the markers for guiding the watershed algorithm, an algorithm based on a gradient vector field (GVF) is adopted to detect the locations of these
nuclei. The gradient vector field in each pixel is defined as:
\[
\tilde{F}(x, y) = \frac{\partial I(x, y)}{\partial x} i + \frac{\partial I(x, y)}{\partial y} j - \pi / 2R,
\]
where \(I(x, y)\) is Gaussian filtered image. The gradient vector \(\tilde{F}(x, y)\) of each pixel points to the local maximum of the filtered image. The markers of the watershed algorithm can be further identified as convergent pixels considered as local maximums. With these markers, the standard watershed algorithm can be improved for the segmentation accuracy especially for the overlapped nuclei. The local maximum usually resides at the center of neural cells. Some nuclei are attached to each other but rarely their central regions. Therefore, to segment the overlapped nuclei, markers will be necessary to employ watershed algorithm to identify the nuclei boundaries including the ones for attached nuclei. These local maximum regions, serving as markers, will be further used in watershed algorithm to depict the accurate nuclei boundaries.

3) Neurite image processing

As the nuclei channel images provides the total of neuron cells, we can obtain information such as the total average length and intensity of the neurites per neuronal cell from the neurite channel image together with the nuclei channel information. The pipeline of proposed neurite tracing method is illustrated in Figure 39. This pipeline is designed for high-throughput applications to assess the state of all neuronal projections in different wells.

![Figure 39 Neurite channel processing pipeline](image-url)
a) **Preprocessing and soma detection**

The soma regions, the bodies of neuron cells, are intensity peaks represented by a cluster of pixels. To enhance the image contrast, we apply morphology operations such as 'Bottom-Hat' and 'Top-Hat' transforms. Then, to detect the soma regions, the intensity values of the neuronal image are converted into a 1-D data sequence. The sequence is then clustered into three groups using the fuzzy c-means clustering method [126]. The group with the highest intensity values is selected to generate the threshold, defined as the minimum intensity value of that group. The soma regions are pixels with higher intensity than the threshold.

b) **Centerline extraction**

The centerline is detected following the similar strategy described in Chapter III.A.3), 'Backbone and Spine Detection'. We employ several Gaussian kernels with different variances with Hessian matrix to identify curvilinear structures with different widths.

As described in Steger, 1998, an ideal ‘bright and dark’ line profile of width $2\omega$ and height $h$ in 1D (one dimension) can be modeled as: $f(x) = \begin{cases} h, & x \leq \omega \\ 0, & x > \omega \end{cases}$

In the 2D case, the curvilinear structure shows characteristics of the one dimension line profile in the normal direction of the line. There are quite a number of algorithms using a $2 \times 2$ Hessian matrix to estimate the normal direction of each pixel and calculate $t_x$ (detailed definition is provided in the later part) based on the direction [101, 107, 127]. By applying this method, we could fine one direction for each pixel. However, for branched regions, most pixels tend to point to different directions, and the eigenvector does not point to the normal direction of the centerline. Thus, $t_x$ always becomes too strong to detect the centerline.

To solve this issue, we employ an improved curvilinear detector based method to detect all centerline pixels including single lines and branched areas simultaneously. To reduce computational cost, we quantize all directions from 0 to $2\pi$ into 16 directions. Each direction is notified by an index from 0 to 15. There is a 22.5° angle between every two neighbors of quantized directions. Therefore, we can build a steerable filter composed of a class of basic
filters, whose response at an arbitrary orientation is synthesized as a linear combination of basic filters:

\[ f^\theta(x, y) = \sum_{j=1}^{M} k_j(\theta) f^\theta_j(x, y) \]

where \( M \) is the number of basic filters, \( f^\theta_j(x, y) \) is \( j \) th basic filter, \( \theta_j \) is the \( j \) th basic angle, \( j = 1, 2, \ldots, M \), and \( k_j(\theta) \) is the interpolation functions. We further design two categories of steerable filter, \( G'(x, y, \theta, \sigma) \) and \( G''(x, y, \theta, \sigma) \), based on the first and second order derivatives of the circularly symmetric Gaussian function \( G(x, y, \sigma) = (2\pi\sigma^2)^{-1/2} \exp\left(-\frac{x^2 + y^2}{2\sigma^2}\right) \), where \( \sigma \) represents neurite width. It has been shown in [128] that \( G'(x, y, \theta, \sigma) \) can be represented by synthesizing two basic filters with basic angles \( \theta_1 = 0 \) and \( \theta_2 = \pi / 2 \) linearly, while \( G''(x, y, \theta, \sigma) \) can be represented by synthesizing three basic filters with basic angles \( \theta_1 = 0 \), \( \theta_2 = \pi / 4 \) and \( \theta_3 = \pi / 2 \) linearly. These two steerable filters are defined as:

\[ G'(x, y, \theta, \sigma) = k_1(\theta) \times G_x(x, y, \sigma) + k_2(\theta) \times G_y(x, y, \sigma) \]

and

\[ G''(x, y, \theta, \sigma) = k_{11}(\theta) \times G_{xx}(x, y, \sigma) + k_{22}(\theta) \times G_{yy}(x, y, \sigma) + k_{12}(\theta) \times G_{xy}(x, y, \sigma) \]

with \( k_1(\theta) = \cos(\theta) \), \( k_2(\theta) = \sin(\theta) \), \( k_{11}(\theta) = \cos^2(\theta) \), \( k_{22}(\theta) = \sin^2(\theta) \), \( k_{12}(\theta) = \sin(2\theta) \),

\[ G_x(x, y, \sigma) = -\frac{x}{\sqrt{2\pi\sigma^3}} \exp\left(-\frac{x^2 + y^2}{2\sigma^2}\right), \quad G_y(x, y, \sigma) = -\frac{y}{\sqrt{2\pi\sigma^3}} \exp\left(-\frac{x^2 + y^2}{2\sigma^2}\right), \]

\[ G_{xx}(x, y, \sigma) = \frac{1}{\sqrt{2\pi\sigma^3}} \left(\frac{x^2}{\sigma^2} - 1\right) \exp\left(-\frac{x^2 + y^2}{2\sigma^2}\right), \quad G_{yy}(x, y, \sigma) = \frac{1}{\sqrt{2\pi\sigma^3}} \left(\frac{y^2}{\sigma^2} - 1\right) \exp\left(-\frac{x^2 + y^2}{2\sigma^2}\right), \]

and \( G_{xy}(x, y, \sigma) = \frac{1}{\sqrt{2\pi\sigma^3}} \frac{xy}{\sigma^2} \exp\left(-\frac{x^2 + y^2}{2\sigma^2}\right) \).

In the 1D, \( t_x \) for each pixel can be defined as:

\[ t_x(x, y, \theta, \sigma) = -\sigma^{1/2} \frac{f'(x, y, \theta, \sigma)}{f''(x, y, \theta, \sigma)}. \]
where \( f'((x, y, \theta, \sigma)) \) and \( f''((x, y, \theta, \sigma)) \) are the first and second normalized derivatives at \((x, y)\) with different directions. For each \( \theta \), we find a direction \( \sigma \) satisfying the criterion [101]:
\[
\sigma = \arg \min_{\sigma} (f''((x, y, \sigma))).
\]
Therefore, this formulation can be redefined as:
\[
t_i(x, y, \theta, \sigma) = -\sigma_{\theta}^{1/2} \frac{f'(x, y, \theta, \sigma_{\theta})}{f''(x, y, \theta, \sigma_{\theta})}
\]
where \( f'(x, y, \theta, \sigma_{\theta}) \) and \( f''(x, y, \theta, \sigma_{\theta}) \) can be estimated by convolving the image with steerable filters described in previously defined filters and multiplying by the normalized coefficients.

For each pixel \((x, y)\), we calculate a vector \( t(x, y) \) whose length is 16, and \((x, y)\) can be considered as a centerline candidate if we can find a set of \( \theta \) satisfying the following condition:
\[
\theta = \{ \theta_i \mid t_i(x, y, \theta_i, \sigma_{\theta_i}) \leq 0.125, i = 0, 1, 2, \ldots, 15 \}
\]
and \( -l''(x, y, \theta, \sigma_{\theta_i}) > thr \), where \( thr \) is a user specified threshold and \( -l'' \) can be treated as the strength of the line.

With the above definitions, we will describe how to find the direction for each centerline candidate. Since each neurite has a local minimum width along its normal direction and it has been proven that \( \sigma \) of the Gaussian kernel is proportional to the width [108], the normal directions of each candidate are obtained by the finding local minimum of \( \sigma_{\theta} \) [129]:
\[
\sigma_{\theta}(x, y) = \min_{\theta} \sigma_{\theta}(x, y), \theta \in [\theta - \Delta \theta, \theta + \Delta \theta],
\]
where \( \Delta \theta \) is defined as \( \pi/8 \). This resolution at \( \pi/8 \) is selected considering that all the possible directions in a 2D are quantized into 16 directions. Note that this is a tradeoff between resolution and computational cost because higher resolution achieves better precision but requires dramatically increased running time and memory.

The end points of each neurite centerline can be identified as points with only one 8-adjacent neighbor. A safeguard procedure to further confirm an ending points is to check whether the local line direction of ending points can be computed by turning the calculated direction by \( \pi/2 \) or \(-\pi/2\) to ensure it points outwards from the centerline. To link the breaking points, for each ending point of neurite line section, we check whether there is a centerline point in the local line direction in its neighborhood of radius \( R \). If so, Bresenham line
drawing algorithm [109] is applied to link these two points. To avoid false positives, the strength map is also checked at every step of connection to ensure true positives. The threshold of the strength map is determined by experiment. Once a smaller value is reached, the linking at this end point is terminated. The branching point is determined as described in spine detection part.

4) Neurite outgrowth quantization

We provide a statistical quantification of the total neurite length in one image, which is subsequently used to calculate Average Neurite Length (ANL) as the statistical feature of neurite outgrowth in each well. ANL is defined as the ratio between Total Neurite Length and Total Neuron Cell Numbers per image. ANL is a statistical parameter, which averages the neurite lengths in the entire neuronal field and makes the analysis results robust to slight changes in the neuron culture and staining as well as local variations in cell density and errors in tracing of individual neurites due to high cell density. The mathematical definition of ANL is:

$$\text{ANL} = \frac{R \sum_{i=1}^{N_s} l_i}{N_c}$$

where $R$ is the resolution of the image, $l_i$ is the length of $i$-th neurite segment. $N_s$ and $N_c$ are the number of neurite segments and number of cells respectively. Since both of the total neurite length and neuron cell number are statistical results averaged over entire image, ANL is a robust measure of neurite out-growth, which is highly accurate and reproducible even in high density cultures.

With the calculated ANL for each well in the screening, we could employ this statistic to estimate the effect of screened compounds. We define a z-score based on the ANL of the whole screening data to select the candidate compound hits. The z-score is calculated to remove the plate effect and the final score of each channel for each compound is obtained by medium value of the z-scores. Since the most effective drugs/compounds may have the largest z-scores, the distribution maps of z scores can be used in statistical analysis of compound screening. The hit selection process is performed using a pre-selected threshold ($\bar{z} + 1.5 \times SD$) to choose hits with high final scores. Based on ANL in each well, the z-score calculation steps are as follows:
(1) Calculate the $\text{Avg}^-$ and $SD^-$ of each plate;

(2) Check if $\text{Avg}^+ > \text{Avg}^-$;

(3) Calculate $z$-scores $z = (x - \text{Avg}^-) / SD^-$;

(4) Check $z$-scores, and see whether $z^- \approx 0$ and $1.5 \leq z^+ \leq 2.0$.

where $\text{Avg}^+$ and $\text{Avg}^-$ are average values of the positive and negative control group respectively, $SD^-$ is the standard deviation of negative control group. $z^+$ and $z^-$ are the average $z$-scores of the positive and negative control group respectively. Positive control group is the first column in the $z$-score map (Figure 40), which has none of $A\beta$ compound treated; while negative control group is the rightmost column (Figure 40), which is only treated with $A\beta$. The rest wells are treated with compounds according to the library plate map. The candidate compound hits are selected based on neurite loss quantization from the microscopy images.

**Figure 40** A screening and $z$-score map of an example screening 1,040 compound from NINDS custom collection library II
5) **Nuclei and neurite detection results**

![Figure 41](image)

**Figure 41** Image processing demo figures for both Nuclei and Neurite channels, with segmented nuclei and labeled neurites shown as green lines.

Figure 41 illustrate the nuclei detection and neurite tracking results with our algorithm. From the nuclei channel results, illustrated by Figure 41(a), we could conclude that our pipeline is able to automatically detect the nuclei structure, especially for the touching nuclei and nuclei in a cluster. It provides accurate estimate of the total cell numbers. As demonstrated by Figure 41 (b), the proposed pipeline in neurite channel could trace the neurite outgrowth in an accurate manner. It is capable of robustly detecting neurites with different widths and strength, and identifying line structures in branching regions. The results are smooth as well. We have compared our method with other available software packages, including NeuronJ [104] and Neurite Tracer [130]. In general, NeuronJ, Neurite Tracing and proposed approach are all developed based on line-pixel detectors. NeuronJ provides very accurate centerline extraction. However, it is a semi-automatic method and requires the users to specify the starting and ending points, which make it incapable of large-scale screening. NeuriteTracer is also a semi-automatic approach. Semi-automatic labeling algorithms do not address ‘branching problem’ since the users always consider branch point as an ending or a starting point. Proposed approach introduces Gaussian kernels with different widths to improve the traditional algorithm in order to
have a better accuracy in line detection. Besides, our framework also integrates information from nuclei channel and neurite channel, which automatically offers statistical results for assessing neurite outgrowth from hundreds of neurons. Based on the discussion above, proposed system outperforms the existing software in high content neuron-based imaging.

B. A HCS study to identify inhibitors of neurite loss induced by Aβ peptide

In this section, we will illustrate how the neurite detection and quantification could be employed in the large-scale screening studies with an example of identification of small molecule inhibitors of neurite loss induced by Aβ peptides. In this example, we design the high content screening from end to end, including array design, imaging, image analysis, statistical analysis of the screening results. We will describe these parts in detail, except for the image analysis discussed above, to provide an illustrative drug development example for AD.

1) Background

In contrast to traditional drug discovery approaches, which heavily focus initial drug development efforts primarily on compound potency and selectivity, the early stage of drug discovery employed advanced high throughput screening (HTS) to screening a large number of compounds to identify molecules that affect the activity of specific drug targets of interest. To facilitate large-scale compound screening, semi-automated imaging and screening platform and automated image analysis and statistical analysis are essential in the high content screening system.

Loss of synaptic connections and neuronal projections has been shown to be a common feature of many neurodegenerative diseases including Alzheimer’s disease. Chemical compounds targeting neurite loss prevention or neurite outgrowth stimulation could represent a promising novel treatment in AD. In addition, many drugs have potentially useful effects that go beyond their original use. For example, aspirin was originally used as a pain reliever, but it has now been shown to reduce the risk of heart disease and stroke as well. Therefore, in this study, to identify small molecule inhibitors of neurite loss induced by Aβ peptide, we conduct a screening
with NINDS custom collection compound library II containing 1,040 known bioactive small molecule compounds. While the collection of compounds used in this study is much smaller than the number of compounds usually tested by pharmaceutical companies, it is chemically diverse. This made it plausible that one or more categories of the drugs might be effective against neurodegenerative disorders.

2) **Method**

a) **Screen design**

NINDS custom collection compound library II containing 1,040 known bioactive small molecule compounds was used in the primary screen. The details of this library can be viewed on the ICCB-Longwood, Harvard Medical School’s webpage (http://iccb.med.harvard.edu/screening/compoundibraries/bioactives_ninds2.htm). The compounds were robotically added to neurons at a concentration of 25 μM with all compound plates screened in triplicate. Each plate included untreated and Aβ1-40 treated control wells. Only plates with significant difference (at least p ≤ 0.1, usually p ≤ 0.05 using 2-tailed student t-test) between control groups were used for hit selection. Z-scores were calculated based on degree of protection as compared to Aβ1-40 treated control. The following criteria were used for hit selection: (1) average and median z-score for each hit compound ≥1.5 (p ≤ 0.05), (2) average z-score for each hit ≥ z-score difference between untreated and Aβ1-40 controls for that specific plate, and (3) visual inspection of hit compound wells confirmed high image quality. This resulted in a total of 42 primary hits (4%). These were re-examined in a secondary screen, under the same conditions, except the compounds were used at 5 concentrations (2.5 nM, 25 nM, 250 nM, and 2.5 μM) to generate an estimated EC50 as described below.
Figure 42 Representative images before and after treatment in neurite channel: (a) control or untreated; (b) neuron treated with Aβ1-40; (c) neuron treated with Aβ(1-40) and Ibuprofen (0.1 nM) (d) neuron treated with Aβ(1-40) and Ibuprofen (1.0 μM)

b) EC50 calculation

For EC50 calculations, neurons were treated with Aβ as above in the presence of either 4 (2.5 nM, 25 nM, 250 nM, and 2.5 μM) or 9 (0.1 nM, 1.0 nM, 10 nM, 50 nM, 100 nM, 500 nM,
1.0 µM, 5 µM, 50 µM and 100µM) concentrations compound as described in the text. EC50 values were calculated by non-linear regression using GraphPad Prism software. For “EC50 (µm) (from 0 to 100%)” column, values represent the concentrations of compound that provide 50% neurite protection relative to the untreated control, which was set as 100%. In ‘EC50 (µm) (from min to max)’ column values represent concentrations of compounds providing half protection between the lowest and highest points of each compound curve. N/A indicates lack of fit. Two example curves are illustrated in Figure 43.

![Ibuprofen and Nabumetone EC50 Curves](image)

**Figure 43** Examples of EC50 curves of drug response

### 3) Result

#### a) High-content screen for compounds suppressing Aβ1-40 induced neurite degeneration

To identify small molecules that can suppress Aβ1-40 induced degeneration of neuronal processes, we screened the 1,040 compound known bioactives NINDS custom collection library II, in primary mouse cortical neurons in the presence of Aβ1-40 (25 µM). Neurite lengths were quantified using the neurite detection and quantification method described in previous section, in which individual neurons were identified and the average neurite length was determined. The proposed method can accurately calculate a reduction of neurite length in response to Aβ1-40 peptide treatment [131] (Figure 42). To minimize false positives, hits were selected using a high threshold (Average + 1.5 standard deviation) over ANL values for Aβ treated control wells in
each plate (Figure 44). In our primary screen, 42 compounds (~ 0.4%) were identified based on significant (p≤0.05) protection against Aβ1-40 induced-neurite loss (Fig. 2A). To confirm the specificity of the hits, the compounds were re-examined in a secondary screen and the half maximal effective concentrations (EC50) were estimated. The secondary screen confirmed 36 (85%) compounds (Figure 45(a)) that significantly (z-score≥2.15, p≤0.01) protected primary cortical neurons from Aβ1-40 induced-neurite degeneration at micro molar or lower concentrations. Functional clustering of the compound hits identified several drug categories that were enriched when compared to their representation in the NINDS library (Figure 45(b)). Notably, NSAIDs, anti-histamines and alkylating/DNA damaging agents were highly enriched among the hits. We believe compounds that showed statistical significance in both the primary and secondary screens are real hits, however, because the drug category did not show significant enrichment as compared to their representation in the library we did not focus on these classes of inhibitors in molecules. These molecules have a broad spectrum of targets and these will need to be further examined in the future work. These results were promising since all three categories of molecules have been previously investigated as anti-Aβ drugs.

**Figure 44** A representative heat-map derived the screening after processing and calculating average neurite length in a 96-well plate.
### Table

<table>
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<tr>
<th>Compound</th>
<th>Category</th>
<th>Z-score</th>
<th>EC\textsubscript{50} (0-100%)</th>
<th>EC\textsubscript{50} (min to max)</th>
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<td><em>milamycin c</em></td>
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<td>N/A</td>
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<td>N/A</td>
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<td>0.2022</td>
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<td>1.4059</td>
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<td>6.5757</td>
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<td>4.5565</td>
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<td>12.3620</td>
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<td>1.7426</td>
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<td>5.3081</td>
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</table>

### Pie Charts

- **Compound hits**:
  - 14% p = 0.00076
  - 29% p = 0.00022

- **NINDS library**:
  - 3% 13% 7% 7% 14% 14% 7% 14%
**Figure 45** Inhibitors of Aβ-induced neurite loss identified in NeuriteIQ high content screen. (a) Compounds that protect against Aβ (1-40)–induced neurite loss. Forty-two compounds were identified in a primary screen and clustered based on their functional categories. Following the secondary screen, six compounds did not fulfill the criteria (indicated with *). EC50 values were estimated using 2.5 nM, 25 nM, 250 nM, and 2.5 μM of the compounds as described in the methods. (b) Hits were clustered based on their targets or mechanism of action; the enrichment of certain categories is shown as compared to their representation in the NINDS library. The p values based on hypergeometric distributions are indicated next to each category for which significant enrichment was identified.

4) Discussion

The loss of synaptic connections and neuronal projections is a common feature in neurodegenerative diseases such as Parkinson’s and Alzheimer’s [132]. Neurite degeneration precedes cell death, suggesting that neurite loss might be a primary cause of cognitive function decline [133]. Recent reclassification of AD suggests that pathophysiology begins many years before the diagnosis of AD dementia [134]. Therefore, chemical compounds attenuating neurite loss or promoting neurite outgrowth could represent promising new treatments in AD.

HCS using primary neurons is hampered by intrinsic difficulties, such as variability in cell density throughout the well and well-to-well, the need to distinguish between the neuronal and non-neuronal cells present in the cultures, the need for extended culturing of neurons for neurite development, and difficulties in accurately quantifying neurite lengths in complex dense neuronal cultures. The proposed neurite detection and quantification system introduces a number of elements to address these difficulties, such as: a) imaging large well areas at 10X magnification to reduce the effects of differences in intrawell densities, b) use of neuron-specific neurite labeling, c) nuclear staining, which provides means to normalize neurite lengths to cell numbers and to exclude positively labeled debris not connected to nuclei, and d) filtering nuclei not connected to neurites, which eliminates non-neuronal cells. These features in conjunction with statistical analysis of hits relative to the positive and negative controls in each plate and screening the library in triplicate resulted in a highly reliable hit selection. Thirty-six out of 42 screening hits displayed activity in our secondary assay. Overall, these data suggest that the screening procedure provides a reliable tool for discovery of novel neurite-protective molecules.
and putative AD drug candidates. Furthermore, this method can be easily extended to other applications in neurodegeneration.

Our screen of 1,040 compounds from the NINDS collection of known bioactives and FDA approved drugs identified several functional categories of inhibitors, including NSAIDs, anti-histamine drugs, antibiotics, DNA damaging agents, and regulators of cholinergic system and protein synthesis machinery. Importantly, a number of identified molecules have known connections to Aβ neurotoxicity. For example, previous work showed that cyclophosphamide, a DNA cross-linking agent and a hit in our screen, decreased tau phosphorylation at Ser-396/404 site [135]. This result is consistent with the notion that aberrant neuronal cell cycle re-entry may lead to tau hyperphosphorylation, and thus inhibiting cell cycle progression may be one target of therapeutic intervention. Anti-histamines also showed promise in preclinical trials for the treatment of human cognitive disorders [136]. The discovery of six NSAIDs as suppressors of neurite loss was particularly intriguing, since this class of molecules has attracted significant attention as potential AD drugs. While clinical data has been generated, the results were generally not conclusive. Some studies showed benefits of certain NSAIDs in the improvement of cognitive functions or delaying onset of AD. However, other studies showed no improvement [137]. Intriguingly, while neuroinflammation, which accompanies development of AD, has been the primary reason for testing NSAIDs, several studies have suggested that the positive effects of this class of drugs are not related to inflammation (or Aβ processing, see below) [138, 139]. Multiple additional mechanisms of neuroprotection for NSAIDs have been proposed. These include inhibition of γ-secretase activity, Aβ secretion, and Aβ aggregation (especially by ibuprofen [140]) as well as stimulation of neurite outgrowth by some NSAIDs (ibuprofen and indomethacin), but not by naproxen [141, 142]. It is unclear which of these mechanisms reflect the activity of COX enzymes as opposed to other targets of particular structural classes of NSAIDs [143]. Importantly, our data show for the first time that a wide range of NSAIDs can attenuate Aβ induced neurite loss in the presence of processed exogenous Aβ and thus strongly indicate that: 1) NSAIDs target COX proteins to modify cellular signaling responses to Aβ and 2) NSAIDs target Aβ signaling, rather than Aβ generation/processing to attenuate neurite loss. Based on our data we believe that NSAIDs may represent a therapeutically viable option to prevent or delay Alzheimer’s at the earliest stages of the disease. This is consistent with many
epidemiological reports that positive effects of NSAIDS when used on for long-term treatment (US Veterans Affairs Health Care System Study [144], Cache County Study [145], Cardiovascular Health Cognition Study [146], Chicago Health and Aging Project [147]). It is also quite interesting to note that in several clinical trials involving patients who already display mild to moderate cognitive decline (Alzheimer's Disease Cooperative Study (ADCS) group [148], NSAIDs showed no benefit. We will further validate and investigate NSAIDs target in our following works.

C. Chapter summary

In this chapter, we proposed an automated neurite image processing, quantification and statistical analysis pipeline. In order to achieve the fully automated, fast, and accurate processing, a number of novel computational methods are employed in this application, such as the modified curvilinear structure detector, and the target identification method with statistical analysis. Increases in the ease and speed of neurite quantification due to automation will enable investigators to extend the application from studies of limited numbers of cell samples to comparisons involving a large population of cell samples. Such population-based studies provide strong statistical power and enable many innovative studies and large-scale explorations to be carried out, for example, genome wide screening for effectors on pre- or post-synaptic activities.

With the proposed method, we screened NINDS custom collection compound library II containing 1,040 known bioactive small molecule compounds, for reagents protecting Aβ 1-40 induced degeneration of neuronal processes. Our method accurately measured a reduction of neurite length in response to Aβ1-40 peptide treatment and allowed us to assess the rescuing effect of each compound. To minimize false positives, hits were selected using a high threshold (1.5 Standard Deviation) over average neurite length values for Aβ-treated control wells in each plate. 42 compounds (~0.4%) were identified based on significant (p≤0.05) protection against Aβ1-40 induced-neurite loss. To confirm the specificity of the hits, the compounds were re-examined in a secondary screen and the half maximal effective concentrations (EC50) were calculated through dose-dependent experiments. The secondary screen confirmed 36 (85%) compounds that significantly (z-score≥2.15, p≤0.01) protected primary cortical neurons from
Aβ1-40 induced-neurite degeneration at micromolar or lower concentrations. Functional clustering of the compound hits identified several drug categories that were enriched when compared to their representation in the NINDS library, highly supporting the reliability of the screening assay. This further validated the utility of the proposed strategy and highlighted its value for novel target identification with large-scale studies.
VI. CONCLUSIONS

To understand neurodegenerative diseases and develop treatment for this category of one of the less understood diseases, image based large scale studies are recently developed in high-throughput or high content manners. As part of these large scale studies, image analysis, including image processing, objects recognition, segmentation and quantification, is of great significance in identifying statistically important factors, discovering genetic regulation relationships, establishing pathways as well as drug discovery. However, the lack of system level informatics tools designed specifically for the large scale studies on neurodegenerative disease results laborious manually processing and quantification, low efficiency and inconsistency in manual results and so on. In this work, we developed a system level methodology to automate the image analysis process for the large-scale screening studies on neurodegenerative disease. As illustrated in Figure 46, this system covers most structures related to synapse functions, which are considered as the most important structures in information processing, transmission and storage in the neuron network. These structures include presynaptic vesicles, dendritic spines in post synapses and information transmission channel, the neurites comprised by axons and dendrites. The system is able to identify all three structures from multi-scale images with minimal or without human intervention. We proposed novel algorithms to perform automated, accurate, fast and batch processing capable segmentation and quantification on the multi-scale image data. We illustrated the capability of the system by providing a screening example of neurite to identify potential drugs for Alzheimer’s disease. The results show that accurate, consistent and reliable results can be automatically obtained for three different image scales by the system, to free scientist and researcher from laborious manual processing and counting objects. This system advances the large-scale studies of neurodegenerative disease to a phase of automated imaging and informatics acquirement.
In Chapter III, we proposed a novel strategy for spine detection and tracking for in vivo neuron images, which can be also applied to process in vitro data. The sample data used in this chapter is noisy and low contrast in vivo image data of live mice of an AD model. The pipeline can automatically label and quantify small spines and match spine pairs at different time points to track neuron dynamic changes from the real-time in vivo data. The algorithm treats dendrite backbones and spines as line structures and detects them by using curvilinear structure detector. The proposed adaptive local binary fitting (aLBF) level set method can accurately identify the dendritic structures boundaries using the previously detected curvilinear structures as the initialization. Then, we proposed a maximum likelihood estimation based tracking algorithm to track the evolution of spines at different time points as well as to detect spine elimination and formation. The algorithm is highly automated and efficient, and requires minimum human interaction. As mentioned in the Discussion section of Chapter III, since current method projects 3D data into 2D using maximal intensity projection to enable weak spine detection, it loses the
rich space information in 3D. Perhaps a 2D-3D hybrid method, which uses projected image to reconstruct a 3D skeleton and detects boundaries in 3D using the skeleton as initialization, could solve the weak spines detection in 3D without information loss and achieve better results. This is one of our further work direction.

To analyze image data generated by large-scale experiments on synaptic vesicle assay, automated processing and statistical analysis of the synaptic vesicle image will provide significant statistical power and save biologists from manually labeling and counting vesicles. In Chapter IV, we presented a quantization pipeline to analyze a synaptic vesicle image stained by FM dye. We can measure the neuron activity by detecting active vesicles releasing neurotransmitter event. We proposed a pipeline of local adaptive threshold on a MSVST enhanced map to detect both vesicles with high intensity background and weak vesicles with low intensity. In addition, we developed novel strategy to solve the overlapped spots issue for tiny objects such as synaptic vesicles. When compared with a popular supervised method, the proposed pipeline provides better detection accuracy and time efficiency. With detection and segmentation results, we extract different types of image features to quantify each detection vesicle. A quantified score was defined for each input image to measure the activity of the neuron in a whole image. This score allows the statistical quantification of the neuron activity, which is measured by differences between before and after stimulation. This pipeline is fully automated, time efficient, and capable of batch processing. This module in the system emphasizes the screening related the presynaptic functions and processes, which is an essential part in neurodegenerative disease research.

In addition, research on Alzheimer's disease indicates that there is strong relationship between neurite degeneration and cognitive functions. This motivates the use of high content screening to investigate chemical compounds targeting neurite loss prevention or neurite outgrowth stimulation at a large scale, also demands a new generation of bioinformatics tools to support accurate quantization and data modeling. In Chapter V, we proposed a strategy to integrate optimized neuron image analysis and drug scoring methods into one coherent bioinformatics pipeline to support Alzheimer’s disease drug discovery. We developed automated pipelines to quantify dendrite, to address statistically significant features and to infer screened compound outcomes with images collected from both nuclei and neurite channel. The nuclei are
detected with mark controlled watershed method. The neurites, considered as line structures, are identified using the similar algorithm in dendritic spine detection with curvilinear structure detector. In addition, loss of neuronal projections is modeled in vitro in primary mouse cortical neurons treated with the amyloid beta peptide. We illustrated the screening and image analysis with an example of screening 1,040 compounds in the National Institute of Neurological Disorders and Stroke custom collection compound library II. Compound hits are selected by quantization results of neurite loss and outgrowth. Forty-two hits are selected using the integrated pipeline to screen the 1,040 compounds through two stages of screening. Functional analysis studies indicate that there within the 42 hits there is an enrichment of anti-histamine drugs and nonsteroidal anti-inflammatory drugs (NSAIDs), that are able to attenuate beta amyloid induced neurite loss. These findings will lead to further development of anti-inflammatory drugs being used in treatment of Alzheimer’s disease. We will further investigate the screened and identified compound to validation our data analysis results by lab experiments, both biological and chemical, in the further.
Bibliography


