Intravital Multiphoton Imaging of Immune Cells

Jo Keeble, Chi Ching Goh, Yilin Wang, Wolfgang Weninger, and Lai Guan Ng

Abstract. Intravital multiphoton microscopy (MP-IVM) is a powerful imaging approach that allows direct visualization of cells within their native environment in real time. Multiphoton imaging of immune cells has been performed in different tissues, and these studies have revealed intriguing insights into the unique migratory and interactive behavior of immune cells in the steady-state and during disease conditions. Here we provide an overview of a MP-IVM model of the mouse ear skin, as well as the benefits, limitations and pitfalls of this approach. We also discuss the prospects of intravital imaging in the areas of image analysis, data management and standardization.

1 Introduction

One of the fundamental characteristics of the immune system lies in the highly dynamic nature of its cellular components. For instance, responses to a pathogen typically require long-range migration of cells, short-range communication by local chemotactic signaling, and direct cell-cell contact. Thus, direct visualization of these dynamic events by intravital microscopy is able to provide essential in-sights into how immune cells exert their functions in the context of intact organs or tissues. Several forms of intravital microscopy, including brightfield, epifluorescence and laser scanning confocal microscopy, have long been adopted by immunologists and have generated substantial insights into leukocyte trafficking. However, intravital

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observations using these linear-absorption microscopy techniques have been limited to translucent tissues or superficial regions of non-translucent organs. These limitations are now over-come by intravital multiphoton microscopy (MP-IVM, also known as 2-photon microscopy), which provides greater depth penetration as the result of its localized nonlinear signal generation. Since the first application of multiphoton immune imaging in 2002 [Miller et al. 2002,Bousso et al. 2002], MP-IVM has rapidly evolved beyond merely an observational approach to address complex immunological questions at a quantitative level. MP-IVM is now routinely performed in a variety of tissues including lymph nodes, skin, brain, bone marrow, kidney, liver and even lung (reviewed in [Germain et al. 2006]). In this chapter, we first briefly introduce the methodology of MP-IVM, then provide an overview of a model for MP-IVM imaging of mouse ear skin ([Ng et al. 2008, Roediger et al. 2008]). Finally, we provide a broad overview of the future developments re-quired to overcome some key challenges facing intravital imaging. Our intention is to use the skin model to make the practical aspects of MP-IVM more accessible to physicists, mathematicians and computer scientists, with the aim of facilitating inter-disciplinary interactions between immunologists and experts in the field of software development. We believe that the limitations and pitfalls of MP-IVM discussed in this chapter will provide a useful framework for developing algorithms and analysis tools that can be specifically applied to multiphoton imaging data.

2 Multiphoton Microscopy

Unlike conventional fluorescent microscopy, multiphoton micros-copy relies on the near simultaneous absorption of two (or more) photons by a fluorophore, which together provide sufficient energy to excite and subsequently emit fluorescence (Figure 1). Given that the possibility of a fluorophore simultaneously absorbing more than one photon is extremely low at normal light intensity, multi-photon microscopy uses a high-powered femtosecond-pulsed laser beam that is focused through the objective lens to generate 'photon crowding' around the focal plane. Due to its non-linear excitation, multiphoton microscopy features the following advantages over conventional fluorescent microscopy: (1) Excitation is confined to a small volume around the focal point. This enables intrinsic optical sectioning and minimizes the effects of photobleaching and/or phototoxicity above and below the focal plane. (2) Longer wavelengths of multi-photon excitation result in less light scattering and absorption through tissues, thus enabling a deeper penetration of up to 1 mm depending on tissues; Excitation wavelengths used for multiphoton imaging are approximately double the wavelengths used for one photon microscopy (within the range of 700-1020 nm). The laser can also be coupled with a synchronously pumped optical parametric oscillator (OPO), which can tune the laser beam to even longer wave-lengths (1080-1500 nm) for excitation of red and far-red fluorophores. (3) Due to the broad multiphoton excitation spectra, multiphoton microscopy enables simultaneous excitation and detection of several fluorophores using a single

wavelength; and (4) Due to the multiphoton generation of second and third harmonic signals (SHG and THG), components of the extracellular matrix, such as collagen and elastin, can be visualized without the need for fluorescent labeling ([Witte et al. 2011, Zoumi et al. 2002]). This provides a useful reference, which facilitates the localization of fluorescent immune cells within the context of a whole organ or tissue.

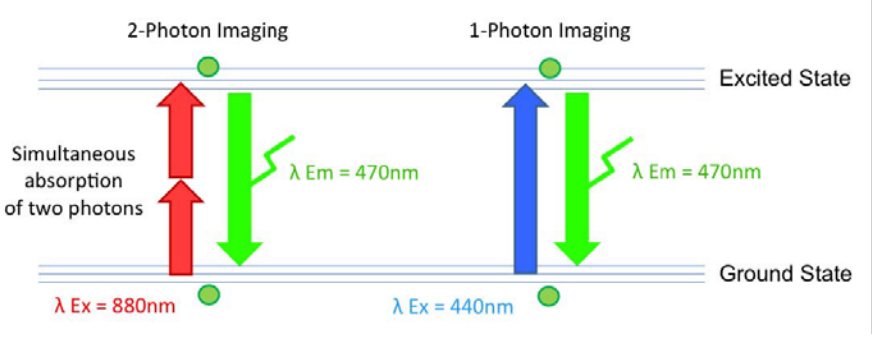

Fig. 1 Two-photon imaging versus one-photon imaging. Multi-photon microscopy relies on the simultaneous absorption of two photons that together provide the sufficient energy to excite the fluorophore of interest. In practical terms, this means that the wavelengths used for multi-photon imaging are approximately double the wavelengths used for conventional fluorescent microscopy. The wavelength of light emitted from an excited fluorophore as it returns to its ground state is identical for two-photon and one-photon imaging.

3 Intravital Skin Imaging Model

3.1 The Skin: An Important Immunological Site

The skin is the largest organ of the body and provides protection against the invasion of foreign pathogens by forming a physical barrier to the external environment, and is also home to several subsets of immune cells. The skin can be divided into two anatomical compartments: the epidermis, a relatively thin layer of cells composed primarily of keratinocytes, and the underlying dermis, which is rich in collagen-producing fibroblasts and contains a network of blood and lymphatic vessels. In the mouse skin, epidermal Langerhans cells [Banchereau et al. 1998] and dendritic epidermal T cells [Havran et al. 1988] are found in tight association with the surrounding keratinocytes in the epidermis. The dermis harbors macrophages, mast cells, dermal dendritic cells and memory T cells, and even scarce neutrophils that are dispersed throughout the collagen-rich interstitial space [Gebhardt et al. 2009], [Ng et al. 2008, Sumaria et al. 2011]. Upon infection or injury, circulating inflammatory cells such as neutrophils are recruited in response to pro-inflammatory

cytokines released by skin resident cells. These inflammatory cells orchestrate innate immune responses to serve as first line of defense for eliminating invading pathogens [Nathan 2006]. Meanwhile, dendritic cells recognize and take up foreign antigens, and subsequently migrate to lymph nodes to initiate the adaptive immune response [Banchereau et al. 1998]. Maintaining the homeostasis of these immune cells is essential for the structural and functional integrity of the skin. Thus, a better under-standing of these processes could lead to the identification of therapeutic targets for inflammatory diseases and may facilitate vaccine development.

3.2 The Mouse Ear Skin Model

The most commonly used sites for MP-IVM of the skin in mice are the ear and the hind footpad [Zinselmeyer et al. 2008, Ng et al. 2008, Matheu et al. 2008], [Peters et al. 2008, Sen et al. 2010]. Our laboratory mainly focuses on using the mouse ear skin. The mouse ear skin is an optimal imaging site as it is easily accessible and re-quires no surgical procedures prior to imaging, thereby preserving its physiological state. Its distal location also limits the effects of respiratory movements that can hinder imaging. Preparation for imaging starts with the removal of hair, which is highly auto-fluorescent and contributes to background signal. We have previously shown that hair removal does not interfere with the behavior of immune cells in the skin [Ng et al. 2008]. Albino mice are the best choice for imaging as the auto-fluorescent melanin, which is found in black-coated mice, may compromise the image quality. Although reducing laser power may dampen these background signals, this is sub-optimal as it results in reduced tissue penetration and decreased of signals when imaging deeper into the skin. For the actual experimental setup, after hair removal, the anesthetized mouse is placed onto a custom-built microscope stage (Figure 2) where the ear is immersed in PBS/glycerin (70:30 vol:vol) and covered with a glass cover slip. Care must be taken not to induce inflammation through the preparation, which may occur through physical trauma or dysregulated temperature. To ensure interstitial leukocyte activity is kept physiological, the temperature of the ear platform must be maintained around 35°C. The animal is kept at 37°C via a heating pad. In addition, adequate depth of anesthesia must be monitored closely to ensure that the animal is under a complete surgical anesthesia during the experiment (see [Roediger et al. 2008]).

3.3 Visualizing Immune Cells in the Vasculature and Skin

In order to visualize immune cells within the skin by MP-IVM, the cells of interest must be fluorescently tagged. This can be achieved by the endogenous expression

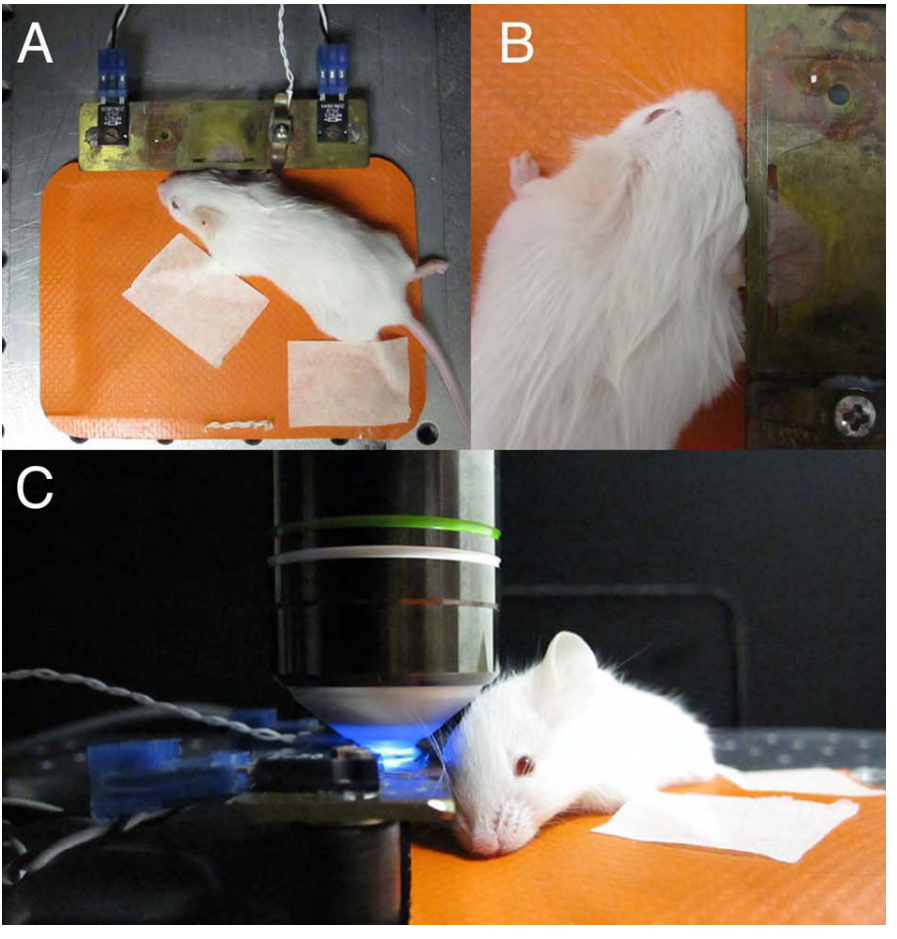

Fig. 2 Stage setup for intravital ear imaging. (A) The intravital imaging stage is a custom-built metal platform with a heating pad to maintain the body temperature of the anesthetized mouse at 37°C. The ear is positioned, dorsal surface up, on a metal strip with heating elements that maintains the temperature of the ear at 36°C. (B) Close up view of prepped ear on the metal strip. The ear is immersed in PBS/glycerin and covered with a glass cover slip. (C) The stage is placed on the microscope for imaging.

of a fluorescent protein under the control of an immune cell-specific gene promoter. The introduction of such an expression system into mice generates immune cell-specific fluorescent transgenic mice. Currently, there is a wide array of fluorescent transgenic mice available, for this chapter we only outlined the mouse strains that are useful for skin imaging (see Table 1). Alternatively, fluorescently-labeled antibodies can be administered that recognize and bind to immune cells in vivo, or immune cells can be isolated and exogenously labeled with fluorescent compounds such as Cell Tracker Orange (CMTMR, invitrogen), before being adoptive transfer into

recipient mice [Bonasio et al. 2006, Mempel et al. 2004]. To visualize blood vessels, quantum dots, fluorescent dextrans or Evans blue dye can be used. As shown in Figure 3, using our ear skin model, we were able to visualize dendritic cells, collagen fibers and blood vessels in the ear dermis of a CD11c-YFP mouse (a fluorescent transgenic mouse expressing yellow fluorescent protein in all CD11c+ cells, which are the dendritic cells).

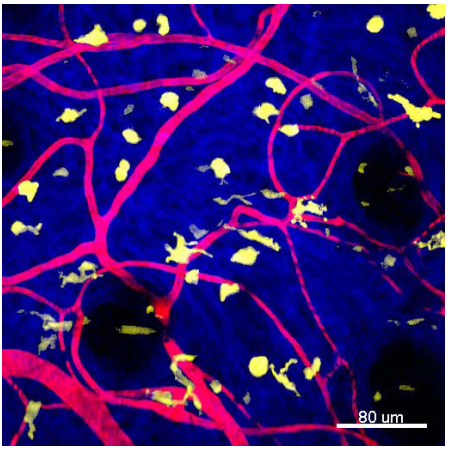

Fig. 3 Multiphoton imaging of the dermal dendritic cells within the ear skin of a CD11c-YFP mouse. Dermal dendritic cells (yellow) can be seen widely distributed within the highly vascularized (blood vessels shown in red), collagen-rich (blue, SHG) dermis.

Table 1 Immune Cell Specific Fluorescent Transgenic Mice.

Mouse Strain	Fluorescent Immune Cells	References
LysM-GFP	Neutrophils (GFPhi) Monocytes (GFPint)	[Faust et al. 2000]
CD41-YFP	Megakaryocytes and Platelets	[Varas et al. 2007]
CD11c-YFP	CD11c+ Dendritic Cells (DCs)	[Lindquist et al. 2004]
Langerin-GFP	Langerhans Cells	[Kissenpfennig et al. 2005]
CX3CR1-GFP	Monocytes Subsets of NK cells and DCs Microglial cells	[Jung et al. 2000]
FoxP3-IRES -mRFP (FIR)	Foxp3+ T regulatory cells	[Wan et al. 2005]
IL-4 GFP- enhanced transcript (4get)	IL-4 producing immune cells	[Mohr et al. 2001]

Data acquisition and parameter extraction from intravital multiphoton imaging
The major strength of multiphoton imaging is the direct visualization of immune cell activities within intact organs and tissues. To under-stand the dynamics of the

immune system, three-dimensional (3D) image stacks are acquired longitudinally over time through time-lapse imaging (Figure 4). Special software is required to process and view the four-dimensionality (x,y,z and time) of these data. To date, the most widely used commercial software packages for data processing and analysis are Imaris (Bitplane) and Volocity (PerkinEl-mer). The output of these 4D datasets is most often presented as ”ex-tended focus view” movies, in which the information from multiple z-planes is compressed into one single plane. Visual inspection of the movie is the first step to examine the cellular behavior of the fluorescent objects in the image stacks. Although this can provide very useful information, it is still important to obtain quantitative in-formation. Tracking analysis based on the extended focus view movies is not recommended because these data sets do not contain in-formation from the z-axis. Ideally, to measure cell motility within an intact tissue/organ, 3D tracking should be performed. For optimal measurements, several aspects of image acquisition have to be care-fully designed and recorded, these include: size and depth of the scan field, time interval between acquisition and the number of fluorochromes. In the end, various parameters (Table 2) can then be extracted from the tracking analysis to characterize cell motility and interactions.

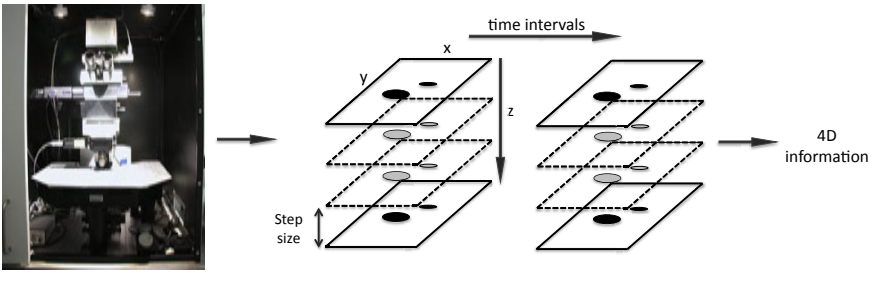

Fig. 4 Basics of Image Acquisition. Multi-photon imaging gives 4-D information (x, y, z plane over time, t) of cell morphology and motility. The area, depth, step size and imaging time can be determined prior to acquisition.

3.4 Data Processing and Presentation

Data sets generated from multiphoton imaging can be enormous in file sizes, as these files contain multi-dimensional information. The major challenge is how to best present dynamic cellular data in 2D print media such as articles in scientific journals. One possible way for such representation is to show snapshots of selected time-lapse images (Figure 5). This is often complemented with movie files up-loaded to the journal’s website, which can provide a better dynamic view of the cellular activities. Although time-lapse images and movie files contain information for x-y dimensions, they lack the depth information on the z-axis. Because of this shortcoming,

Table 2 Parameters used to characterize cell motility and interaction (adapted from [Cahalan et al. 2008]).

Parameters	Description	Unit
Instantaneous velocity (v)	Velocity calculated as displacement/time during a single time step	μm/min
Mean velocity (v)	Mean velocity of a cell over several time steps (usually the entire imaging period)	μm/min
Contact time	Time for which a cell is in contact with a defined cell or structure	sec:min
Path length	Cumulative distance traveled by a cell over a given time	μm
Displacement (D)	Straight-line distance of a cell from its starting point after any given time	μm
Motility coefficient (M)	M = $displacement^2/4t$ (for 2-D measurements) or $displacement^2/6t\mu m^2$/min (for 3-D measurements); analogous to the diffusion coefficient for Brownian motion	μm^2/min

this can lead to incorrect visual perceptions, for instance the image may show that two objects are in close contact, but in reality they were only moving passed each other on different z-planes. Thus, it is important not to draw conclusions about cellular dynamics merely base on the visual inspection of images/movies. There is no question that dynamic imaging offers exciting ways to visualize immune cell behavior in vivo, however, results from extensive quantitative and statistical analyses must still form the basis for making conclusions.

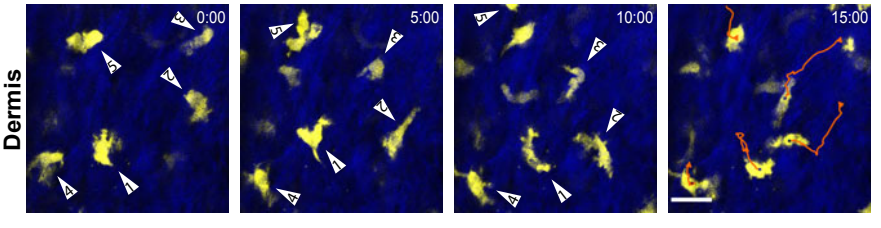

Fig. 5 Tracking cell motility using Imaris, a scientific 3-D/4-D Image Processing and Analysis Software. Five dermal dendritic cells (yellow, labeled 1 to 5) were tracked using Imaris (shown here are four consecutive, representative time frames). Orange lines indicate the 2-D tracking of these cells. Extracted from [Ng et al. 2008, Roediger et al. 2008].

3.5 Intravital Multiphoton Imaging Considerations

In previous sections, we have outlined the benefits of using MP-IVM to study immune cells in the skin, as well as caveats and pit-falls associated with it. For the following section, we will provide a broad overview of MP-IVM areas requiring

software developments to overcome current challenges not only for skin imaging but also for other MP-IVM studies.

4 The Missing Link in Multiphoton Microscopy: Software Development

In the past few years, hardware development in multiphoton micros-copy has accelerated to the point where it is now possible to purchase an off the shelf system that is highly reliable, compact and user friendly. As this technology slowly becomes common-place like conventional laser scanning confocal microscope , the main focus for future developments will be on bioinformatics tools. With the continuing efforts in hardware and optics developments, one can envision that future multiphoton imaging experiments will become more ambitious in terms of the numbers of the detection channels, resolution, acquisition speed, volume and duration. This multidimensional approach will no doubt generate large volumes of digital information, and special considerations should be given to find better solutions for storing, processing and analyzing these data. At the practical level, software that is capable of handling large volumes of image data sets to allow visualization, segmentation and quantification of multi-dimensional image sets, and provide methods for annotating raw and analyzed data in a standard format will be extremely useful. To date, no such widely functional software exists. In the following section, we will discuss several possible future developments that may help to achieve this goal.

4.1 Image Acquisition and Data Management

One challenging aspect for managing multi-dimensional images is keeping track of important contextual information related to these files. Currently, most acquisition software allows exportation of such information through Open Microscopy Environment (OME) or Metadata file formats [Swedlow et al. 2003]. However there is still no standardized metadata format for describing the hardware (e.g. objective lens and filter sets) or acquisition parameters (e.g. scan field and resolution) between microscope manufacturers. Implementations of standardization in the future will no doubt facilitate inter transferability of image-based data between different platforms and laboratories.

4.2 Spectral Unmixing

Given the advent of transgenic mice expressing fluorescent proteins as well as new techniques for labeling cells/structures in vivo, one would expect that multi-color

MP imaging be routinely performed. However, most of the MP imaging studies have only used 2 to 3 color detection. The limitation lies in the fact that most of the commonly used fluorescent proteins and/or dyes have overlapping emission spectra, leading to spectral bleed-through into multiple detection channels. As a result, it is then difficult to attribute a given color to a specific immune cell type or cellular structure. Spectral unmixing could be one approach to solve this problem [Dickinson et al. 2001, Hiraoka et al. 1991, Lansford et al. 2001]. However, it is important to note that current spectral unmixing algorithms were not designed for the separation and quantification of depth resolved spectra in complex biological tissues [Ducros et al. 2009]. Future efforts should focus on understanding how scattering and absorption effects in biological tissues may influence the emission spectra of various fluorophores. Such an understanding will be instrumental for the development of spectral unmixing algorithms that are suitable for the separation and quantification of spatially and spectrally over-lapping fluorophores in depth (within tissue) with high resolution.

4.3 Image Analysis

The principal challenge posed by analyzing images from multiphoton imaging is to translate pixel-based representation data to meaningful object-based (e.g. immune cells) data represented by the pixels. Recognizing and classifying cells in images, a process called segmentation, can be performed by software packages such as Imaris (Bitplane), Volocity (PerkinElmer) and ImageJ. Often this process is highly labor intensive, as it can only be done in a semi-automated manner. The difficulty of performing automated segmentation of images from intravital imaging lies in the multi-dimensionality of the data sets, i.e. cell volumes and movements over time. There is no common algorithm for segmenting cellular structures and cell tracks. Algorithms that work well for one application may not be good for another one. A major goal for future development should be providing a set of tools capable of extracting cell-based data from many different types of data sets generated by intravital imaging. One approach is to create organ- or tissue-specific sets of algorithms for image analysis. This approach, however, is possible only with the implementation of standardized imaging (see below).

4.4 Towards Standardized Imaging

Moving forward, one long-term goal of intravital imaging is to ac-quire and convert image-based data into a standardized, quantitative cell-based and anatomical format. If this goal is accomplished, it will allow direct comparison of data generated from different laboratories, and more importantly it can be used as a framework for cell- and anatomical-based databases. However to achieve this, major efforts need

to be made in standardizing several aspects of intravital multiphoton imaging, these include standardization of the imaging protocol, data extraction and presentation. If a universal system of standardized imaging is implemented, then one could imagine that data generated from MP-IVM can be digitized into a standard for-mat. This will form a powerful tool for immunologists to perform functional imaging, as comparison of normal and mutant phenotypes (e.g. genetically modified, gene-deletion or over-expression) could then be performed easily at cellular resolution.

4.5 Data Interpretation and Presentation

Although cell migration and interaction are major components of immune cell function, it remains a big challenge to ”convince” non-imaging immunologists about the biological relevance of the numerical information extracted from imaging experiments. There is no best solution for this, however, if new methods can be implemented to better present the analytical data from these studies, it may help to bridge the perception gap. As mentioned above, cellular dynamics data are often represented by time-lapse images or movies, which lose most of their multi-dimensional information. Methods have been devised to provide more multi-dimensional information for the viewer. For instance, one scheme implemented was color coding of a cell based on its z-position in the tissue [Miller et al. 2002]. Future works should focus on how to create a simple intuitive interface that allows the viewer to perform rotation, zooming and simple 3D visualization of movie files without the need of specialized image analysis software.

5 Concluding Remarks

Intravital MP imaging is a powerful approach for investigating immune cell activities within their native environment. Although this is a relatively new field, it is now possible to perform dynamic, multi-dimensional imaging to simultaneously track cell populations at depths of up to several hundreds microns inside tissues over a long period of time (up to few hours). Despite its vast potential, there are still many limitations associated with MP imaging. We believe that interdisciplinary efforts from biologists, chemists, physicists, mathematicians and computer scientists will be needed in order to usher in a new era of functional immune imaging.

With the above-mentioned developments in mind, we hope that future software will be able to overcome major challenges for analyzing datasets obtained from in vivo imaging studies. These challenges include:

- A low signal-to-noise ratio due to the dynamics of the observation;

- The sharpness of the biological structure contours due to the similarity of the radiometry of the observed tissue;
- The variability of the shape and size of the biological structures.

We believe that overcoming these challenges is key to developing a software package that can automatically segment and track cells in a multidimensional manner.

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