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Spinning Disk Confocal Imaging of Neutrophil Migration in Zebrafish

Pui-ying Lam^{1,4}, Robert S Fischer^{2,4}, William D. Shin^{2,4}, Clare M Waterman^{2,4}, and Anna Huttenlocher³

¹Program in Cellular and Molecular Biology, University of Wisconsin-Madison; Madison, WI 53706; USA

²Cell Biology and Physiology Center, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD 20892; USA

³Departments of Pediatrics and Medical Microbiology and Immunology; University of Wisconsin-Madison; Madison, WI 53706; USA

⁴Whitman Center, Marine Biological Laboratory, Woods Hole, MA 02543; USA

Abstract

Live-cell imaging techniques have been substantially improved due to advances in confocal microscopy instrumentation coupled with ultrasensitive detectors. The spinning disk confocal system is capable of generating images of fluorescent live samples with broad dynamic range and high temporal and spatial resolution. The ability to acquire fluorescent images of living cells *in vivo* on a millisecond timescale allows the dissection of biological processes that have not previously been visualized in a physiologically relevant context. *In vivo* imaging of rapidly moving cells such as neutrophils can be technically challenging. In this chapter, we describe the practical aspects of imaging neutrophils in zebrafish embryos using spinning disk confocal microscopy. Similar setups can also be applied to image other motile cell types and signaling processes in translucent animals or tissues.

1. Introduction

The zebrafish (*Danio rerio*) immune system is remarkably similar to its mammalian counterpart and it has therefore gained tremendous popularity as a vertebrate disease model of both innate and adaptive immunity (reviewed in [1, 2]). Zebrafish embryos are largely translucent for the first few days of development, allowing for noninvasive *in vivo* imaging and studies of cell behavior in a physiologically relevant context.

Here, we describe methods for *in vivo* live imaging of neutrophils in zebrafish embryos. Neutrophil imaging is usually performed at 2 to 3 days post fertilization (dpf). At this stage of development, adaptive immunity has not sufficiently matured, allowing the direct study of innate immunity in isolation. Neutrophils at this stage are considered to be fully

functional, as they can directionally migrate (chemotaxis) to wounds or infections and perform immune functions to fight off pathogens. In addition, neutrophils migrate spontaneously (random migration) in the interstitial tissue within the head region (reviewed in [2]). The ability to image both neutrophil chemotaxis and random motility in the same organism allows for the dissection of the molecular regulators required for basic motility and directional sensing. One thing to note is that neutrophils at this developmental stage are not terminally differentiated, since they retain the ability to undergo cell division under some conditions and possess an elongated nucleus instead of the multi-lobulated nucleus observed in human neutrophils.

This protocol describes the use of spinning disk confocal microscopy (SDCM) for live imaging of genetically encoded fluorescent reporters expressed in zebrafish embryos. The benefits of imaging using SDCM over conventional single-point laser scanning confocal microscopy (LSM) including acquisition speed, detection efficiency, resolution, reduced photobleaching, and improved signal-to-noise ratio, both *in vitro* and *in vivo*, has been discussed elsewhere [3–5]. One common concern regarding high-speed imaging is the trade-off between speed and resolution. In our experience, acquiring a Z-stack time series using an SDCM system with optimal camera setup offers improved temporal and spatial resolution as compared to laser scanning confocal microscopy (Fig. 1). One of the major differences between the acquisition speed of the LSM and SDCM is that the former collects signals from the specimen one pixel at a time through a single pinhole. The speed of the LSM thus depends on the line frequency of the scanner, which is limited by the galvanometer mirrors that move the laser beams, along with the number of lines needed for the field of view imaged. However, because the galvanometer speed is fixed, the limitation on image acquisition speed in practice is the dwell time for each XY position (pixel) to produce sufficient signal over background. The detector for LSM is typically a photomultiplier tube (PMT). Although PMTs have a huge capacity for amplification of signal, they also have relatively low quantum efficiency (~10–15% is typical depending on wavelength). Thus, in order to increase speed for a given signal, one must increase excitation laser power, which can result in faster photobleaching and/or increased phototoxicity with LSM [5]. In addition, PMT amplification applies to both signal and noise, thus reducing the overall SNR in LSM images.

In contrast to point scanning with an LSM, a spinning disk confocal utilizes a rotating Nipkow disk with thousands of pinholes arranged in a series of nested spirals to simultaneously illuminate and collect signals from the entire specimen field, thus leading to significantly faster frame rates than LSM systems. Perhaps more significantly, SDCM systems use a chargecoupled device (CCD) camera or camera as a detector, which have both higher quantum efficiencies (~60%) and lower noise than PMTs, resulting in significantly higher SNR and increased sensitivity [6, 7]. Due to the improved SNR and sensitivity, SDCM imaging has the added advantage of requiring lower levels of excitation laser light, leading to less photobleaching and/or phototoxicity of the imaged samples. In recent years, electron multiplying CCDs (EMCCDs) have been introduced that have even greater quantum efficiency (~90%) and low-noise signal amplification resulting in exceptionally high SNR and sensitivity.

When imaging any live specimen by fluorescence microscopy, high expression of fluorescent fusion proteins can produce cytotoxicity or unintended phenotypic effects, including developmental defects in zebrafish. However, live cells expressing fluorescent fusion proteins expressed at approximately endogenous levels of the target protein are often very dim. With samples in which a fluorescent signal is relatively weak, the enhanced sensitivity of SDCM is clearly advantageous. In addition, low fluorescence emission intensity can be compensated for by using longer exposure time; however, this too will decrease the speed advantages of using a SDCM. Further improvements in sensitivity and SNR can be gained through pixel binning, at the cost of lateral resolution. For example, 2×2 binning will increase the SNR by a factor of four, but reduce the lateral resolution by 50%. An EMCCD camera can sometimes abrogate the need for pixel binning, but it should be noted that native pixel sizes vary with camera type (*see* Note 1 and ¹⁸).

A major advantage of using SDCM for live, fast motile cell imaging lays in the image quality, which arises from the aforementioned high SNR due to the use of CCD and EMCCD camera technologies. One notable downside of SDCM for intravital imaging is pinhole cross talk, which can degrade image quality. This effect is particularly problematic with specimens that have high light scattering properties [5]. Newer model spinning disk heads with greater pinhole spacing can mitigate this problem to some degree. Another potential downside of standard SDCM systems relative to LSM is that they do not have the inherent ability to select a region of interest (ROI) for high power illumination as is required in photobleaching or photoactivation experiments. However, ROI illumination can be achieved via separate illumination paths. Despite these potential drawbacks, SDCM is a powerful system for high-speed three-dimensional imaging of fast-moving cells like neutrophils.

In the following sections, we provide protocols for performing live spinning disk confocal microscopy of transgenic zebrafish expressing fluorescent-tagged proteins in neutrophils. This includes protocols for optimizing neutrophil-specific protein expression, preparation of transgenic fish for imaging, microscope setup, and image acquisition.

¹Photometrics Evolve 512 EMCCD and CoolSNAP HQ2 CCD are the common high-performance cameras of choice for SDCM. Sensitivity and resolution are the main criteria for choosing a suitable camera. Evolve EMCCD offers a higher sensitivity but a relatively big 16 μm × 16 μm pixel size. CoolSNAP HQ2 has a 6.45 μm × 6.45 μm pixel size; however, binning may be needed for image acquisition to reduce illumination intensity and exposure time needed. It is highly recommended to have a demonstration of different cameras to find out which one is better suited for your specific application. For our applications, we selected the Evolve EMCCD taking into consideration the speed of neutrophil migration and fluorescent levels of live cells (also *see* Note 18). One thing to note is that because the Evolve EMCCD is very sensitive, it can pick up cosmic ray signals stochastically that appear sporadically as very bright transient dots/comets within the image [19]. These transient dots/comets are very bright and are easily distinguishable from the real signal generated by fluorescent protein expression.

¹⁸EM gain technology multiplies the charge signal from each pixel on a CCD array to a level above the read noise. Therefore, EMCCD cameras can further improve image acquisition speed by reducing the exposure time needed compared to a CCD camera. It can also reduce the amount of excitation illumination needed that may cause photobleaching and phototoxicity of the cell and therefore can extend the possible imaging time of the sample. This is important when performing long-term time-lapse acquisition, especially with Z-stack acquisition of each time point. However, the cost of EMCCD cameras is much higher than traditional CCD cameras. If an EMCCD camera is used, adjust EM gain settings, preferably to the camera optimal setting. Increasing EM gain will increase signal intensity but may introduce noise at the same time. In the case where a bright field image is needed, EM gain for bright field acquisition can be reduced to zero to decrease graininess. Alternatively, 2×2 binning on a CCD camera with small pixel sizes (e.g., 6.45 μm in the case of the Photometrics HQ2) will improve its sensitivity but will still lack the signal amplification afforded by an EMCCD.

2. Materials

2.1 Zebrafish embryos

1. Zebrafish husbandry according to The Zebrafish Book [8]. Available online http://zfin.org/zf_info/zfbook/zfbk.html. Accessed 14 February 2013.

2.2 Microscope supplies & components

This is just a reference of the setup we use to acquire the images presented in the figures (Fig. 2 and 3). Other vendors also provide similar systems.

1. Inverted microscope (Zeiss Observer Z1) (*see* Note 2)
2. Spinning disk (Yokogawa CSU X1) (*see* Note 3)
3. EMCCD camera (Photometrics Evolve 512 EMCCD) (*see* Note 1 and ¹⁸)
4. Objective (LCI Plan-Neofluar 63×/1.3 Imm Corr DIC M27; water or glycerine immersion) (*see* Note 4)
5. Immersion medium (Carl Zeiss Immersol W 210; distilled water is also suitable for short-term imaging; *see* Note 5)
6. Heating stage/heating chamber (PECON Incubator P, compact S1 and Heating Insert P S1; heating is optional depending on room setup and stage of embryo)
7. Scanning stage with Z-piezo (*see* Note 6)
8. Multi laser module (laser unit 488 / 20 mW (OPSL laser) and 561 nm / 20 mW (DPSS laser)) (*see* Note 7)

²The inverted microscope stand provides a stable base for minimizing vibration as well as providing long-term stability for time-lapse imaging. As most microscope models will have manual parts, automation of the stand is not necessary for spinning disk confocal image acquisition. Motorized components of the stand conveniently allow for fast and seamless switching between preconfigured settings for sample viewing and image acquisition. The stand should include a filter-cube turret to allow switching between different dichromatic mirrors for specimen viewing and image acquisition, a multi-position objective lens nose piece for switching between magnifications, transilluminator for phase/DIC imaging/viewing, and an epilluminator for wide-field excitation of the sample for viewing. Having multiple imaging ports provides flexibility for introducing illumination, mounting digital cameras, and sample observation. Incorporating electronic shutters in the transillumination and epifluorescence illumination path will help to minimize photobleaching and phototoxicity when imaging/viewing the sample.

³The Yokogawa CSU ×1 spinning disk confocal unit is a dual-disk scan head design that consists of a disk with microlenses that are in conjugate with the pinholes of the Nipkow disk. This has several advantages for high temporal and spatial resolution live cell imaging over other spinning disk confocal systems. Single Nipkow disk systems only transmit 5% of the incident illumination, whereas the microlenses in the second disk of the Yokogawa CSU ×1 increase the illumination efficiency theoretically up to 40%. Slit-scanning systems do not reject out-of-focus fluorescence along the axis of the slit and thus suffer from higher backgrounds. Compared to previous models (CSU 10, CSU 22), the CSU ×1 has increased illumination throughput and improved beam expanding optics providing flatter field of illumination. The CSU ×1 is available as a manual or motorized version and has a wide range of configurations such as 2 camera port and bright field bypass module. For our application, we chose the basic manual version with 6 position electronic emission filter wheel.

⁴Imaging deep in the sample and far away from the coverslip with an oil immersion objective can suffer from optical artifacts such as spherical aberration. Utilizing a high NA water immersion objective with a correction collar can minimize these induced spherical aberrations caused by mismatched refractive indices of the sample and immersion medium. In addition, the correction collar not only allows one to compensate for coverslip thickness variations but also changes in refractive medium due to temperature.

⁵For extended time-lapse imaging experiments, Carl Zeiss Immersol W 210 instead of distilled water is used as a water objective immersion medium. This reduces evaporation of the immersion medium and allows uninterrupted long-term imaging.

⁶A piezoelectric Z-stage allows higher Z-focusing speeds than a stepper- or servomotor-driven Z-stage. In our experience, it can cut down on the time for a Z-stack acquisition by nearly half. As an alternative, a piezoelectric-driven objective lens mount can be used. Note that piezo devices have much smaller focus range limits (typically 100 μm, some as high as 300 μm) compared to motor-driven devices (tens of millimeters).

9. Emission filter (BP 525/50 DMR 25; FF02-617/73-25) (*see* Note 8)
10. Vibration isolation table (Newport Vision IsoStation)
11. Glass bottom petri dish (*see* Note 9)
12. Zen 2011 (Carl Zeiss) image acquisition software

2.3 Buffers and other reagents (Image Acquisition Components)

1. 60× E3: To make 1L, add 17.2g NaCl, 0.17.2g NaCl, 2.9g CaCl₂ and 4.9g MgSO₄. Fill to 1L with reverse osmosis (RO) water. Adjust pH to 7.2 with NaOH.
2. 1×E3: Dilute 60× E3 with RO water to 1×. Add 0.1% methylene blue.
3. E3/PTU: 0.003% 1-phenyl-2-thiourea in 1× E3.
4. 20× Tricaine: 400 mg tricaine (MS-222) powder, 97.9 ml RO water, ~2.1 ml 1 M Tris (pH 9). Adjust pH to ~7. Store at 4°C.
5. 1× Tricaine: Dilute 20× Tricaine to 1× in E3/PTU
6. Water bath at 42°C.
7. Prepare 0.75--1% low melting point agarose (L.M.A.) in E3 solution for embryo mounting. Microwave to melt the L.M.A. The solution remains fluid at 37°C but quickly hardens when cooled. Maintain the melted L.M.A. in a 42°C water bath.

2.4 Image Processing Components

Image acquisition software usually contains some image viewing and/or imaging analysis functions. Additional commonly used software includes but is not limited to the following:

1. ImageJ (free software; <http://rsbweb.nih.gov/ij>. Accessed 14 February 2013).
2. FIJI (free software; <http://fiji.sc>. Accessed 14 February 2013).
3. FluoRender (free software; <http://www.sci.utah.edu/software/127-fluorender.html>. Accessed 14 February 2013).

⁷Developments in diode lasers and diode-pumped solid state (DPSS) lasers have made them a cost-effective alternative to gas lasers. They are small and quiet, have low heat loads and large range of wavelength selection, and don't require bulky water coolers. Most microscope manufacturers provide fiber-coupled laser combiners to deliver the laser beam, which use an acousto-optic tunable filter (AOTF) or TTL modulation to quickly select, shutter, and modulate the wavelength power. When choosing from the wide selection of available laser lines, the wavelength should optimally excite the fluorescent protein. Common laser wavelengths used for exciting fluorophores are 405 nm for DAPI, 442 nm for CFP, 488 nm for GFP, 514 nm for YFP, 561 nm for RFP, and 647 nm for Cy5. In addition, when considering power selection of individual lines, it should be noted that for fiber-coupled laser combiners, 40--60% of the illumination power (wavelength dependent) is lost due to fiber coupling efficiency. For our application, 20 mW has proven to be sufficient for live zebrafish imaging (*also see* Note 17).

⁸Individual band-pass emission filters, denoted by the center wavelength and bandwidth, should be chosen based on the spectral properties of the fluorophore. When selecting band-pass filters, it is important that the bandwidth be wide enough for efficient emission collection of the fluorophore, as well as provides sufficient blocking to eliminate the excitation source and signal from other fluorescent channels. For our filters, we chose a center wavelength of 525 nm with a 50 nm bandwidth (BP 525/50 DMR 25) and a 617 nm center wavelength with a 73 nm bandwidth (FF02-617/73-25). Manufacturers, such as Chroma Technology Corp and Semrock, Inc., can provide suggestions for filter selection based on the fluorophore spectral properties.

⁹Glass bottom dishes are commercially available, but an inexpensive and relatively easy alternative is to make them in house. Drill a 16--18 mm hole in the center of a 35-mm tissue culture dish bottom. Apply a thin layer of Norland Optical Adhesive 68 on the outside surface around the hole. Place a 22-mm round #1 coverslip on top of the adhesive and press down slightly. The adhesive is cured by UV light. Overnight exposure of UV light in a tissue culture hood or approximately 1 hour on a UV transilluminator is sufficient to cure the adhesive.

4. Bitplane Imaris (<http://www.bitplane.com/go/products/imaris>. Accessed 14 February 2013).
5. MetaMorph (<http://www.moleculardevices.com/products/software/meta-imaging-series/metamorph.html>. Accessed 14 February 2013).
6. Volocity (<http://www.perkinelmer.com/pages/020/cellularimaging/products/volocity.xhtml>. Accessed 14 February 2013).

3. Methods

3.1 Neutrophil-specific protein expression

For imaging purposes, transient mosaic expression is usually sufficient. However, in the case where high levels of expression of the transgene affect neutrophil function, generation of a transgenic line may be desired, as gene expression can be lower and specific transgenic lines with low copy number of the transgene can be identified (*see* Note 10). Preparation of transient expression or transgenic zebrafish embryos that express the gene of interest in a neutrophil-specific manner requires the use of a neutrophil-specific promoter such as the myeloperoxidase (*mpx*) [9, 10] or the lysozyme C (*lyz*) promoter [11, 12] (*see* Note 11):

1. Construction of plasmid: Use DNA expression vectors containing minimal Tol2 elements for efficient integration [13] flanking either *mpx* or *lyz* promoter, the gene of interest and an SV40 polyadenylation sequence (Clontech Laboratories Inc.).
2. Preparation of plasmid DNA: Transformation of plasmid into regular cloning competent cells, e.g., *E. coli* DH5 α is sufficient with the exception of plasmids containing the *mpx* promoter (*see* Note 12). DNA extraction and purification is achieved using a commercially available miniprep kit (Promega Wizard *Plus* SV Minipreps DNA purification system) (*see* Note 13).
3. Preparation of Tol2 transposase mRNA: transposase is prepared by *in vitro* transcription (Ambion mMESSAGE mMACHINE) followed by column purification (Qiagen-RNeasy Mini Kit).
4. Microinjection of zebrafish embryos is a pretty standard routine procedure. Different laboratories will have slightly different setups. For basic setup information and injection procedures, please refer to The Zebrafish Book [8] and a video demonstration published elsewhere [14].

¹⁰To make a transgenic line, injected embryos displaying mosaic transgene expression are raised to sexual maturity (as early as 2.5 months). F₀ founders are screened by outcrossing with wild-type zebrafish and checked for transgene expression in the neutrophils in the F₁ embryos at 2–3 dpf. The positive F₁ transgenic progeny can then be used for imaging or propagation of the transgenic line.

¹¹Since the *mpx* promoter is much larger than that of the *lyz* promoter (8 Kb vs. 4.1 Kb, respectively), it is easier to express larger transgenes (i.e., >3 Kb) driven under the *lyz* promoter in our experience. The *mpx* promoter drives gene expression mainly in neutrophils, although there may be very low levels of expression in macrophages as well [9]. The *lyz* promoter drives gene expression in neutrophils at later stages (> 2 dpf) [12]. However, in earlier stage embryos (i.e., 1 dpf), expression can be seen in primitive macrophages [20].

¹²Cloning with the *mpx* promoter is better achieved using bacterial strains specific for unstable DNA inserts, e.g., Stb13 (Invitrogen).

¹³Not all DNA extraction and purification procedures/kits produces high enough DNA quality for microinjection. Injecting poor quality DNA will result in embryo death. For small amounts of DNA preparation, the Promega Wizard *Plus* SV Minipreps DNA purification system yields good DNA quality in our experience.

5. Expression of construct is obtained by injection of 3 nL of 12.5 ng/ μ L DNA plasmid with 17.5 ng/ μ L Tol2 transposase mRNA into the cytoplasm of one-cell stage embryos.

3.2 Preparation of 35-mm glass bottom petri dish

Embryos will stick to polystyrene and glass surfaces without coating and this will create unwanted wounds on the embryos. Submerge the 35-mm glass bottom Petri dish (*see* Note 9) in 1% BSA or 1% skim milk for 10 minutes. Rinse several times with RO water and air dry before use.

3.3 Preparation of samples

Imaging is usually performed at 2–3 days post fertilization (dpf). To prevent pigmentation, exchange regular media with E3 containing 0.003% 1-phenyl-2-thiourea (PTU) as early as 15 hours post fertilization and no later than 1 dpf before pigment starts appearing. Keep the embryos in the E3/PTU medium throughout. Before imaging, remove chorion using ultrafine forceps if embryos are still unhatched. Mounting of an embryo in L.M.A. which will stabilize the embryo and prevent movement is required for high-magnification imaging, since even the slightest movement by an embryo will affect image quality. Proceed to the mounting procedures as follow:

1. Put embryos in 0.2 mg/mL tricaine in E3/PTU for 1 minute.
2. Transfer 1–2 embryos onto a glass bottom Petri dish using a plastic or glass transfer pipette. Remove as much E3 as possible without drying out the embryo using a P100 pipette or transfer pipette. Any exposure of the embryo to air is enough to create injury on the epithelium and may affect neutrophil behavior.
3. Add in ~80 μ L of the melted L.M.A. directly onto the drop of liquid containing the embryo. In order to achieve an even consistency of the L.M.A. around the embryo, remove the L.M.A. and add in new L.M.A.. Repeat the removal and addition of L.M.A. steps again and let it settle. Embryos usually lay down on the bottom of the plate laterally at this stage and are ideal for imaging. Otherwise, adjust the angle of the embryo before the L.M.A. hardens. Wait for approximately 5 minutes at room temperature to ensure that the L.M.A. has hardened (*see* Note 14).
4. Slowly add in 2–4 mL E3/PTU with tricaine. Check if embryos are still attached to the bottom and have not floated. If floating occurs, perform the mounting steps again.

3.4 Microscopy, Image Acquisition and analysis

Imaging is performed at the desired developmental stage, typically at 2–3 dpf. Imaging of spontaneous neutrophil motility is performed in the head region. The typical average speed

¹⁴A small amount of L.M.A. just covering the embryo is enough for securing the position of the embryo on the glass bottom dish. The water tension of a small drop of L.M.A. helps hold the embryo close to the bottom of the dish. A larger volume of L.M.A. may result in an agarose gap between the embryo and the glass bottom, which is not obvious under low-power magnification. As higher magnification and higher NA objectives usually have a shorter working distance, this may make a substantial difference in image quality.

of neutrophils undergoing random motility that we have observed is between 80--130 nm/s. Directional migration can be performed with different stimulants, i.e., tail fin or ventral fin wounding using a needle or surgical scalpel [12, 15], CuSO₄ induced neuromast damage [16], and LTB₄ injection [17] or incubation [18] etc. Common applications include imaging the dynamics of the cytoskeleton such as F-actin (Fig. 2); subcellular localization of organelles such as the microtubule organizing center and the nucleus (Fig. 3); subcellular localization of proteins; subcellular localization of signaling molecules and protein activity using various bioprobes and ratio analysis; and morphological and tracking analysis during cell trafficking, etc.

Different SDCM setups and image acquisition programs can be used. Here, we describe a general inverted microscope setup and the acquisition parameters to be considered before image acquisition. There is no fixed parameter for each acquisition as every sample is different and adjustments are often required to determine the optimal settings. The key is the balance between the speed of acquisition, phototoxicity and image SNR. The following is a general procedure of setting up a Z-stack time series for single or multiple channel image acquisition:

1. Turn on heating stage and set at 28.5 °C.
2. Apply a small drop of immersion medium on the objective (*see* Note 5).
3. Secure the glass bottom Petri dish with samples on the microscope stage. Make sure that the dish is seated flat and firmly in the stage, as this will minimize focus drift during imaging. If stage clips are present, clip the sample in tightly.
4. Locate target cell using eyepiece (*see* Note 15).
5. Choose a suitable emission filter for acquisition (*see* Note 16).
6. Adjust laser power to as low as possible while still obtaining a good SNR image, especially for long time imaging (*see* Note 17).
7. Adjust optimal exposure time (usually around 50--100 msec) and EM gain (around 300--500 for Evolve EMCCD) (*see* Note 18). Note that exposure time and laser power will be interdependent. In general, lower laser power at the cost of longer

¹⁵Ideally, find neutrophils that are close to the objective lens (i.e., not deep into the embryo) and have a normal morphology, i.e., elongated cell shape and display pseudopod formation. Rounding of neutrophils is a common sign of abnormality due to transgene overexpression. If neutrophils have multiple fluorescent proteins expressed, find neutrophils that have similar fluorescent intensity of both fluorophores.

¹⁶When performing ratio image analysis or imaging more than one fluorescence channel, caution should be taken to check for bleed-through between fluorescence channels. If this happens, separate single band-pass emission filters in an electronic filter wheel should be used instead of a multiband emission. Although the time needed for the filter wheels to switch between filters limits the acquisition speed of multicolor images on a single camera, sequential acquisition of different channels is necessary to produce an accurate co-localization and/or ratio quantitative analysis. It is recommended to acquire the longest wavelength emission probe first to avoid possible bleed-through (also *see* Note 22). Careful control of total signal for each channel is also important in limiting the contribution of cross talk. In addition, the intensity of fluorescence emission varies from one channel to another, in order to acquire two channels with comparable exposure time and signal intensity, it is best to adjust laser power without introducing excessive phototoxicity.

¹⁷We usually use 30% laser power with our laser unit for imaging experiments. Too high of a laser intensity will result in unhealthy neutrophils or even cell death (also *see* Note 7). Ideally, set the initial maximum signal to 50% of the dynamic range of the camera bit depth. Fiber optic coupling efficiency and final output power at the objective will vary depending on the microscope setup, so empirical testing or direct power measurements should be done to optimize imaging quality and embryo health.

exposure times will result in less phototoxicity and photobleaching, assuming that the time resolution for Z-stack collection is not limiting for the experiment.

8. Set spinning disk revolution speed to maximum (*see* Note 19).
9. Set the Z-range with a little buffer distance above and below the cell's initial position, as the cell is actively migrating in a three-dimensional tissue.
10. Set the step size for Z-acquisition (*see* Note 20).
11. Set time interval and total duration of the acquisition (*see* Note 21).
12. Set the mode of acquisition as all channels per Z-slice (*see* Note 22).
13. Start acquiring image.
14. Common post-acquisition image analysis programs include but not limited to freeware like ImageJ/Fiji and FluoRender or commercially available Bitplane Imaris, Volocity, and MetaMorph. Each program has a different capacity and details of such are out of the scope of this chapter.

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¹⁹In practice, it is best to synchronize the spinning disk speed and camera exposure time so that the pinholes traverse an integral number of frame crossings during exposure. When the disk speed is not optimal, striped patterns can be observed especially for shorter exposure times (smaller than 50 msec). As it takes time for the spinning disk to change its speed, the disk speed should be set constant throughout the acquisition. For this reason, it is best to set similar exposure times for different channels during multiple channel acquisition.

²⁰Ideally, as a minimum requirement, a Nyquist theorem satisfying step size is preferred, which is equal to half the Z resolution (depth of field/optical depth). This is determined by the objective used and any additional magnification in the light path, as well as the wavelength of excitation used. As an example, for the microscope setup and objective that we use (LCI Plan-Neofluar 63×/1.3 Imm Corr DIC M27), we usually set the step size as 0.4 μm.

²¹In general, 0.5 to 1 minute intervals for a duration of 30 minutes using a 20× objective is sufficient for imaging neutrophil long distance migration. Allowing the specimen to experience maximal “dark periods” (no laser exposure) during the collection will generally result in less phototoxic effects over longer imaging times. For imaging neutrophil pseudopod dynamics, acquire images for 10 minutes continuously at maximal speed or at minimum with a time interval of 10 to 30 seconds using a 60× objective.

²²Caution should be taken in choosing to acquire all channels per Z-slice or whole Z-stack per channel. It is recommended to use all channels per Z-slice, as it will acquire an image of each channel for each Z-plane sequentially, resulting in a more accurate temporal comparison between channels. However, switching between filters for each Z-plane will increase the overhead time.

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Fig. 1. Comparison of laser scanning confocal (LSM) and spinning disk confocal (SDCM) on *in vivo* neutrophil imaging

Maximum intensity projection image of a neutrophil expressing GFP-UtrCH (green) and Lifeact-Ruby (magenta), which labels stable F-actin and all F-actin, respectively. (A–B) Image acquired using an LSM (Olympus). Olympus LUMFL 60×/ 1.1NA Water objective (A) or Olympus PlanApo N 60×/ 1.45NA Oil objective (B) was used with S.U. 200, optimal step size and 3.8× zoom in. (C–D) Image acquired using SDCM (Zeiss). LCI Plan-Neofluar 63×/ 1.3NA Water objective (C) or Plan Apo 63×/ 1.4NA Oil objective (D) was used with 300 EM gain, 55 msec exposure for both channels and 0.4 μm step size. SDCM images

showed superior image quality compared to that of the LSM images. The difference between using an oil objective with a higher NA than the water objective is less significant. Scale bar: 10 μm .

Fig. 2. Spinning disk confocal imaging of F-actin cytoskeleton dynamics

Time-lapse maximum intensity projection image of a neutrophil undergoing random motility in a zebrafish embryo *in vivo*. The neutrophil expresses GFP-UtrCH (green) and Lifeact-Ruby (red), which labels stable F-actin and all F-actin, respectively. A SDCM equipped with an EMCCD camera was used for the acquisition. LCI Plan-Neofluar 63×/1.3 Water objective; 300 EM gain; 55 msec exposure (GFP-UtrCH), 36 msec exposure (Lifeact-Ruby); emission filter BP 525/50 and FF02-617/73-25; 0.4 μ m step size. Acquisition was made as fast as possible and consecutive images are shown in the panel. Scale bar: 10 μ m.

Fig. 3. Spinning disk confocal imaging of organelle subcellular localization

Time-lapse maximum intensity projection image of a neutrophil expressing γ tubulin-GFP (green) and a nuclear probe, mCherry-histone H2B (red). The Microtubule Organizing Center (MTOC-arrow) is localized in front of the nucleus during neutrophil motility *in vivo*. Scale bar: 10 μ m.