Leica SP8 Digital Light-Sheet Microscope

The new Leica SP8 digital light-sheet (DLS) system is capable of rapidly imaging (>100 frames/second) live or fixed organisms (zebrafish embryos), organs (*Drosophila* brain, plant roots), and tissues and rendering them as three-dimensional structures. The rapid image acquisition also allows developmental, physiological, and other biological processes to be monitored over time. Further, DLS technology results in little or no phototoxicity. In addition, because the microscope is equipped with 405, 488, 552, and 638 nm diode lasers, a broad range of fluorophores can be imaged. The sample preparation does require a little practice to learn and a dedicated window of time to perform, but can be quickly mastered and is simple to accomplish. Below is a description of the principles of DLS microscopy and the basics of sample preparation.

I. Principle of light-sheet microscopy

In standard confocal microscopy, an excitation beam is emitted from an objective and excites the fluorescent molecules in a sample. The signal produced by the fluorescent molecule enters the same objective and is recorded by a detector. Because the excitation beam represents a discrete point when it hits the sample, it must be scanned across a region of the sample to produce an image. This point-by-point scanning process can result in a substantial acquisition time (from hundreds of milliseconds to seconds) for a single image. Moreover, although the signal is collected only from the focal plane of interest, the excitation beam penetrates the entire sample in the Z-axis, resulting in photobleaching outside of the focal plane, as well as possible phototoxicity to the organism. Light-sheet microscopy addresses both of these issues.

In light-sheet microscopy, the excitation beam has been adjusted to enter the sample from the side (i.e., orthogonal to the objective recording the signal) and therefore excite only the focal plane of interest, substantially reducing the problem of photobleaching. Moreover, the excitation beam occurs in the form of a light-sheet and therefore transects the sample across the entire focal plane. In combination with a fast, sensitive camera, this use of a light sheet rather than point-by-point scanning allows the image to be acquired quickly. Both the reduced photobleaching and quick acquisition allow one to conveniently collect Z-stacks and acquire 3-dimensional renderings with minimal photobleaching and with little or no effect on the living organism.

II. Set up of the Leica SP8 DLS microscope

In standard confocal microscopy, a single objective is used to emit the excitation beam and collect the signal produced from it. By comparison, in light-sheet microscopy, two objectives are involved in the process of producing a light-sheet and recording the florescent signal. In the case of the Leica SP8 DLS setup, an illumination objective below the sample emits a laser light for sample excitation. Instead of hitting the sample, however, it hits a set of polished metal mirrors (TwinFlect mirrors) attached to an imaging objective above the sample. Importantly, the mirrors themselves are positioned at the same level as the sample. The first mirror deflects the beam to the opposite mirror in a plane orthogonal to the optical axis of the microscope, and a scanner moves the beam across the first mirror to produce a light sheet, which enters the sample and illuminates only the focal plane of interest. Emission from the illuminated plane enters the imaging objective and is recorded by a detector.

III. Preparation of samples for the DLS microscope

There are two considerations to keep in mind when preparing samples for light-sheet imaging. First, the light-sheet microscope uses a special water-dipping objective (10× or 20×) to collect the signal produced by the light-sheet. Hence, the sample needs to be immersed in water and must be mounted or embedded in a media (e.g., low-melt agarose) with the same refractive index as water. Second, the gap between the polished mirrors is 5 mm. Therefore, the sample width (i.e., sample plus embedding media) should ideally be no more than 2.5 mm. Although the gap can be wider (up to about 5 mm), it becomes increasingly difficult to maneuver the sample without hitting the mirrors with it. By contrast, since the sample can be moved along this groove between the mirrors, the sample length is restricted only by the dimensions of the sample container.

In general, a 35-mm imaging dish with a 20 mm #1.5 glass base works well with the DLS set-up (Figure 1A). The cover-slip at the dish bottom allows the sample to be imaged by the light-sheet microscope, and the dish can hold both the sample/embedding media (e.g., agarose) and immersion media (water). Here, we describe two methods of preparing samples using glass-base imaging dishes and low-melt agarose (1-2%). In both protocols, transfer pipettes are used for the addition, transfer, and/or mixing of samples, media, or water.

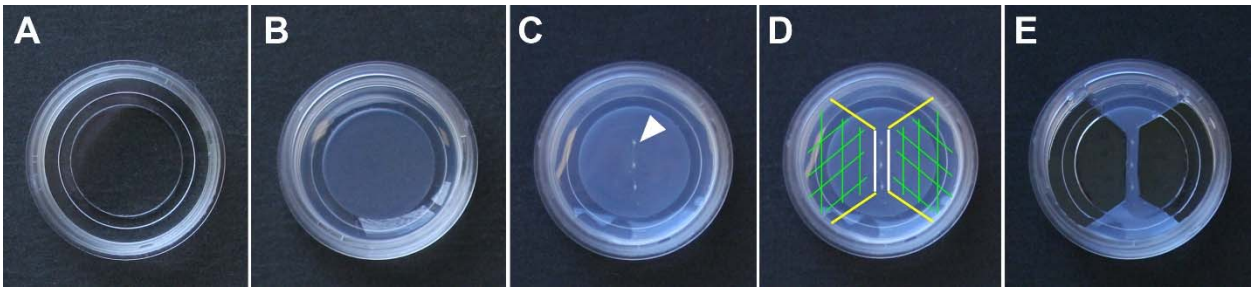

Figure 1. Sample preparation using agarose as the embedding medium. A. A 35-mm glass-base imaging dish. B. A 1-2 mm layer of 1% agarose has been poured into the bottom of the glass-base imaging dish and allowed to set. C. Three samples (zebrafish embryo) arranged in a line and centered within the dish. D. Cut-lines for preparing the samples for light-sheet imaging. E. Final appearance of the dish following the removal of unneeded agarose.

In the first method, the sample is embedded in low-melt agarose within the dish, and unneeded agarose is subsequently trimmed away. As a first step, the dish is filled with 1-2 mm of warm low-melt agarose as a foundation for the samples (Figure 1B). After the agarose sets (5-10 minutes), two to three specimens (e.g., zebrafish) and enough agarose (2-3 mm) to cover them are added to the dish. The samples can be added directly after adding the second measure of agarose or added to the agarose in a 1.5-mL microcentrifuge tube prior to adding it to the dish. When working with live samples, one needs to make sure the temperature of the agarose solution does not exceed 37°C. Under a dissecting scope, a single-hair brush (or similar device) is then used to position the samples to the middle of the coverslip and align them in single row (Figure 1B, white arrowhead). If relevant, the samples should be oriented along the dorsal-ventral axis so that the region of interest is facing upwards. It is important to do this quickly before the agarose hardens.

The next step is to remove the low-melt agarose until the samples are positioned in a narrow strip of it (~2.5 mm) within the center of the sample. This narrow strip contains the embedded samples and will be

fit between the TwinFlect mirror set. To remove the agarose, use a scalpel with a blade (blade size of 11 to 22) to incise two parallel lines on either side of the samples (Figure 1D, white lines). Ideally, the distance between these lines should be no more than 2.5 mm. Importantly, these lines should not extend to the rims of the dish. Instead, about 5-10 mm from the rim on each side, the cut lines should be angled out on both sides of the strip to produce a triangular anchor for stabilizing the interior strip (Figure 1D, yellow lines). Once the cuts are made, cross-hatch incisions can be made within the agarose to be disposed of (Figure 1D, green lines). As you cut through the agarose, you will notice that is quite fragile. Care should be taken to prevent the middle strip from becoming dislodged from the dish surface. If loose agarose remains and becomes difficult to remove, a hand pipette can be used to add water and then pull the water back up into it. By repeating this process, one can remove excess floating agarose. Once the sample has been prepared (Figure 1E), it can be mounted on the microscope stage, and double-distilled water can be added to the dish (to a level of ~¾ of the dish depth) to ensure the TwinFlect mirrors are fully immersed in water.

In the second method, the samples are embedded in FEP (fluorinated ethylene propylene) tubing with the FEP tubing affixed to the rims of the dish using TwinSeal or vacuum grease. With this technique, it is necessary to add the samples to melted agarose in a 1.5-ml microcentrifuge tube (or similar container). As a first step, cut off a length of FEP tubing slightly shorter than the internal diameter of the imaging dish. The samples can be pulled up into the FEP tubing by mounting the tubing on a 200 µL yellow pipette tip and attaching the tip to a 200 µL pipette. Air bubbles inside of the tubing should be avoided since they can interfere with image acquisition. Once the agarose has set, place mixed TwinSeal or vacuum grease on opposite ends of the dish and position the FEP tubing so that its ends are affixed/embedded in the anchoring material and centered within the dish (Figure 2). As with the previous method, water can be added to the dish once it is placed on the microscope stand. FEP tubing is highly suitable for DLS owing to its refractive index (1.344) closely matching that of water and to its low level of autofluorescence in the green and red channels.

Figure 2. Sample preparation using FEP tubing and Twinseal.