Chapter 9

3D Imaging for Cleared Tissues and Thicker Samples on Confocal and Light-Sheet Microscopes

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Abstract

Advances in fluorescence microscopy, specifically the development of confocal and light-sheet microscopes, have enabled researchers to harness tissue clearing techniques to image-stained intact tissue samples in 3D. Using these techniques, tissue structure and biomarker distributions in 3D structures are preserved, thus allowing researchers to gain a wealth of spatial information about their tissue of interest. However, the execution of imaging these larger tissue samples can be challenging. Broadly speaking, tissue clearing techniques unify the refractive indices inside tissue samples, thus enabling deep tissue imaging on a confocal or light-sheet microscope. Here, we provide an overview to tissue clearing and 3D immunohistochemistry staining in general and discuss some difficulties that researchers may encounter when using these techniques. We then focus on imaging CLARITY-processed samples with both confocal and light-sheet microscopes and optimizing the acquisition parameters, before noting potential issues that may come up in imaging.

Key words CLARITY, 3D IHC, Tissue clearing, Confocal microscopy, Light-sheet fluorescence microscopy, 3D imaging, immunohistochemistry

1 Introduction

At first glance, the 3D acquisition of whole or thicker tissue samples appears to be a simple task: Place a sample in the microscope, set your boundaries and z-stack, and acquire the image. However, because of tissue complexity, prior to image acquisition, several steps need to be executed properly to ensure quality imaging. These steps include sample fixation, tissue clearing, immunostaining (if necessary), and image acquisition [1–3]. There are various challenges associated with each of these steps. Failure to address all of these challenges can negatively affect the quality of your 3D imaging.

Sample fixation is a straightforward process that involves standard fixation with 4% paraformaldehyde (PFA) or 10% neutral buffered formalin (NBF) (*see* **Note 1**). Generally, complete fixation

Alexander E. Kalyuzhny (ed.), *Signal Transduction Immunohistochemistry: Methods and Protocols*,
Methods in Molecular Biology, vol. 2593, https://doi.org/10.1007/978-1-0716-2811-9_9,

of tissue sections or whole organs takes between 16 and 24 h, depending on how thick or permeable the tissue is. Under-fixation results in fragile tissues and loss of biological components (DNA, RNA, and protein), which do not maintain their structure and will decompose over time, while over-fixation can result in increased autofluorescence [4].

Tissue clearing techniques are, broadly speaking, chemical techniques to render tissues transparent. This is done by unifying the refractive indices throughout the tissue by removing light-scattering and/or opaque components (*see* **Note 2**) [1–3, 5–7]. They have gained popularity in recent years due to advances in fluorescence microscopy, enabling higher-resolution imaging into intact biological specimens despite their opacity [1–3, 5, 6].

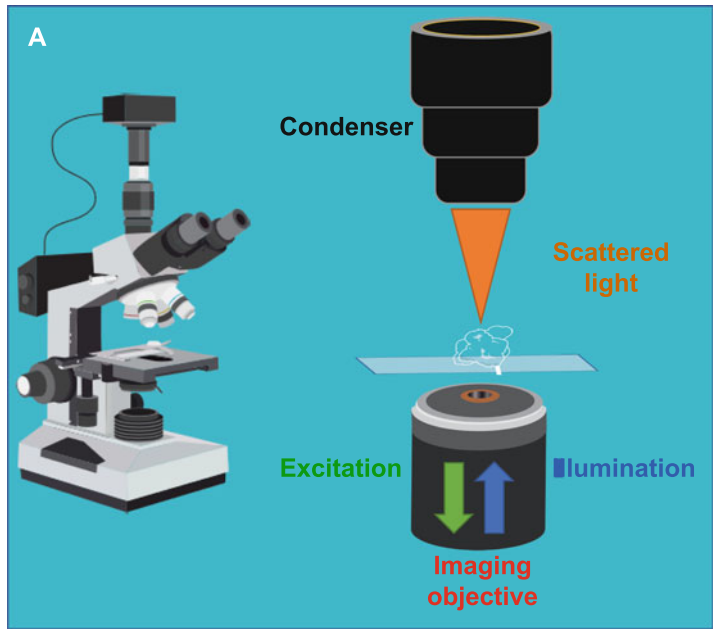
To image-cleared tissues, samples are endogenously and/or genetically labeled or tagged with fluorescent markers or immunostained with antibodies. The fluorescently stained tissues can be imaged with specialized fluorescent microscopes such as confocal, light-sheet, or two-photon. The combination of tissue clearing with fluorescent staining allows information about biomarker distributions and other various spatial information to be captured in 3D without the need for physically sectioning (i.e., cutting) the tissue into thin slices, as is done in traditional histology workflows. Thus, by employing tissue clearing techniques, it is possible to preserve the tissue structure while imaging deeply into the tissue at subcellular resolution, gaining a wealth of spatial information (*see* **Note 3**).

The major challenge in clearing biological samples is determining whether the sample has been successfully and completely "cleared." As previously stated, clearing works to create a uniform refractive index within the sample. This can be done by the removal of light-scattering elements, the use of chemical solvents, or a combination thereof. The final evaluation of whether a sample has been appropriately cleared occurs through imaging of the tissue sample immersed in a refractive index (RI) matching solution. Prior to imaging, some tissue samples which may look completely cleared may in fact be overcleared, leading to structural degradation and signal loss. On the other hand, other tissues may look only semi-transparent, or even opaque, before RI matching and may in fact be completely cleared. In techniques such as CLARITY, only light-scattering lipids are removed during clearing, leaving behind collagen, fibers, extracellular matrix, and other lipids, which can impact how visually clear the samples appear prior to being placed in RI matching solution. Furthermore, clearing times vary not only on the thickness of the tissue sample but also the sample composition [3]. Some tissues may also require additional preprocessing to clearing due to the presence of light-absorbing components such as pigmentation and heme. Hard or calcified tissues will require demineralization (*see* **Note 4**). Furthermore, there are different

clearing benchmarks for imaging on a confocal microscope versus imaging on a light-sheet microscope. This is due to differences in imaging modalities for each platform. Samples that may be cleared well enough for 3D imaging on a confocal microscope (Fig. 1a) may not meet the standard for a light-sheet microscope where the laser must cleanly pass through the entire sample (Fig. 1b).

The main challenges in the immunostaining stage are (1) determining whether the antibodies are compatible with the specific tissue clearing technique and (2) getting uniform, deep penetration of the stain into the thick tissue sample. Similar to Western blots, ELISAs (enzyme-linked immunoassays), and flow cytometry, tissue clearing is another application that requires antibody validation for 3D immunohistochemistry (IHC). Furthermore, since each tissue clearing technique uses a different combination of chemical treatments and workflow, each technique should be considered separate applications within the tissue clearing group. Thus, antibodies need to be validated for the specific clearing method in use. To fully and uniformly stain thicker tissue sections and samples, the staining process may take multiple days depending on the size of the sample. This is because staining is often a diffusion-based process and antibodies are relatively large molecules. Moreover, because the sample sizes are so much larger than what is typically used for traditional IHC workflows, higher concentrations and volumes of antibodies are often necessary. In particular, antibody concentrations must be optimized carefully: overly high concentrations of antibodies may lead to incomplete staining, due to penetration being limited by steric hindrance, while low concentrations of antibodies result in incomplete staining due to antibody depletion [8]. An optimal antibody concentration will be sufficient regardless of varying expression levels within the tissue sample.

The tissue imaging stage, which is our focus here, has a wide variety of challenges as well. First and foremost, the microscope system needs to allow for deep tissue imaging, which requires that the various focal planes can be resolved. Thus, both conventional bright-field and fluorescence microscopy are often insufficient, offering only a limited ability to image beyond the superficial depths into thick tissue samples. 3D deep tissue imaging requires access to a confocal, two-photon, or light-sheet microscope [1–3]. Additionally, though generally not a problem, the microscope objectives must be compatible with the refractive index of the clearing technique. This is extremely important for dipping objectives that will be submerged in the RI solution. To obtain high-resolution images, objectives must not only have high magnification but also have high numerical apertures (NA). However, there is a general tradeoff that higher magnification objectives correspond with lower working distances (WD) of the objective. This limits how thick the tissue sample can be for full acquisition [1]. The microscope stage setup or chamber size can also limit the maximum size of the sample on your imaging platform.



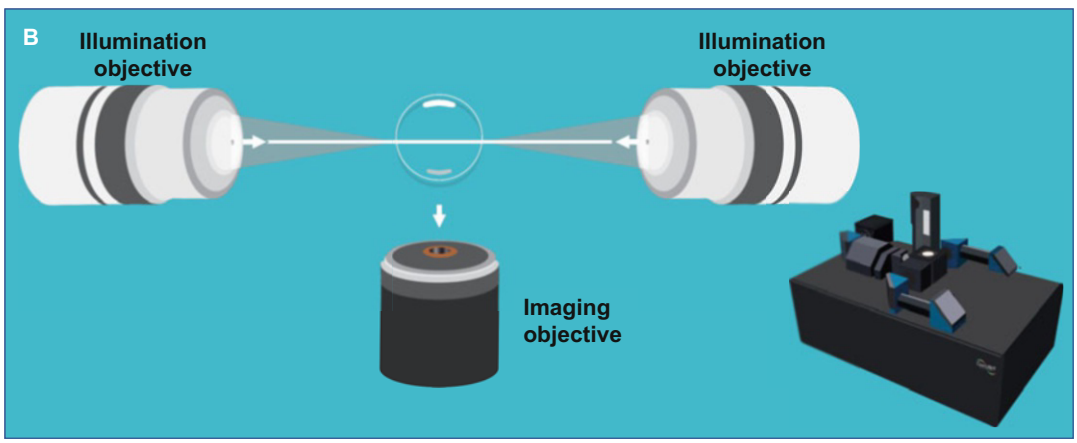


Fig. 1 (**a**) A confocal laser scanning microscope excites a sample by focusing a beam of laser light through one field of view as a time using point-by-point signal acquisition. The beam of light is focused at individual depths one level at a time. The sample is illuminated, and excitation is captured from the same microscope objective. The serial capture of optical focal planes via fluorophores is considered a major advantage and can provide high-resolution and contrast images of biological samples. (**b**) A light-sheet microscope excites a sample by focusing a sheet of laser light through an entire sample. The selective plane illumination minimizes photobleaching, particularly in thicker samples. However, if the sample is not uniformly cleared, then the light sheet cannot pass through the sample unobstructed. As a result, fluorophore excitation and imaging can be affected. Some advantages of light-sheet microscopy include fast image acquisition, minimized photobleaching, and high-resolution images

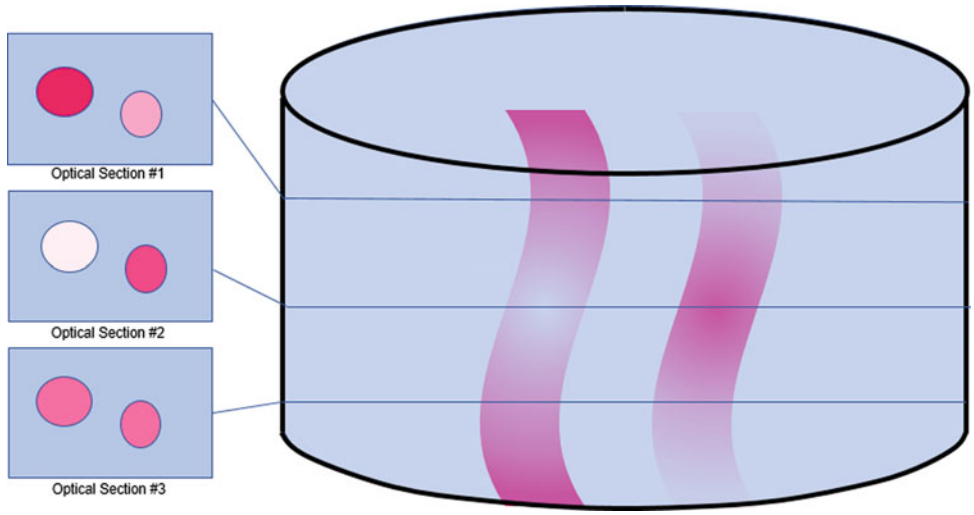


Fig. 2 Biological samples may have areas of variable biomarker expression. The sample variations may occur within a single FOV through the sample depth or across the same focal plane in different FOVs

Finally, while a good signal-to-noise ratio results in crisp images, large tissue samples will often have a wide range of expression levels throughout the sample. To detect the full dynamic range of the sample, it is necessary to survey the entire sample for regions of low signal to determine the minimum laser power and exposure time necessary to capture those regions (*see* **Note 5**), as well as regions of high signal to determine the maxima for laser power and exposure time (*see* **Note 6**). In some cases, large variations in expression level may occur within a single field of view (FOV) or in either the same or different planes of focus (Fig. 2). In these cases, it may be necessary to adjust the display parameters during post-processing to be able to visually appreciate the large range in signal strength. In general, it is easier to make sense of signal variation in more limited regions of interest (ROIs) rather than in larger samples such as whole organs because the differences in signal strength across the ROI tend to be less extreme. Furthermore, having fewer optical slices and smaller ROIs decreases the nonrelevant signal in the image. Additional imaging challenges specific to the biological tissue being evaluated should also be considered (*see* **Note 7**).

2 Materials

Here, we focus on how to image samples that have been cleared using the CLARITY tissue clearing technique, first developed by Chung et al. [9], due to its successful usage in many different tissue types and its high preservation of tissue structural integrity and

signal. We choose to focus only on a single tissue clearing technique, for a reference point, as each tissue clearing technique utilizes different refractive indices to achieve the transparency necessary for deep 3D imaging; however, the steps are broadly applicable:

1. Fixed cleared immunostained tissue sample: 4% PFA or 10% NBF fixation (*see* **Note 1**); tissue clearing application using ClearLight Bio Tissue Clearing Kit stated components: CLARITY Lipid Embedding Solution—high or low lipid (*see* **Note 8**), thermal initiator, wash buffer 1, CLARITY lipid clearing solution, and wash buffer 2. For 3D IHC immunostaining: blocking solution and antibodies (primary and secondary).
2. Refractive index (RI) matching solution: RapiClear CS—SunJin Labs, Hsinchu City, Taiwan.
3. Refractometer.
4. Low-melt agarose 1% or 4%.
5. Leica SP8 confocal microscope (inverted).
6. Luxendo (Bruker company), MuVi SPIM CS light-sheet microscope.
7. 3D visualization or analysis software: Examples include, but are not limited to, Bitplane Imaris, Fiji Image J, or Fiji BigDataViewer.

3 Methods

Before starting the 3D imaging acquisition, there are a few necessary topics to address:

1. Know your microscope. This is essential for establishing the immunostaining multiplex for the cleared tissue sample. Note the laser lines, filters, excitation, and emission abilities of your specific microscope to ensure proper excitation, signal separation, and detection of the endogenous, injected, tagged, or labeled targets of interest.
2. Select the appropriate microscope objective and imaging magnification. All microscope objectives have a NA and WD. It is important to take the thickness of the cleared sample into account when initiating sample imaging. For example, if the sample is 5-mm thick, but a 10x objective with a WD of 3 mm is being used, it will not be possible to image more than 3 mm into the sample due to the microscope objective WD limitation. The NA of the objective will also play a role in imaging resolution (the ability to distinguish between two nearby neighboring features).

3. Choose a suitable magnification based on the sample size and biomarkers of interest. Resolving microstructures and specific cell populations, such as immune cells or neuronal soma, requires higher magnification (20–40×) imaging. However, whole organs and larger cleared tissue samples are often limited to lower magnification (4–8×) imaging due to their size. Thus, smaller structures within the sample cannot be resolved. Higher magnification objectives have smaller FOVs; therefore, a greater number of FOVs are required for the same region at a lower magnification, leading to larger data file sizes.
4. If using a light sheet for imaging, light-sheet alignment may be necessary, as noted by the microscope manufacturer. Prior to light-sheet alignment, it is recommended to check the RI of the RI matching solution in the imaging chamber. This can be done using a refractometer (*see* **Note 9**). If the sample image does not appear as sharp as anticipated, this could be due to misalignment of the light sheets, which may require fine alignment of the light-sheet laser based on your expected signal as noted by your manufacturer. Alternatively, there may be a mismatch in the refractive indices of the sample and RI matching solution in the imaging chamber (*see* **Note 10**).

3.1 3D Imaging on a Confocal Microscope

1. Mount your sample (*see* Fig. 3).
2. Place your sample on a thin glass coverslip (22 × 22 mm). We recommend using #1 thickness (0.13–0.17 mm) for the coverslip. This will allow you to maximize the objective working distance and minimize the amount of glass the laser will need to pass through (*see* **Notes 11** and **12**).
3. Find your sample (Fig. 3). The sample can be easily seen for this imaging platform and thus can be found visually. Manually place the sample over the imaging objective and locate your focal plane by adjusting the imaging depth.
4. Set your scanning region (tile scan vs FOV). A tile scan can encompass an entire sample or focus on selecting multiple concatenated fields of view. To establish your image boundaries, make sure your confocal has been set to "tile scan" for concatenated FOVs. This will result in an imaging area that is a rectangle or square. If you are interested in imaging several FOVs that are scattered throughout your sample and not all connected, then you will want to be in "Mark and Find" or equivalent mode (*see* **Note 13**).
5. Set up your z-stack (i.e., establishing the visible sample thickness). Initially, the sample thickness can be established by scrolling through the sample and setting the points when you can first see your sample. This is the beginning of your z-stack that is typically closest to the imaging objective. Scroll through the

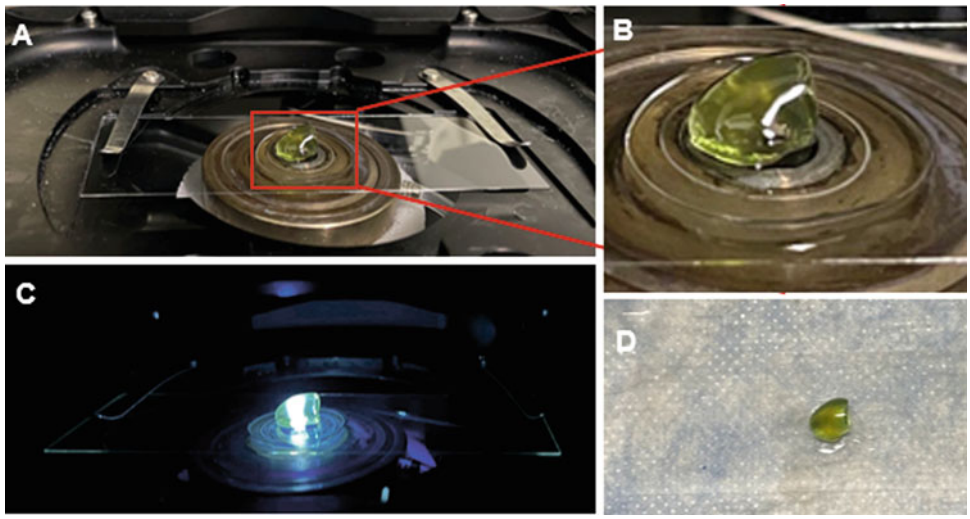


Fig. 3 Sample mounting and imaging on an inverted confocal microscope. (**a**) The cleared sample has been placed on a #1 glass coverslip directly over the imaging objective. (**b**) Red box enlarged. The imaging field is clearly visible, and the sample can be oriented by physically looking at the sample. (**c**) The sample is illuminated by the laser when in the FOV. (**d**) The cover glass must be able to support the size and weight of the cleared sample, and the sample should be placed on the cover glass according to the desired imaging orientation

sample as deep as possible until the signal is lost. This is the end of your z-stack. Once the initial boundaries have been set, you will now go back through the z-stack to review and refine those points. The end of your z-stack should not extend past the point you are unable to differentiate between individual tissue components. Reviewing should proceed in a timely fashion to avoid inadvertent photobleaching of the sample. The confocal microscope allows for laser power or gain adjustments depending on if image acquisition is occurring sequentially or nonsequentially. For nonsequential acquisition (with laser power modification), you can modify both gain and laser power as you move deeper into the sample. Typically, increasing the laser power will result in an increased signal deeper within the sample and will allow for an increase in imaging depth for the z-stack. The ability to moderate the gain (increase or decrease) as the laser power is increased allows for the modulation between the signal-to-noise ratios. For sequential acquisition (with laser power modification), you will only be able to modify the laser power as you move deeper into the sample. Increasing the laser power will result in an increased signal deeper within the sample and will allow for an increase in imaging depth for the z-stack; however, any laser increases should be tempered by overall increases to the FOV background to maintain the best signal-to-noise ratio. In the case where your confocal

microscope utilizes exposure time, a balance between the laser power and exposure time should be established to determine additional changes to the z-stack endpoint.

6. Review your sample tile scan and z-stack to avoid under- and overexposing your sample. Briefly, it is important to scroll through the planned tile scan to ensure the image acquisition settings are not based on an area where the signal is extremely low or extremely bright. 3D image acquisition is about finding the right balance and compromise (*see* **Note 14**). Set your image display to visualize the high/low dynamic range for digital imaging, such as over−/underexposure or heat map. This will provide a clear indicator when your image may be oversaturated and determine the overall signal intensity. Turn on your z-compensation or software equivalent function. Mark the beginning and end of the z-stack, as well as multiple depths in between. For beginners, it is recommended you start with a higher laser power and decrease laser settings, as necessary, while moving deeper into the sample. This may need to be done in 2–3 FOVs if the sample thickness or specific signals vary throughout the sample. If only one FOV is being acquired, you are ready to proceed to image acquisition settings. For larger areas, the entire tile scan will need to be checked. Once the z-compensation stack has been set, the overall sample will need to be reviewed. If your tile scan is small (i.e., 4 × 4 or smaller), individual FOVs can be checked; however, if you are planning on a larger ROI or whole sample imaging, it is recommended that you look at average areas, for example, checking the center of four tiles moving through the whole sample to give a representative view as demonstrated in Fig. 4. Start at the beginning of the z-stack and check each depth for overexposed areas. Reduce the laser power as necessary, but do not increase the laser backup as this may result in oversaturation for a previously reviewed area.
7. Set and/or modify your image acquisition settings. This is the final step before the execution of the 3D imaging tile scan stack. The point of this step is to ensure that the final settings result in a reasonable imaging acquisition that will not negatively affect the sample (*see* **Note 15**). For example, it is highly recommended that imaging acquisition times do not exceed 24 h and the final file size is manageable for the available computing workstations. On the confocal microscope, several factors affect the overall acquisition time. Imaging depth and tile scan area were previously set and are determined by the sample and areas of interest. Sequential imaging and nonsequential imaging are based on the sample multiplex. Other factors that affect the acquisition time are resolution format (512 × 512, 1024 × 1024, 2048 × 2048, etc.), imaging speed, line average, and step size. Step size is the least complex variable

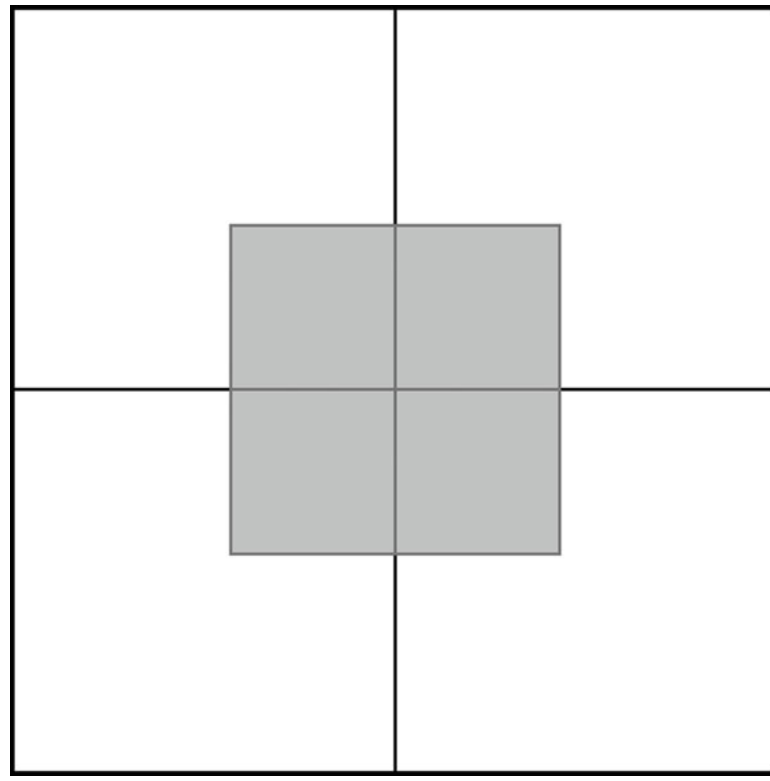


Fig. 4 Example of tile scan imaging review to expedite image review process. The white squares represent individual FOVs in the tile scan. The gray square represents the placement of the region reviewed for the sample

to adjust. Increasing the step size will decrease the acquisition time by reducing the number of slice images taken per z-stack. Utilizing the microscope system's optimized step size is a good place to begin if you are new to confocal imaging, but the larger the sample or imaging area and/or depth, the greater the step size will need to be. If the acquisition time remains long after modifying the step size, revisit the sample tile scan size and/or imaging depth for further reduction. These factors also impact final file size (*see* **Note 16**).

8. Initiate 3D sample imaging and check image progress. Air objectives will rarely present issues; however, if water or oil objectives are utilized, it will be important to maintain the necessary levels for contact between the objective and cover glass. Prolonged imaging with lasers will affect the water and oil levels. For water objectives, the use of a micropump or replenishing system will be essential. Oil objectives will not evaporate as quickly, but the viscosity of the oil may be compromised with prolonged imaging. We recommend assessing this aspect for your system prior to performing sample acquisition.

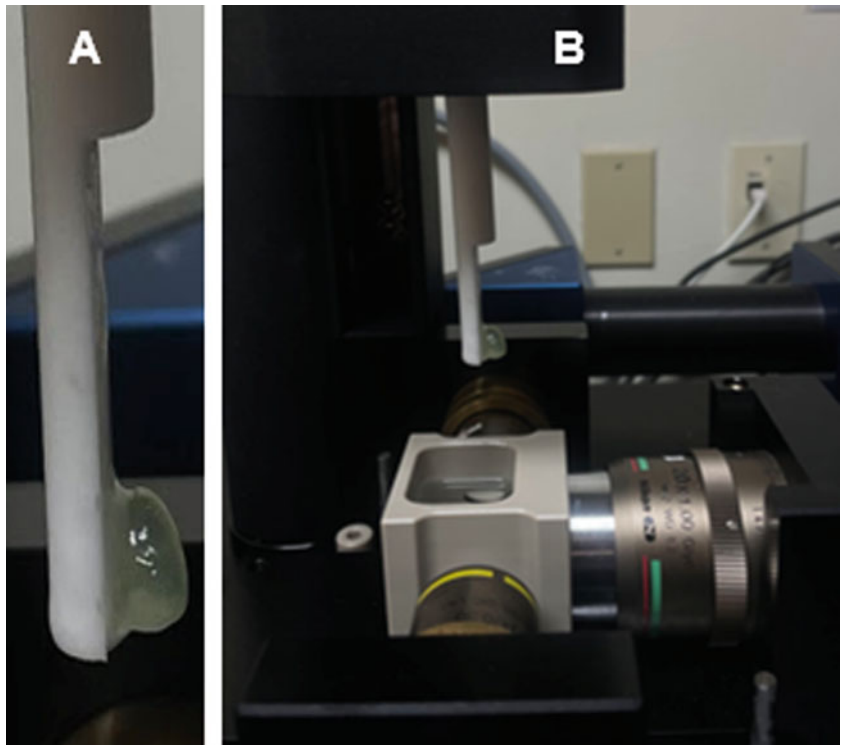


Fig. 5 Sample mounting and imaging on MuVi SPIM light-sheet microscope. (**a**) Cleared sample is solidly adhered to the sample mount using 1% or 4% low-melt agarose. (**b**) The sample mount is vertically inserted into the sample mount holder and will be lowered into the imaging chamber

9. Image process and visualize your 3D image. Some confocal software allows for tile stitching after the image acquisition; however, your data will need to be uploaded into a 2D/3D visualization software for subsequent analysis. A review of the tile scan stitching is always recommended.

3.2 3D Imaging on a Light-Sheet Microscope

1. Mount your sample (Fig. 5). Attach your sample to the sample mount using either 1% or 4% low-melt agarose (*see* **Notes 11** and **17**). Use a thin layer of agarose as your adhesive. The higher the agarose percentage, the quicker the agarose will set (*see* **Note 12**). The sample will be immersed in a chamber filled with your refractive index solution of choice. For CLARITY-cleared samples, the RI is ~1.54.
2. Find your samples (Fig. 6). The sample will be placed in a chamber that is often obscured by either the imaging objective or the sample mount. To find your sample, you will need to make sure the sample is placed within the laser light sheet. This can initially be eyeballed for placement before lowering the samples into the chamber; however, you will need to use the

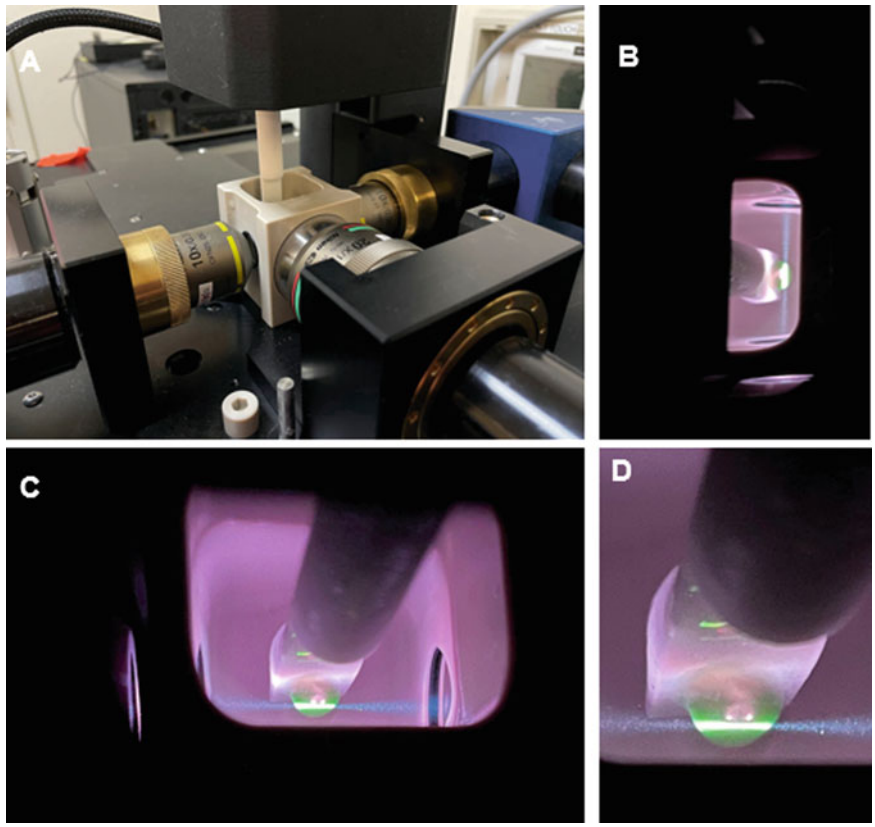


Fig. 6 Locating your sample in the MuVi light sheet. (**a**) Sample placement is obscured by the light-sheet illumination and imaging objectives on the sides and by the sample mount holder from the top. (**b**) Selecting angles from above the sample chamber may allow you to locate your sample. (**c**) One single light sheet should be visible when correctly aligned. The sample will need to be in the light sheet to see it through the imaging objective and on the imaging monitor. (**d**) The light sheet should be able to pass through the sample unobstructed

imaging objective to locate the sample. In certain light-sheet setups, the sample is placed in the imaging chamber first, and the dipping imaging objective is lowered on top of the sample.

3. Set your scanning region (tile scan vs FOV). A tile scan can encompass an entire sample or focus on selecting multiple concatenated fields of view. To establish your desired boundaries on a light sheet, you will set a z-stack and mark your X and Y boundaries by moving your sample to the desired location within the imaging objective and save the locations within the z-stack window. Move the sample in X and Y along the marked image boundaries, making sure that the area of interest is captured in the tile scan. Find the appropriate X and Y locations for the forward-most Z plane of the sample, and check that the foremost point of the sample (farthest from the mount) is included in the z-stack. At both edges of the sample in Y,

check the other end of Z (closest to the mount) to ensure the desired thickness of the sample is captured while avoiding imaging the mount (*see* **Note 18**).

4. Set up your z-stack (i.e., establishing the visible sample thickness). Initially, the sample thickness can be established by scrolling through the sample and setting the points when you can first see your sample. This is the beginning of your z-stack that is typically closest to the imaging objective. Scroll through the sample as deep as possible until the signal is lost. This is the end of your z-stack. Once the initial boundaries have been set, you will now go back through the z-stack to review and refine those points. The end of your z-stack should not extend past the point you are unable to differentiate between individual tissue components. Most light sheets utilize exposure time; a balance between the laser power and exposure time should be established to determine additional changes to the z-stack endpoint.
5. Review your sample tile scan and z-stack to avoid under- and overexposing your sample. Briefly, it is important to scroll through the planned tile scan to ensure the image acquisition settings are not based on an area where the signal is extremely low or extremely bright. 3D image acquisition is about finding the right balance and compromise (*see* **Note 14**). When reviewing your z-stack tile scan on the light sheet, the display histogram will be the reference point to establish the signal-to-noise ratio for your biomarker of choice. Each channel will need to be reviewed. To modify the signal appropriately, adjust the laser and/or exposure time(s). Increasing the exposure time will impact the overall image acquisition time. Be mindful of increases to laser power as the lasers will be traveling through the imaged sample (Fig. 7). The necessary imaging channels should have been added following light-sheet alignment with the appropriate calibration for each channel (*see* **Note 19**). Moving through the sample in X, Y, and Z, check various areas in the sample in all of the channels to ensure that the laser and exposure settings are appropriate for the entire sample.
6. Set and/or modify your image acquisition settings. This is the final step before the execution of the 3D imaging tile scan stack. The point of this step is to ensure that the final settings result in a reasonable imaging acquisition that will not negatively affect the sample (*see* **Note 15**). For example, it is highly recommended that imaging acquisition times do not exceed 24 h and the final file size is manageable for the available computing workstations. Imaging on a light sheet is known to produce very large datasets on the order of terabytes. Depending on your workstation memory and storage capacities, this can be detrimental to image processing and analysis

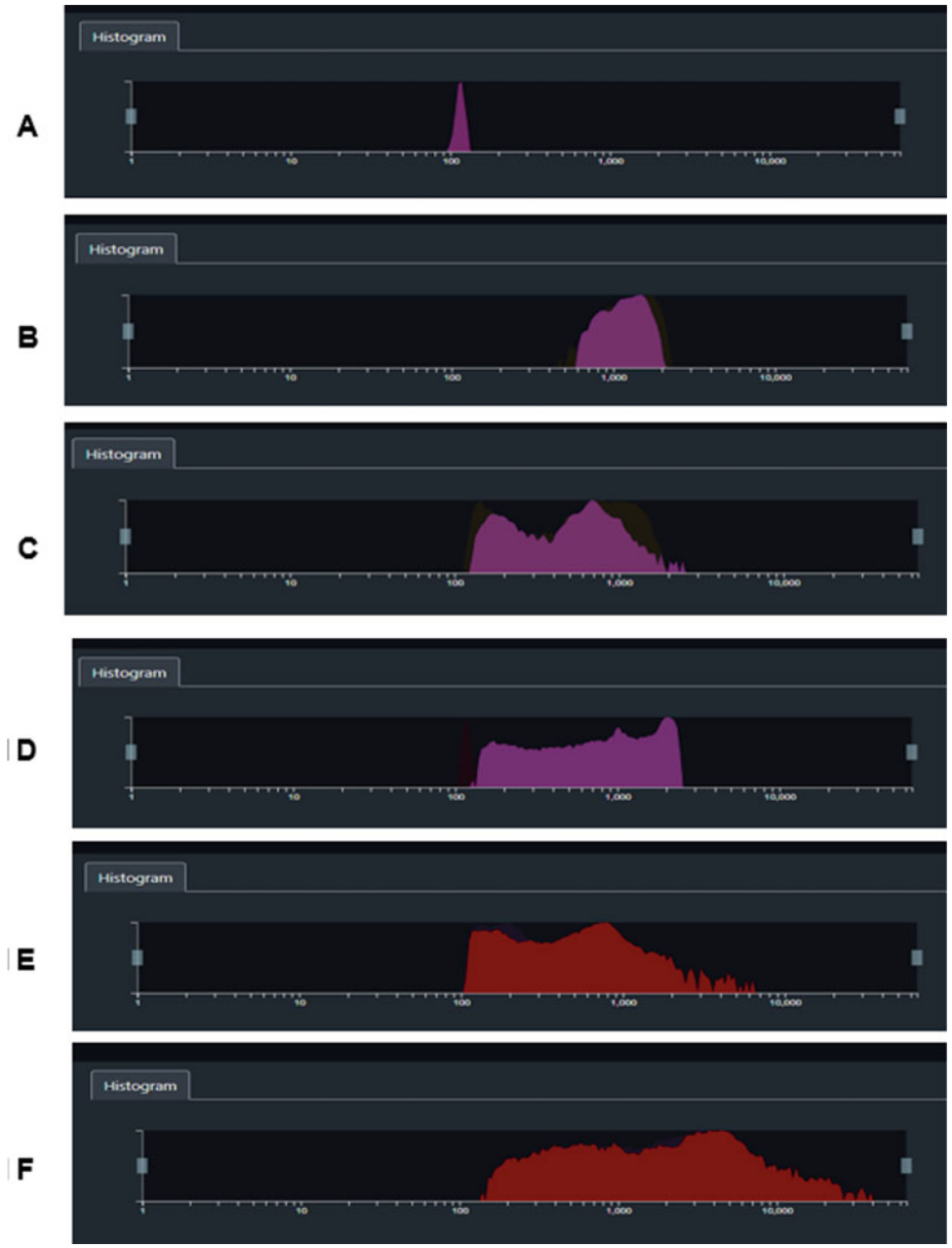


Fig. 7 Determining your image acquisition settings on a light sheet using histograms. (**a**) A very low signal is shown. This is typically a background signal. (**b**) Good sample signal is demonstrated with no background noise. (**c**) This histogram has better signal with good signal-to-noise separation. (**d**) A high background is observed with no signal-to-noise separation visible. (**e**) There is good biomarker signal; however, higher background noise is present. (**f**) The sample is possibly overexposed. A high background is observed, no signal-to-noise separation is present, and signal expression is very high

post-acquisition. For example, if the initial acquisition settings are estimated to produce a 10 TB dataset, and that is beyond your system capabilities, it would be prudent to adjust the final settings accordingly. Increasing the step size, adjusting the FOV overlap, and/or reducing the depth for the z-stack are all helpful and simple alternatives (*see* **Note 16**).

7. Initiate 3D sample imaging and check image progress. A sample will need to be properly mounted in your light sheet. Periodically check on the image acquisition to make sure your sample has not fallen off your mount or is shifting during image acquisition.
8. Image processing and visualizing your 3D image. Most light-sheet software has specific software built to process the large datasets that are generated. Downsampled datasets should be generated initially to check the quality of the image acquisition. If the downsampled dataset appears in order, proceed to the full-resolution dataset for further downstream analysis.

4 Notes

1. Deviations from the noted fixation methods can be impactful on 3D IHC imaging. For example, other fixative alternatives may interfere with the tissue lipid clearing, which may result in hazy imaging at increased tissue depth or impede antibody penetration into the tissue samples.
2. Various tissue clearing techniques have been developed since the first tissue clearing protocol was published in the early 1900s by Werner Spalteholz [10], and each has their own strengths and weaknesses [2, 5, 6]. Organic solvent-based tissue clearing techniques, such as the 3DISCO/iDISCO/uDISCO (3D Imaging of Solvent-Cleared Organs) family [11–14], BABB (Benzyl Alcohol and Benzyl Benzoate) [15], and PEGASOS (PEG-Associated SOlvent System) [16], aggressively clear tissues but have been noted to shrink the tissue samples, due to the need to dehydrate the samples, and compromise endogenous fluorescent signals. Aqueous tissue clearing techniques, such as the CUBIC (Clear, Unobstructed Brain/Body Imaging Cocktails) family [17–21] and the Sca*l*e/Sca*l*eS/Sca*l*eA2 (a hyperhydration tissue clearing technique that uses detergents and sorbitol) family [22, 23], typically preserve endogenous fluorescence better than organic solvent-based techniques but take much longer to clear tissues, sometimes resulting in under-cleared tissues. The third class of clearing techniques are hydrogel-based techniques, which include CLARITY (Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging/Immunostaining/In situ hybridization-

compatible Tissue hYdrogel) [9], PACT (PAssive Clarity Technique) [24, 25], and SWITCH (System-Wide control of Interaction Time and kinetics of CHemicals) [26]. These are generally compatible with endogenous fluorescence, and the incorporation of the hydrogel scaffold helps maintain tissue structural integrity; however, these methods also tend to take longer than solvent-based clearing techniques and may result in sample expansion.

3. Recent advances in fluorescence microscopy, namely, the development of confocal and light-sheet microscopes, have enabled wider adoption of tissue clearing; these imaging modalities allow thin focal planes to be captured with minimal signal from out-of-focus planes (i.e., optical sectioning). This enables crisp 3D images to be assembled and rendered [1, 3].
4. Preprocessing treatments meant to minimize light-blocking or light-absorptive elements often involve harsh chemicals and can damage the tissue sample and/or quench endogenous signals if not handled carefully [1, 2].
5. Underexposure of the sample during image acquisition cannot be effectively fixed with post-processing. Increasing the signal during post-processing increases the sample background overall and may make it difficult to create a good separation between the true signal and the background noise.
6. Overexposed or over-saturated image acquisitions cannot be fixed post-acquisitionally. In such images, neighboring pixels cannot be differentiated, and there will be loss in the overall signal dynamic range in the area.
7. Tissues will often have some amount of autofluorescence from various biological components, including mitochondria, collagen, elastin, and red blood cells [4]. To determine and account for the baseline level of autofluorescence background, control tissues which have not undergone staining can be used. The biological components of red blood cells cause them to naturally autofluorescence over multiple common laser wavelengths [4]. Sample perfusion is the best approach to reduce the presence of red blood cells; yet, in instances when that is not an option, note they are easily identifiable by the size, shape, grouping, and full spectrum autofluorescence [4]. However, autofluorescence can be useful to visualize the sample shape and vascularization or to maintain orientation when viewing the tissue sample without needing to specifically stain for landmark biomarkers.
8. When clearing samples using CLARITY, there are different hydrogel formulations that can be used based on the lipid content of the sample. The CLB Tissue Clearing kit recommends the High Lipid kit for tissues such as embryo, brain,

lymph nodes, calcified tissues, organoids, eyes, tonsil, cell pellets, and spinal cord and the Low Lipid kit for the lung, kidney, skin, cancerous pancreas, liver, intestine, muscle, and heart tissues.

9. Some detection objectives have an RI correction collar that can be adjusted according to the RI measured on the refractometer allowing crisper alignment and imaging.
10. To check if the refractive indices of the sample and the solution in the imaging chamber are the same, use a refractometer to measure each of their respective indices. Ideally, the values of the RI matching solution in the imaging chamber and the RI matching solution the sample was in will be the same or within 0.005 of each other.
11. Make sure your samples are immobilized prior to image acquisition. If sample drifting, wiggling, shaking, or general instability is apparent during setup, this will affect the imaging acquisition. Sample movement during image acquisition will be apparent on the final 3D image.
12. When mounting the sample on a cover glass or light-sheet sample mount, avoid leaving bubbles in the RI matching solution or agarose under or on the sides of the sample as they will be visible in the acquisition and impact the quality of your image.
13. When using "Mark and Find," only the marked FOVs will be imaged. In the case of neighboring FOVs, it is recommended that FOVs overlap to allow for better alignment. In "tile scan" mode, most microscope software default settings include 10–20% overlap between FOVs; however, this is not a default in "Mark and Find."
14. If the image settings are based on an area with weak signal, there is an increased risk for overexposure for most of your samples, particularly if you are imaging a large area. Similarly, if the image acquisition settings are set based on the brightest signal, the risk of underexposing the sample will increase.
15. Large samples involve long imaging times, especially for confocal microscopes which must raster scan over every focal plane to acquire the image data [1–3]. For this reason, photobleaching and sample overheating may become a concern. Generally, the laser power chosen for excitation of the fluorophores should be as low as possible while still maintaining a good signal-to-noise ratio. For weak endogenous signals, signal amplification may be required for robust detection. Low laser power is often paired with longer exposure time; however, lengthening exposure time can also affect the overall acquisition time. Long acquisition times may also result in

evaporation of the immersion water for water objectives or refractive index matching solution in the imaging chamber for light-sheet microscopes.

16. Impacts on data size are related to the following: tile scan size, magnification (which impacts tile scan size), step size, and file format (resolution).
17. Larger samples, for example, whole mouse brains, can be too heavy for 1% agarose and may slide off the mount.
18. Including the mount in the z-stack will result in undesirable autofluorescence in the 3D image that will likely obscure the ability to visualize the samples and markers of choice.
19. Different laser wavelengths can have an optical shift requiring different calibration settings to ensure the beam waist is in the FOV; this becomes especially relevant when looking at UV, visible light, and infrared on the same sample.

References

1. Richardson DS, Lichtman JW (2015) Clarifying tissue clearing. Cell 162(2):246–257
2. Jing D, Yi Y, Luo W et al (2019) J Dent Res 98(6):621–631
3. Gómez-Gaviro MV, Sanderson D, Ripoll J et al (2020) Biomedical applications of tissue clearing and three-dimensional imaging in health and disease. iScience 23(8):101432
4. Whittington NC, Wray S (2017) Suppression of red blood cell autofluorescence for immunocytochemistry on fixed embryonic mouse tissue. Curr Protoc Neurosci 31:2.28.1–2.28.12
5. Ueda H, Erturk A, Chung K et al (2020) Tissue clearing and its applications in neuroscience. Nat Rev Neurosci 21:61–79
6. Tian T, Yang Z, Li X (2020) Tissue clearing technique: recent progress and biomedical applications. J Anat 238(2):498–507
7. Ariel P (2017) A beginner's guide to tissue clearing. Int J Biochem Cell Biol 84:35–39
8. Chen Y, Shen Q, White SL et al (2019) Three-dimensional imaging and quantitative analysis in CLARITY processed breast cancer tissues. Sci Rep 9:5624
9. Chung K, Wallace J, Kim SY et al (2013) Structural and molecular interrogation of intact biological systems. Nature 497:332–337
10. Spalteholz W (1914) Über das durchsichtigmachen von menschlichen und tierischen präparaten und seine theoretischen Bedingungen, nebst Anhang: Über Knochenfärbung. S. Hirzel, Leipzig
11. Ertürk A, Becker K, Jahrling N et al (2012) Three-dimensional imaging of solvent-cleared organs using 3DISCO. Nat Protocols 7:1983–1995
12. Renier N, Wu Z, Simon D et al (2014) iDISCO: a simple, rapid method to immunolabel large tissue samples for volume imaging. Cell 159(4):896–910
13. Pan C, Cai R, Quacquarelli FP et al (2016) Shrinkage-mediated imaging of entire organs and organisms using uDISCO. Nat Methods 13:859–867
14. Cai R, Pan C, Ghasemigharagoz A et al (2019) Panoptic imaging of transparent mice reveals whole-body neuronal projections and skull-meninges connections. Nat Neurosci 22:317–327
15. Dodt HU, Leischner U, Schierloh A et al (2007) Ultramicroscopy: three-dimensional visualization of neuronal networks in the whole mouse brain. Nat Methods 4:331–336
16. Jing D, Zhang S, Luo W et al (2018) Tissue clearing of both hard and soft tissue organs with the PEGASOS method. Cell Res 28:803–818
17. Susaki EA, Tainaka K, Perrin D et al (2014) Whole-brain imaging with single-cell resolution using chemical cocktails and computational analysis. Cell 157(3):726–739
18. Tainaka K, Kubota SI, Suyama TQ et al (2014) Whole-body imaging with single-cell resolution by tissue decolorization. Cell 159(4):911–924

19. Kubota SI, Takahashi K, Nishida J et al (2017) Whole-body profiling of cancer metastasis with single-cell resolution. Cell Rep 20(1):236–250
20. Murakami TC, Mano T, Saikawa S et al (2018) A three-dimensional single-cell-resolution whole-brain atlas using CUBIC-X expansion microscopy and tissue clearing. Nat Neurosci 21:625–637
21. Tainaka K, Murakami TC, Susaki EA et al (2018) Chemical landscape for tissue clearing based on hydrophilic reagents. Cell Rep 24(8): 2196–2210
22. Hama H, Kurokawa H, Kawano H et al (2011) Scale: a chemical approach for fluorescence imaging and reconstruction of transparent mouse brain. Nat Neurosci 14:1481–1488
23. Hama H, Hioki H, Namiki K et al (2015) ScaleS: an optical clearing palette for biological imaging. Nat Neurosci 18:1518–1529
24. Tomer R, Ye L, Hsueh B et al (2014) Advanced CLARITY for rapid and high-resolution imaging of intact tissues. Nat Protocols 9:1682–1697
25. Yang B, Treweek JB, Kulkarni RP et al (2014) Single-cell phenotyping within transparent intact tissue through whole-body clearing. Cell 158:945–958
26. Murray E, Cho JH, Goodwin D et al (2015) Simple, scalable proteomic imaging for high-dimensional profiling of intact systems. Cell 163:1500–1514