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Imaging in focus

Massive volumetric imaging of cleared tissue: The necessary tools to be successful

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ABSTRACT

Recent technological innovations in high-speed microscopes, tissue clearing methods, optics and computation have made it possible to image enormous biologic volumes. These techniques are applicable to a broad range of biomedical disciplines and are becoming a necessary tool for exploring biologic systems that extend beyond cellular microenvironments to entire organs and organisms. Here we broadly discuss the tool sets that are available for cleared tissue imaging and how various choices may influence the planning and feasibility of an experiment.

1. Going big because we can

Much like genomics in the late 1990's began to look past the function of individual genes in favor of global genomic analysis, biologists of all disciplines are beginning to move beyond single cellular and molecular questions to interrogate whole organs and biologic systems. This type of 'big picture' interrogation places into context how organ and organismal systems function collectively. As we are roundly in the era of systems biology, the need to reach beyond traditional boundaries of scientific inquiry is requiring less justification. This applies to all biologic disciplines as we collectively examine the importance of neuronal connectivity within the brain and within the whole body, the distribution and trafficking of immunological populations, the development of complex organs, the mechanisms of infectious disease dissemination and the characterization of rare cell populations to name just a few examples.

The microscope, a most fundamental tool of biologic investigation, has long been at odds with this approach. As a matter of function, the microscope is restricted to probing only the smallest regions of tissue, and for most of its history even this has been a slow and manual process. In the last five years, technology has come of age that enables fast, deep and automated acquisition of very large volumes. These technologies include fast imaging platforms, improved tissue clearing approaches and robotics for controlling all aspects of acquisition. Equally important is the continuing increase in computation and storage capabilities that is vital to supporting data that scale in lockstep with increasing volume. No doubt, massive volumetric imaging will become accessible if not common place for all biologic disciplines in the coming 5 years, much like the increase in the penetrance of high throughput sequencing. This mini review will broadly address the considerations that should be made when curating equipment and designing experiments for large volumetric imaging (ie whole organs/organisms). Specifically, the focus will be on cleared tissue imaging which offers the most flexibility and accessibility to researchers as they begin imaging large volumes.

2. Imaging platform(s)

The cohort of tools that are required to image very large volumes extends far beyond a particular imaging platform. However, the platform that is chosen will pigeon-hole every other parameter of acquisition; including speed, size of sample, depth and resolution. Most modern confocal and multiphoton microscopes are equipped with a robotic sample stage for accurate large area acquisition, and nearly every one of these scopes can accommodate a low-power, low-numeric aperture (NA), long working distance air-objective which is a minimum requirement for most large volumetric acquisitions. Thus, most platforms can be used for large volumetric acquisition. However, at the speed of a standard laser line scanning, acquisition of a single-color whole organ the size of a mouse brain would require at least 1 week at cellular resolution (voxels of $2 \times 2 \times 20\,\mu m$). This makes speed a primary consideration when choosing an imaging platform. Speed is most often achieved through accelerating line scanning with a resonant mirror, widefield psudoconfocal techniques or selective plane light sheet illumination microscopy (SPIM) (Santi, 2011; Watkins and St Croix, 2018) that take advantage of high-speed digital cameras

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(Watkins and St. Croix, 2013). Each of these platforms has advantages/disadvantages that include speed, resolution and ease of use.

3. Resolution

Resolution can be one of the most difficult aspects to rationalize when designing an experiment. Researchers tend to overestimate what is required for their task. Since resolution is the ability to distinguish two objects it is helpful to think of the problem in terms of how compacted and complex in integration are the structures that you wish to distinguish. When determining the trajectory of a sparsely labeled neuron over distance, the required resolution may be surprisingly low, perhaps an order of magnitude lower than the smallest dimension of the neuron itself. The resolution required to distinguish densely packed cell bodies may require resolution near the diffraction limit – similar when sub-cellular localization of structures is required. The required resolution for a given experiment is often best determined empirically. Each of the major microscope manufacturers have excellent online guides which describe the principles of resolution and the importance of proper sampling.

Higher resolution is not always better. This decision should not be considered trivial. As resolution increases so does the time required to acquire the volume by the third power. As in the example above, decreasing the voxel dimensions by a factor of two ($1 \times 1 \times 10\,\mu m$) would push acquisition time on a standard line scanning confocal to 8 weeks. Additionally, the file size of the imaging data will increase by the same factor. Even if you have unlimited time at your disposal, extended scan times can lead to sample deterioration, signal quenching, sample movement and a greater risk of hardware or software malfunction leading to an unwelcome abort of acquisition.

4. Objectives

Since the point of cleared tissue imaging is to look deep into a specimen, a primary requirement is that you use a long working distance objective (for example 5–7 mm for a mouse brain). Nearly all confocal systems will support long working distance air optics. However, the most important factor that determines resolution is the NA of the detection objective(s). Since NA is essentially the open angle of light collection, to be maintained over greater working distances objectives must become larger. To reduce refractive index (RI) difference between the sample and the objective, specialized objectives generally use glycerol as a transition fluid which has a RI of n = 1.47 and is a midpoint for common tissue clearing solutions (n = 1.38–1.56). The RI of glycerol is a healthy compromise between maximizing effective NA and reducing spherical aberration due to a RI mismatch which can be adjusted for by a RI-specific correction collar. Many clearing objectives have ceramic endcaps which allows for direct dipping in potentially corrosive organic solvent-based clearing solutions which make very effective clearing options due to high RI. Another feature of these clearing objectives is that they are generally low magnification (2–25x). This is a prime advantage as it allows acquisition of a low-resolution overview with the same objective that is used to capture the final high-resolution image. The use of long working distance immersion objectives generally requires an upright microscope design which allows the samples to be fully covered in relatively large volumes of solution.

5. Clearing technologies

A diverse array of tissue clearing technologies are available. The field is in a renaissance, and increasingly refined clearing protocols appear regularly in the literature. In fact the choice may be daunting for a novice to the approach so it is suggested that guides are consulted to understand the options that will work best for their applications (Ariel, 2017; Muntifering et al., 2018; Richardson and Lichtman, 2015). Briefly, clearing results when the RI is equalized across a tissue and with an immersion solution. This is most often accomplished by removing lipids in favor of preserving a protein meshwork which has a predictable RI ranging from 1.4 and 1.5. Clearing methods that preserve lipids exist (Ke et al., 2013) but are characteristically less effective at RI matching due to the increased molecular heterogeneity and the required use of clearing solutions with lower RI. Clearing protocols broadly fall into two categories. Dehydration methods clear by using organic solvents to remove lipids and match RI whereas water-based methods remove lipids and clear using aqueous solutions and detergents. Dehydration can cause tissues to shrink up to 8-fold from physiologic size, but these methods are generally the best options for rendering tissues optically clear due to the high RI of the final clearing solutions. Water-based clearing methods are generally better at preserving endogenous fluorescent proteins and in some circumstances can cause tissues to inflate up to 150%. The change in tissue size is an important factor when choosing the imaging system and resolution that is required. When tissues physically change size, much like with expansion microscopy (Wassie et al., 2019), the power required to resolve structures within those tissues changes proportionately. This will influence the calculus around what imaging systems, resolutions and optics must be employed to answer the question at hand.

6. Data acquisition, storage and processing

Large volume imaging produces massive datasets. Careful thought must be given to how data will be moved, stored and processed. A single color, 16 bit, $2 \times 2 \times 20\,\mu M$ mouse brain will require around 60GB of storage space. Doubling the resolution ($1 \times 1 \times 10\,\mu M$) would increase this volume to 480GB and again ($0.5 \times 0.5 \times 5\mu M$) to 3.8 terabytes. The physical scale of these data presents concomitant problems with movement, visualization and processing. The 'cloud' is currently not a viable option as the software packages required for data processing and visualization are largely not supported for remote operation. Additionally, the microscope acquisition computer should not be considered a location for analysis as these are purpose-built machines designed to control the microscope. When analysis is performed simultaneously with image acquisition, it can cause instability. Thus, significant thought must be placed into local data movement pipelines that are connected to the machines that will be used for processing either directly or through high-speed networks. For example, the 3.8 terabyte dataset discussed above would require over 9 h to transfer using a traditional 1 gigabit network. If installation of a high-speed network is not an option, manual movement of data by foot using portable hard drives may be required.

The computer(s) that will be used for processing must be designed to accommodate the software packages to be used for analysis. A good rule of thumb is to have random access memory (RAM) equal to 2x the data set size. This is not possible with extremely large datasets, so it is advisable to maximize the RAM in any given system (128 gigabytes for consumer-grade computers and up to multiple terabytes with professional-grade hardware). Access to abundant fast solid-state drive (SSD) storage is a necessity to enable fast random read/writes (IO). In general, much image processing and rendering is an inherently parallel task and can be carried out using the graphics processing units (GPU), thus a modern GPU capable of CUDA/OpenGL and as much vRAM as you can afford will have enormous impact on processing speed using most current software suites and is truly essential for 3D visualization. Finally, a current multicore processor should be a priority as the CPU is essential for managing GPU tasks and for image processing tasks that are not yet GPU enabled.

Most leading commercial and community developed image analysis packages are only now incorporating methods for dealing with truly massive volumetric data. Each of these software suites has solutions to specific challenges (i.e. visualization, specific analyses, etc), but none have yet developed a framework that enables a truly comprehensive set

of general image analysis tools for large volumetric data. Excellent software options include (alphabetical order): Arivis Vision 4D ("Vision4D", 2015), Bitplane Imaris ("Imaris", 2019), the FIJI (Schindelin et al., 2012) distribution of ImageJ2 (Rueden et al., 2017) which includes many excellent community-developed plugins like Big Data Viewer (Pietzsch et al., 2015), MBF Neurolucidia and Vesselucidia ("MBF Bioscience", 2019), Nikon NIS-Elements ("NIS-Elements", 2019) and Vaa3D (Peng et al., 2010). Even with excellent software packages, as datasets scale above 1TB, a moderately complex set of analyses can take days to finish even with the best equipped workstations. Also, the inherently iterative nature of image processing increases this further.

7. Summary

Large volumetric high-resolution imaging of biologic systems is a truly exciting area of development which will continue to grow in the coming years. While increasingly useful, it is important to give significant forethought to platform planning and tool integration to best match the questions being asked and the technologies being used.

References

Ariel, P., 2017. A beginner's guide to tissue clearing. Int. J. Biochem. Cell Biol. 84, 35–39. https://doi.org/10.1016/j.biocel.2016.12.009.

Imaris [WWW Document] n.d. URL http://www.bitplane.com/imaris (Accessed 21 February 2019) 2019.

Ke, M.-T., Fujimoto, S., Imai, T., 2013. SeeDB: a simple and morphology-preserving optical clearing agent for neuronal circuit reconstruction. Nat. Neurosci. 16, 1154. https://doi.org/10.1038/nn.3447.

MBF Bioscience [WWW Document] n.d. URL https://www.mbfbioscience.com/products (Accessed 21 February 2019) 2019.

Muntifering, M., Castranova, D., Gibson, G.A., Meyer, E., Kofron, M., Watson, A.M., 2018. Clearing for deep tissue imaging. Curr. Protoc. Cytom. 86, e38. https://doi.org/10.1002/cpcy.38.

NIS-Elements [WWW Document], (Accessed 21 February 2019), n.d., URL Nikon Instruments Inc. https://www.microscope.healthcare.nikon.com/products/software/nis-elements 2019.

Peng, H., Ruan, Z., Long, F., Simpson, J.H., Myers, E.W., 2010. V3D enables real-time 3D visualization and quantitative analysis of large-scale biological image data sets. Nat. Biotechnol. 28, 348–353. https://doi.org/10.1038/nbt.1612.

Pietzsch, T., Saalfeld, S., Preibisch, S., Tomancak, P., 2015. BigDataViewer: visualization and processing for large image data sets. Nat. Methods 12, 481–483. https://doi.org/10.1038/nmeth.3392.

Richardson, D.S., Lichtman, J.W., 2015. Clarifying tissue clearing. Cell 162, 246–257. https://doi.org/10.1016/j.cell.2015.06.067.

Rueden, C.T., Schindelin, J., Hiner, M.C., DeZonia, B.E., Walter, A.E., Arena, E.T., Eliceiri, K.W., 2017. ImageJ2: ImageJ for the next generation of scientific image data. BMC Bioinform. 18, 529. https://doi.org/10.1186/s12859-017-1934-z.

Santi, P.A., 2011. Light sheet fluorescence microscopy: a review. J. Histochem. Cytochem. 59, 129–138. https://doi.org/10.1369/0022155410394857.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D.J., Hartenstein, V., Eliceiri, K., Tomancak, P., Cardona, A., 2012. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682. https://doi.org/10.1038/nmeth.2019.

Vision4D [WWW Document], arivis AG 2015; URL https://www.arivis.com/en/imaging-science/arivis-vision4d (Accessed 21 February 2019).

Wassie, A.T., Zhao, Y., Boyden, E.S., 2019. Expansion microscopy: principles and uses in biological research. Nat. Methods 16, 33–41. https://doi.org/10.1038/s41592-018-0219-4.

Watkins, S.C., St Croix, C.M., 2018. Light sheet imaging comes of age. J. Cell Biol. 217, 1567–1569. https://doi.org/10.1083/jcb.201804016.

Watkins, S.C., St. Croix, C.M. (Eds.), 2013. Current Protocols Select: Imaging and Microscopy. John Wiley & Sons, Inc, Hoboken, New Jersey.