CLEARED TISSUE IMAGING AT THE HARVARD CENTER FOR BIOLOGICAL IMAGING

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Jeff Lichtman (l) and Doug Richardson

The Harvard Center for Biological Imaging (HCBI) recently celebrated its five year anniversary. A central pillar in its mandate is to ensure that researchers have access to the most advanced imaging equipment and experimental techniques currently available. As such, tissue clearing was identified as an important advance that needed to be rapidly adopted.

Biological samples are inherently three dimensional. However the biologist's primary tool, the light microscope, historically produced two dimensional images. Over the past few decades fluorescence has emerged as the contrast method of choice for light microscopy and a variety of optical sectioning microscopes have now become widely available. Biologists can now acquire a serial stack of two dimensional images that provide a three dimensional volumetric view of a sample without the need to physically section samples into many thin slices. One serious limitation; however, is that both fixed and living tissue material are not transparent but have a rather milky appearance. This translucence prevents a researcher from obtaining sharp images of sections deep within a volume. The problem is explained by the way light passing through a sample interacts with the molecules that make up the tissue. Light passing through biological tissue is said to be "scattered". The scatter limits the unperturbed penetration of even the most intense and uniform light sources (i.e. lasers) to fractions of a millimeter and similarly impedes the light coming back from fluorescent objects deep within tissue. Therefore, techniques that reduce light scatter would greatly enhance the ability of optical sectioning microscopes to image

deeper into tissue and better elucidate the three dimensional structures and interactions present in biological samples.

Recent advances in three disparate fields have now combined to make three dimensional imaging of thick tissues possible. First, building on a method originally described in the early 1900s a number of new biochemical techniques for "clearing" thick, fluorescently labelled biological samples have recently been published. Second, a number of advances in optical designs (parallelized confocal, light sheet microscopy) now allow researchers to capture large volumes of tissue much more rapidly. Finally, as we enter the era of big data, computational processing power and data storage has reached the point where acquisition and analysis of terabyte-sized datasets is possible.

The realization that there were still many questions and misunderstandings regarding tissue clearing, led HCBI Faculty Director Jeff Lichtman and HCBI Director of Imaging Doug Richardson to attempt to "clarify" this field with an extensive review that has recently been published in the journal Cell.

In the review, Richardson and Lichtman first provide a brief explanation to the underlying physics that determines whether a substance is clear, like water, or cloudy, like milk. As light propagates, it constantly interacts with matter. Specifically, when it comes in contact with a given substance, it can briefly impart some of its energy to the electrons of the atoms and molecules comprising that material. If one of these interactions occurs, the forward propagation of light pauses for a very brief moment and the direction the light is travelling is altered to "scatter" it in all directions. In clear substances, like water, a high concentration of homogenous scatters exist (individual molecules) and those that are excited by the light simultaneously interfere with each other. This synchronous scattering causes deconstructive interference in directions perpendicular to the direction light entered the liquid. In contrast the sequential scattering of light in the forward direction leads to constructive interference allowing light to continue moving in the forward direction. In milky appearing liquids, the presence of inhomogeneities in the number of scatters prevents the lateral deconstructive interference so light propagates in all directions giving the liquid its cloudy appearance.

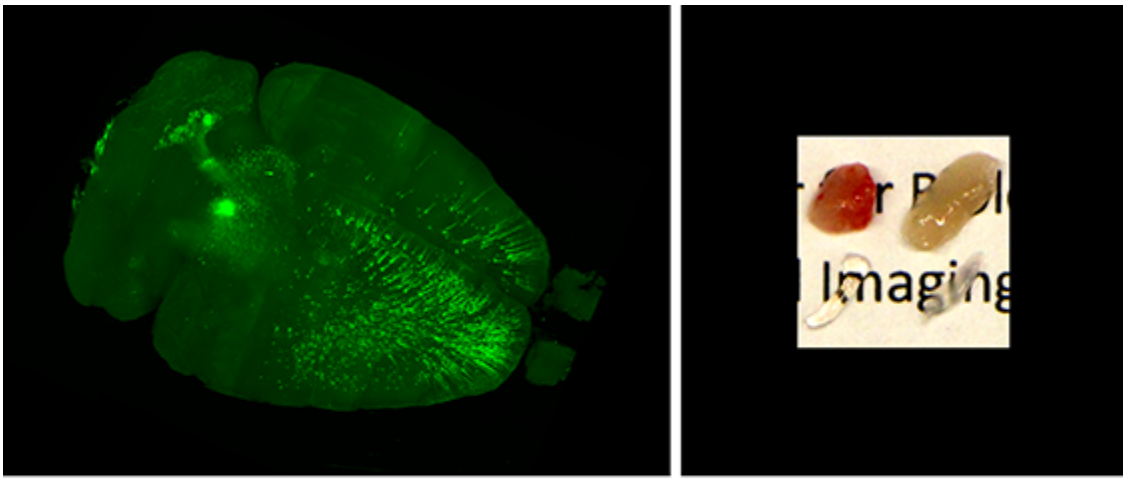
Richardson and Lichtman continue the review by categorizing the many clearing techniques currently available into two categories: solvent-based clearing and aqueous-based clearing. Solvent-based clearing was originally proposed in the early 1900s, but has recently undergone a renaissance. Solvent-based techniques are economical, rapid, and easy to perform; however, they produce a high degree of tissue shrinkage and rapidly quench fluorescent protein emission. Therefore, a number of aqueous-based techniques have also been recently developed to address these issues. Richardson and Lichtman discuss the three main categories of aqueous-based techniques (simple immersion, hyperhydration, and hydrogel embedding), and describe the molecular mechanisms whereby they produce cleared tissue.

Finally, a discussion regarding the optical sectioning modalities available to image large cleared tissues (confocal, 2-photon, and lightsheet microscopies) is included. Specifically, this section describes which samples are best suited to a certain microscope, considerations for proper objective lens selection, and suggestions for data handling and analysis.

Overall, Richardson and Lichtman hope this review will be a useful reference that can guide and inform a novice looking to begin a series of tissue clearing experiments, give researchers an intuitive sense of how to think about new clearing strategies, and also frame a discussion regarding the developments that are needed to further advance the field.

The HCBI has been working closely with Carl Zeiss Microscopy to assist in the development of a modified version of their Lightsheet Z1 microscope that is capable of rapidly imaging cleared tissue. In addition, the HCBI has now acquired the first commercial electrophoretic tissue clearing device in North America from Logos Biosystems to further simplify the tissue clearing pipeline. The HCBI is also working to find answers to the big data questions that tissue clearing presents. Close collaborations with FAS Research Computing have led to solutions for data storage and transfer, while work with Arivis AG has presented methods for visualization and analysis of these huge data sets (recognized by a Microsoft Life Sciences Innovation award in 2014).

The HCBI plans to remain at the forefront of tissue clearing techniques and is looking forward to facilitating a number of biological breakthroughs as more of its member labs adopt these methods.



Imaging Cleared Tissue (left) An adult mouse was injected with an EGFP-encoding rabies virus to label a subset of neurons throughout the brain. Brain tissue was fixed and cleared using a modified CLARITY protocol including an electrophoretic tissue clearing step (Chung et al., 2013). The cleared, intact brain was imaged via lightsheet microscopy. *Sample courtesy of William Menegas from the Uchida lab.*

Clearing Tissue (right) Skeletal muscle tissue from an adult mouse was cleared using the PACT technique (Yang et al., 2014). Freshly dissected skeletal muscle tissue (top left), skeletal muscle tissue dissected from an animal after cardiac perfusion with isotonic buffer (top right), skeletal muscle embedded in hydrogel and passively cleared of lipid (bottom right), and passively cleared tissue equilibrated with Refractive Index Matching solution (RIMs) (bottom left) are displayed. All samples were independently isolated and prepared. The size differences are not indicative of shrinkage or expansion during the clearing process. Scale bar equal to 5 mm. *Samples prepared by Ian Boothby from the Lichtman lab.*