Overview of Tissue Clearing Methods and Applications

Tissue clearing is a broad term that defines techniques that have the final goal of reducing the opacity of biological tis
features deep within a sample while maintaining its original structure. To do that, the sample is treated with a series of
solutions that will make it transparent and, when combined with fluorescent staining and advanced imaging methods,
achieve an unprecedented view of the whole specimen and allow acquisition of detailed information from the intact bi

This Technical Note aims to help researchers select the best clearing protocol for their experiments. We present a com
summary of the most common methods of clearing that have been developed in the past two decades. As such, the no
main points, click on any of those below to navigate to the section of interest:

Movie 1. Mouse brain sectioned at 600µm thickness and imaged with Dragonfly
Part of a montage of a 10 x 12 array of tiles stitched together, each one containing 237 z-steps, to cover 570 µm in thickness. To prepare th
mouse was injected with Rabies-GFP and Rabies-RFP, the tissue was SHIELD fixed, sectioned at 600um, passively delipidated with SDS, and
with antibodies and DAPI. Imaged with Andor Dragonfly 202, using simultaneous 2 colour imaging with a 10X objective. Image credit: Hong

Ph.D. Lab at UCLA, Department of Neurobiology.

Limitations When Imaging Large Tissues

The opacity of thick tissue hinders light penetration and makes it almost impossible to obtain well-resolved images. Re traditionally overcome the inability to **image deep within tissues** by taking a three-dimensional specimen and physica into ultra-thin 2D slices. However, this disrupts the integrity of the sample and limits the amount of information that ca

The limit of imaging depth that's imposed by the natural scatter of biological non-cleared samples can vary from 50 to means that after 50um the image quality starts to degrade, the background intensity increases and as a result, the con signal-to-noise ratio) is reduced. In cleared tissues, on the other hand, the light can traverse millimeters or even centim tissue unhindered. Now imaging mm or cm deep into tissues is possible. Nevertheless, to be able to visualize those thi **essential to use a confocal microscope, such as** Andor Dragonfly (see Movie 1 and Figure 1), which allows rejection light and delivers high-resolution, 3D images with more contrast. For more information on how to image cleared tissue Solution Note: Imaging Cleared Tissues with Dragonfly Spinning Disk Confocal.

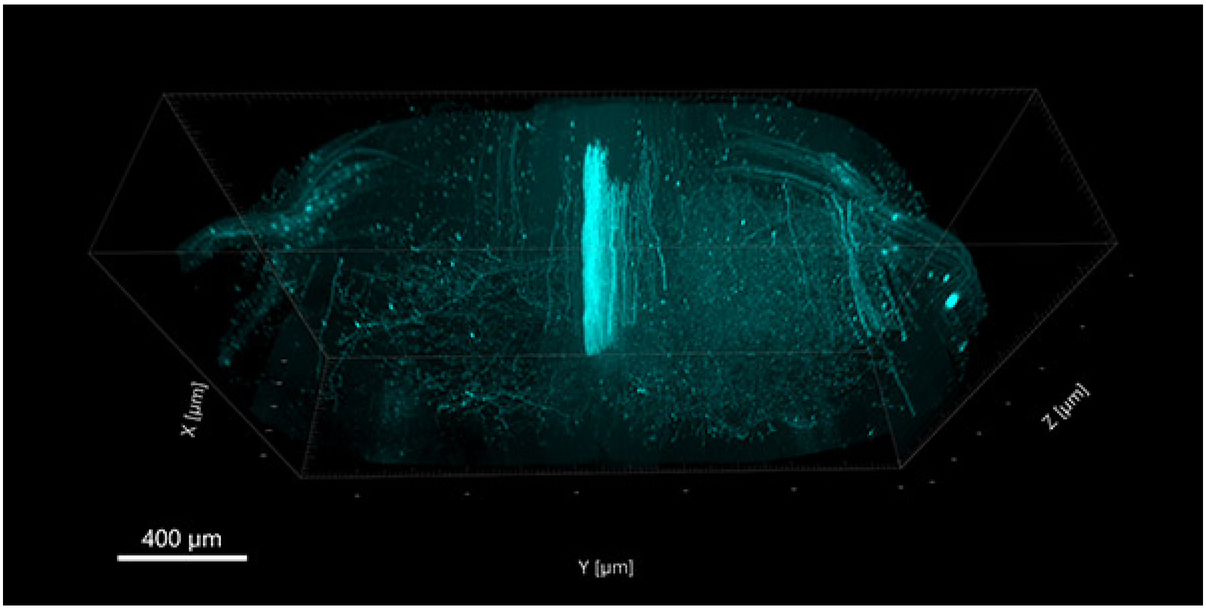

Figure 1. Confocal image of a mouse spinal cord cleared with the iDISCO clearing method.
Montage of a 5x1 array of tiles stitched together, each one containing 2530 z-steps, to cover 1.3 mm in thickness. To prepare the sample, t injected in the motor cortex with an adeno associated virus that expresses the fluorescent protein tdTomato. After spinal cord collection, t immunolabeled with a fluorescent antibody that recognizes the fluorescent protein and then cleared. Imaging was performed on an **Ando confocal Dragonfly in 1 hour and 15 minutes,** with a 25X water-immersion, long working distance objective with a numerical aperture of Kar Men Mah, Vance Lemmon, and Pantelis Tsoulfas; The Miami Project, University of Miami.

How Does Tissue Clearing Work?

Proteins and lipids that form cells and biological tissues have a high refractive index (RI ~ 1.45-1.47), while cytosol, the a inside each cell, has a refractive index closer to water (RI = 1.33). When light passes through these regions with differen indexes it is diffracted (bent) and adsorbed differently causing the ray to be scattered (dispersed). The more cells in the greater the amount of light that gets dispersed, making the tissues appear opaque.

The concept behind tissue clearing methods is to equalize the Refractive Index of all the components inside th the light can pass through the whole sample undisturbed (Figure 2).

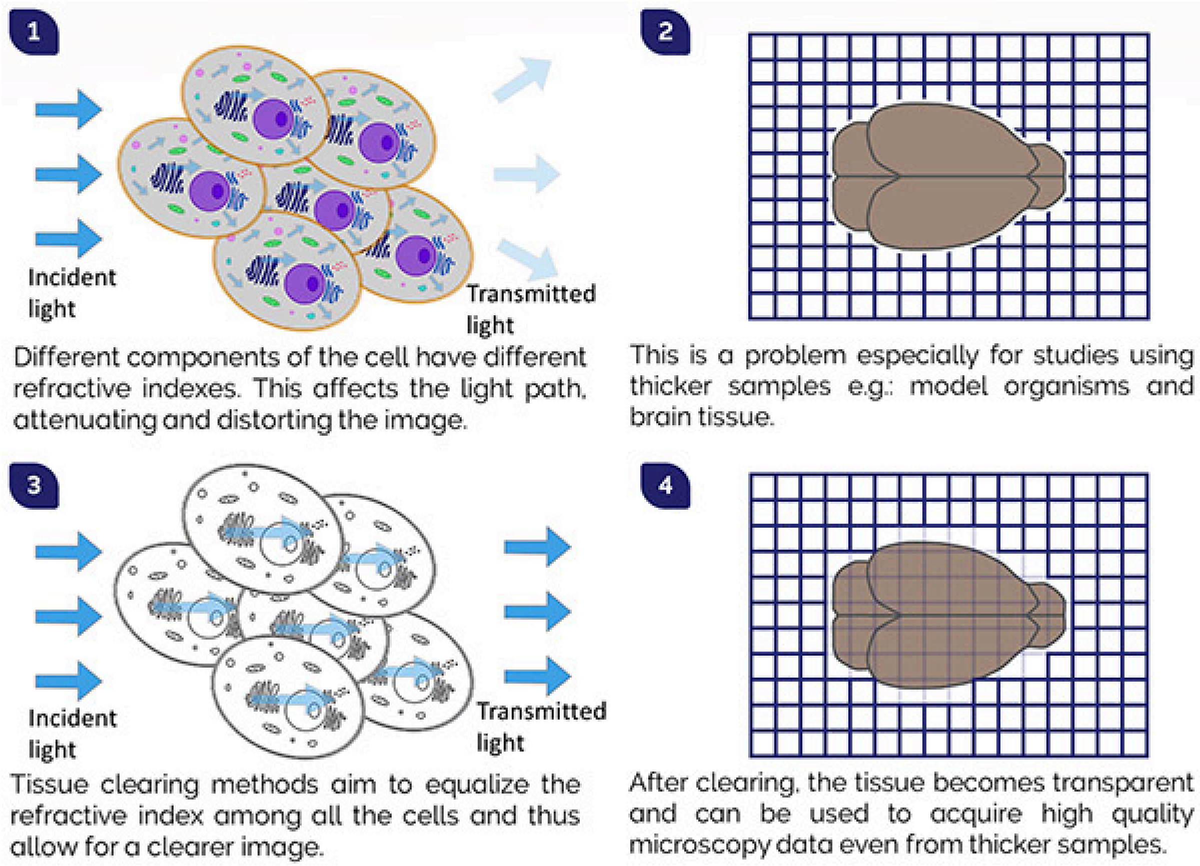

Figure 2. Tissue Clearing Process

1) Differences in refractive indices inside the cells cause the scattering of light in the tissue. 2) The refractive index mismatch results in tissu
a problem when imaging thicker samples (e.g.: whole organs or small model organisms). 3) Tissue clearing reagents aim to reduce light sca
normalizing the refractive index throughout the tissue so that the light can pass undisturbed. 4) Cleared tissues are transparent and can b
entirety.

Overview of Clearing Methods

Since the development of the first modern method to clear tissues in 2007 [1], there has been a proliferation of new cl
each with its own unique advantages and disadvantages. To help researchers to navigate the literature and decide whi
work best to answer their research questions we list the most common clearing methods, highlighting their main chara
are three primary approaches to make biological tissues transparent:

- Organic solvent-based (also called hydrophobic or high refractive index matching techn
- Aqueous and hyper-hydrating
- Hydrogel-embedding

1. Organic solvent-based tissue clearing

Organic solvent-based methods generally require dehydration followed by lipid removal and refractive index matchin
3). The protocols are rapid and robust in clearing tissues but tend to shrink the samples and are unsuitable for lipid stu
compatible with immunostaining but tend to quench fluorescent proteins and require additional signal amplification s
A major disadvantage of these protocols is that the BABB solution (which is used in all of them) is very toxic for human
contact with microscope equipment (e.g.: objectives) it will severely damage it.

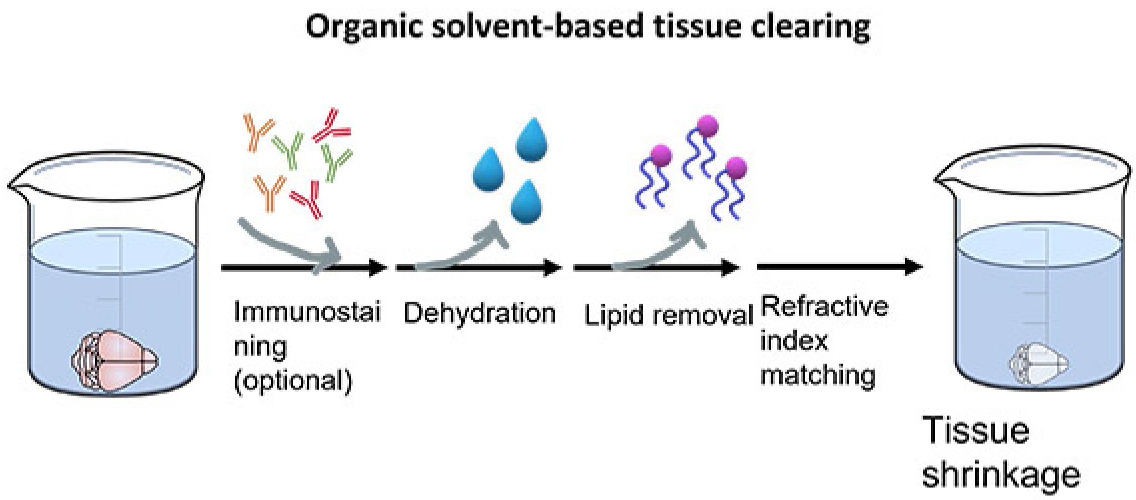

Figure 3

2. Aqueous hyper-hydrating tissue clearing

Aqueous hyper-hydrating protocols such as CUBIC, Scale, SeeDB, allow better retention of endogenous signals but th limited to smaller samples and requires a longer time to achieve complete transparency. Moreover, upon clearing with hydrating agents, the samples tend to expand (Figure 4). The main advantage is that these methods are much less haz compared to all the other clearing protocols and generally compatible with immunostaining and fluorescent proteins.

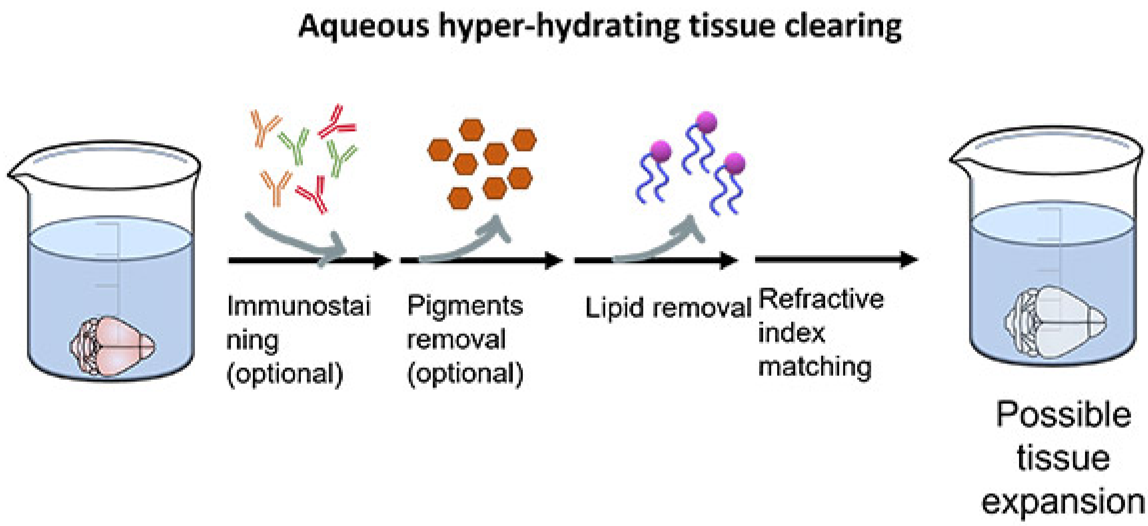

Figure 4

3. Hydrogel-embedding tissue clearing

Hydrogel-embedding techniques generate cross-links between the proteins to create firm scaffolds to avoid damage structure. To achieve this, the sample is embedded in hydrogel and only after it is immersed in strong detergents to re followed by refractive index matching (Figure 5). These methods are technically more difficult and sometimes require a electrophoresis step to accelerate the clearing process and increase the penetration of antibodies. These techniques e preserve the proteins in the sample, as well as RNA and DNA, making them ideal for multiplexed labelling and fluoresc hybridization (FISH) studies. The most common protocols of this type are CLARITY, PACT, PARS, and SHIELD.

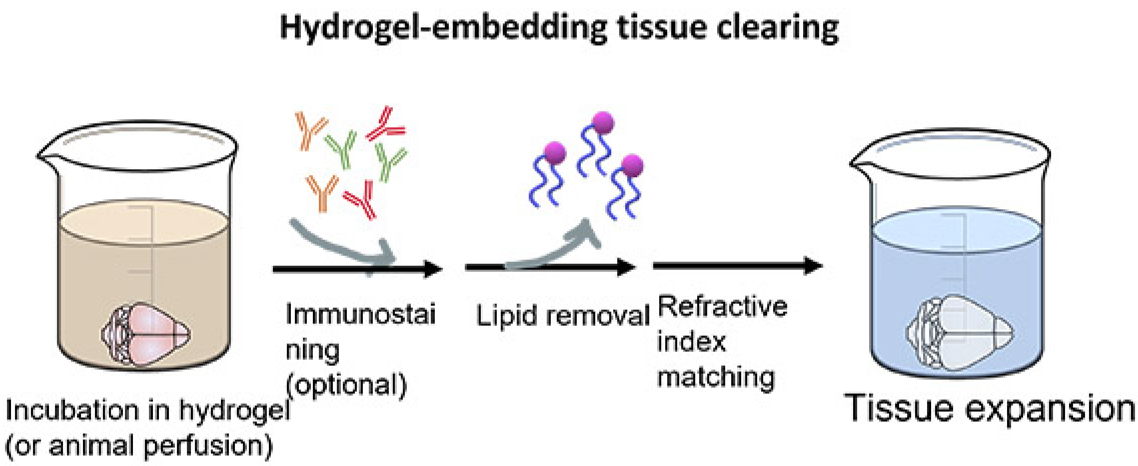

Figure 5

Which is the Best Optimal Tissue Clearing Method For My Application

With the proliferation of multiple tissue clearing methods, it is challenging to choose the most appropriate method wh technical approach. Table 1 aims to be a guide in to help you choose where begin. Nevertheless, researchers should re each sample and application will always require hands-on optimization, so please use the table as a starting guide.

Once the samples are cleared, and the tissue is stained, the next step is to image the large, cleared samples. There are requirements that a microscope needs to have to be an ideal system for such a task. Among others, the microscope sh and fast imaging and deliver high background rejection with an excellent signal-to-noise ratio. Read our solution note t Andor Dragonfly **is the ideal system to image cleared samples.**

Method Type	Method Name	Immunostaining	Fluorescent Proteins	Protocol Time	Morphology	Tissue Size	Refractive Index
Hydrophobic (solvent-based)	BABB	Yes	No	Days	Shrinkage	Young mouse brain	1.55
	3DISCO	Limited	Yes	Hours/ Days	Shrinkage	Adult mouse brain	1.56
	iDISCO(+)	Yes	No	Hours/ Days	Shrinkage	Adult mouse brain	1.56
	uDISCO	Yes	No	Hours	Shrinkage	Whole adult mouse	1.56
	vDISCO	Yes	No	Hours	Shrinkage	Whole adult mouse	1.56
Aqueous (water-based)	Scale AS (S)	Yes	Yes	Days	Expansion (Preserved)	Mouse embryo	1.38
	SeeDB	No	Yes	Days	Preserved	Mouse Brain	1.48
	CUBIC	Yes	Yes	Days	Expansion	Max	1.47

						1-2 mm tissues	
	ClearT	Yes	No	Hours/ Days	Preserved	Mouse embryo	1.44
Hydrogel embedding	CLARITY	Yes	Yes	Days/ Weeks	Expansion	Whole mouse brain	1.45
	PACT (PARS)	Yes	Yes	Days	Small Expansion (Preserved)	Whole adult mouse	1.38 - 1.48
	SHIELD	Yes	Yes	Days	Preserved	Up to 5 mm	1.45

Table 1: Summary of the most common tissue clearing techniques.

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