Insights into Tissue clearing Imaging

Concepts and Implementations

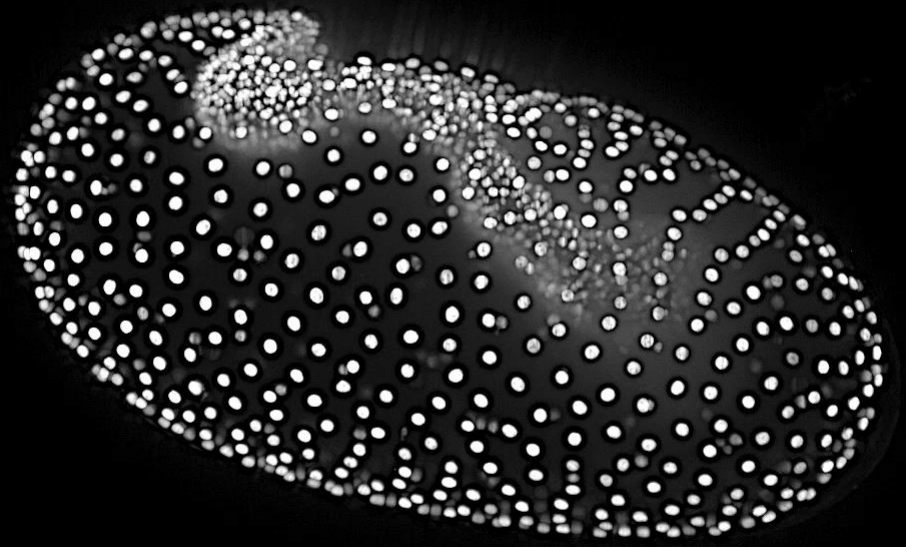
Klaus Weisshart

8 June 2022

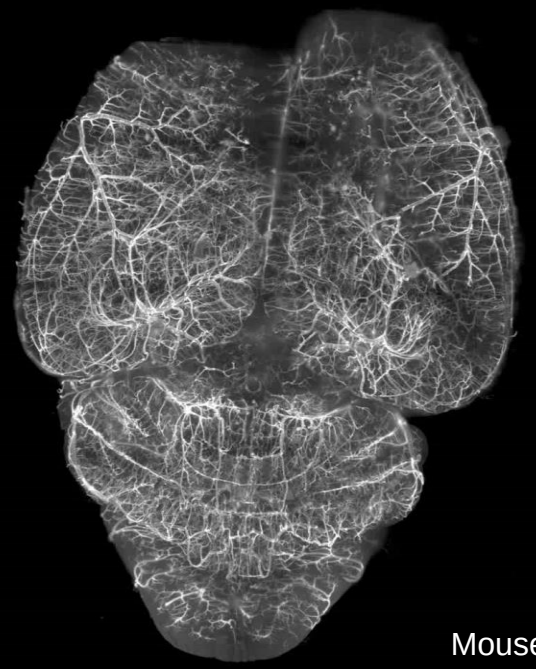
"Sample and Tissue Clearing for Lightsheet Microscopy: *How to Get Best Images with Various Types of Immersion Media and Clearing Agents*"

From Live Organism to Cleared Tissue Imaging

w/ superb optical quality



Tribolium castaneum (Flour beetle)
Multiview z-stack in time series
Nuclear GFP expression
Nipam Patel
U. of California, Berhkeley, USA



Mouse brain
Z-stack in tiling
CellTracker™ CM-DiI Dye for vaaculature
Erin Diel & Doug Richardson
Harvard U. HCBI, Cambridge, USA

Light sheet Cleared Tissue Imaging

Basic Optics

5x/0.1 foc.

10x/0.2 foc.

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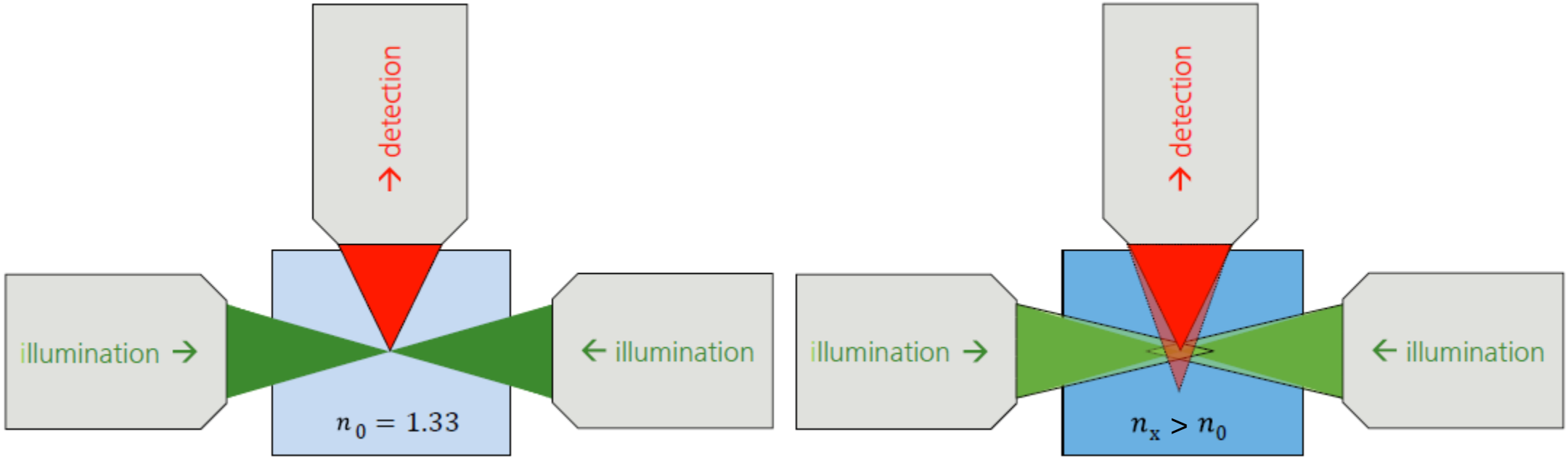
Focus shift introduced by higher RI media



$$\text{refractive index } (\mathbf{n}) = \frac{\text{speed of light in vacuum } (\mathbf{c})}{\text{speed of light in medium } (\mathbf{v})}$$

Focus shift due to refractive index variations. Left: water immersion, right: chamber filled with a medium of higher RI.

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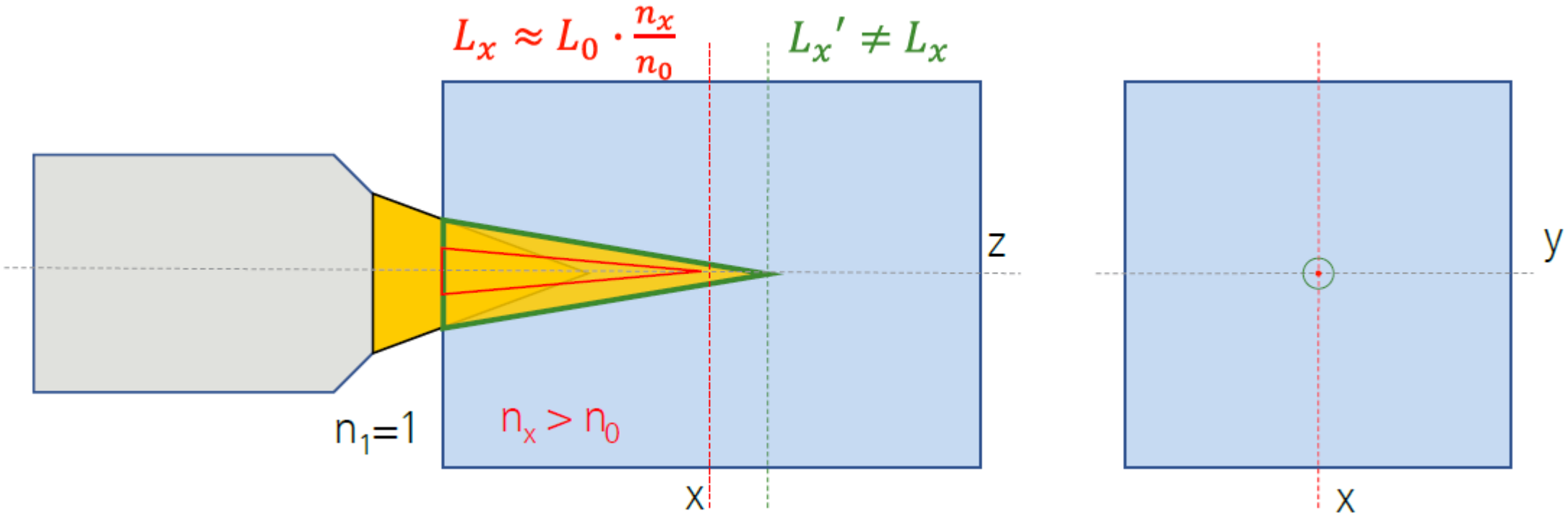
RI mismatch and spherical chromatic aberration



Spherical aberration, when not all rays of a focused cone of light convene in a common focus point. The axial rays meet in the red focus point, whereas the rays at the edge of the cone have a different focus point.

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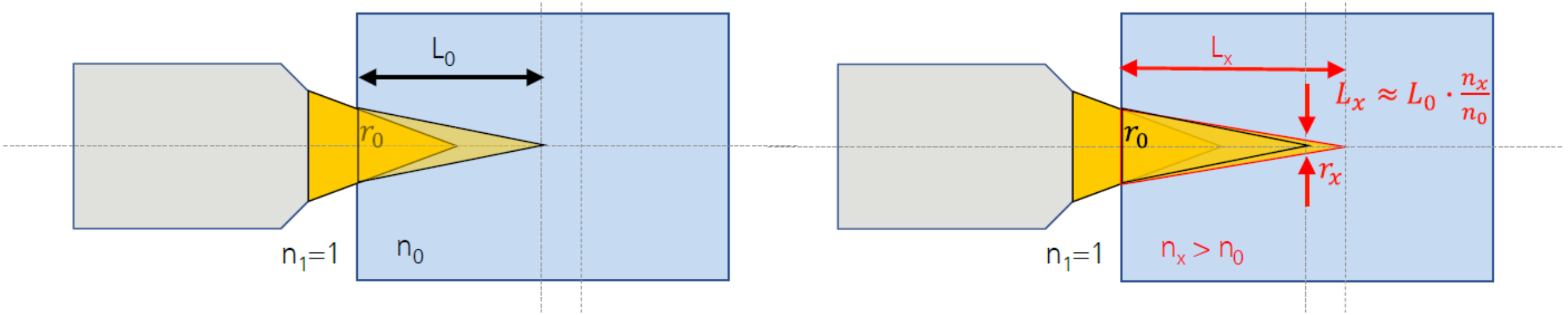
Focus shift introduced by higher RI media



Focus shift occurs in an objective-chamber combination that is designed for one imaging medium (refractive index n_o) and used with another medium (n_x)

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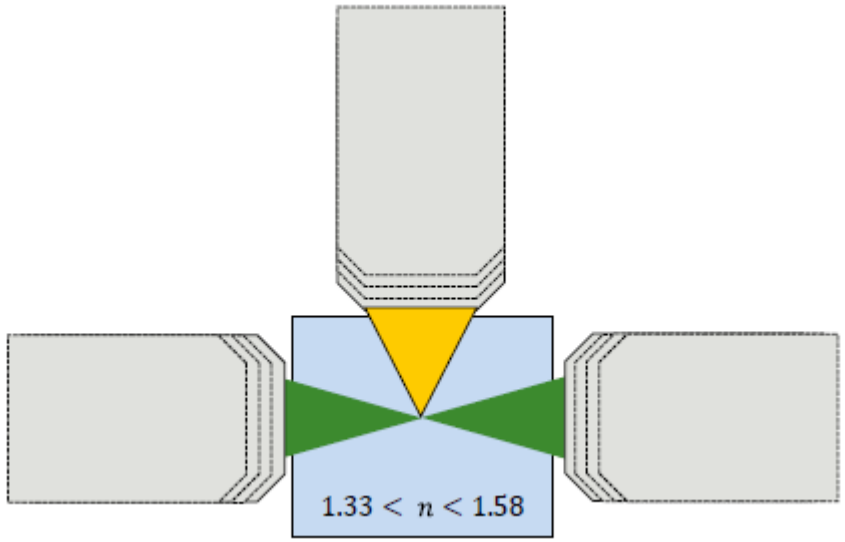
Compensation of focus shift via focusable optics



Compensating focal shift for a wide range of refractive indices (1.33 to 1.58) by focusing the objectives.

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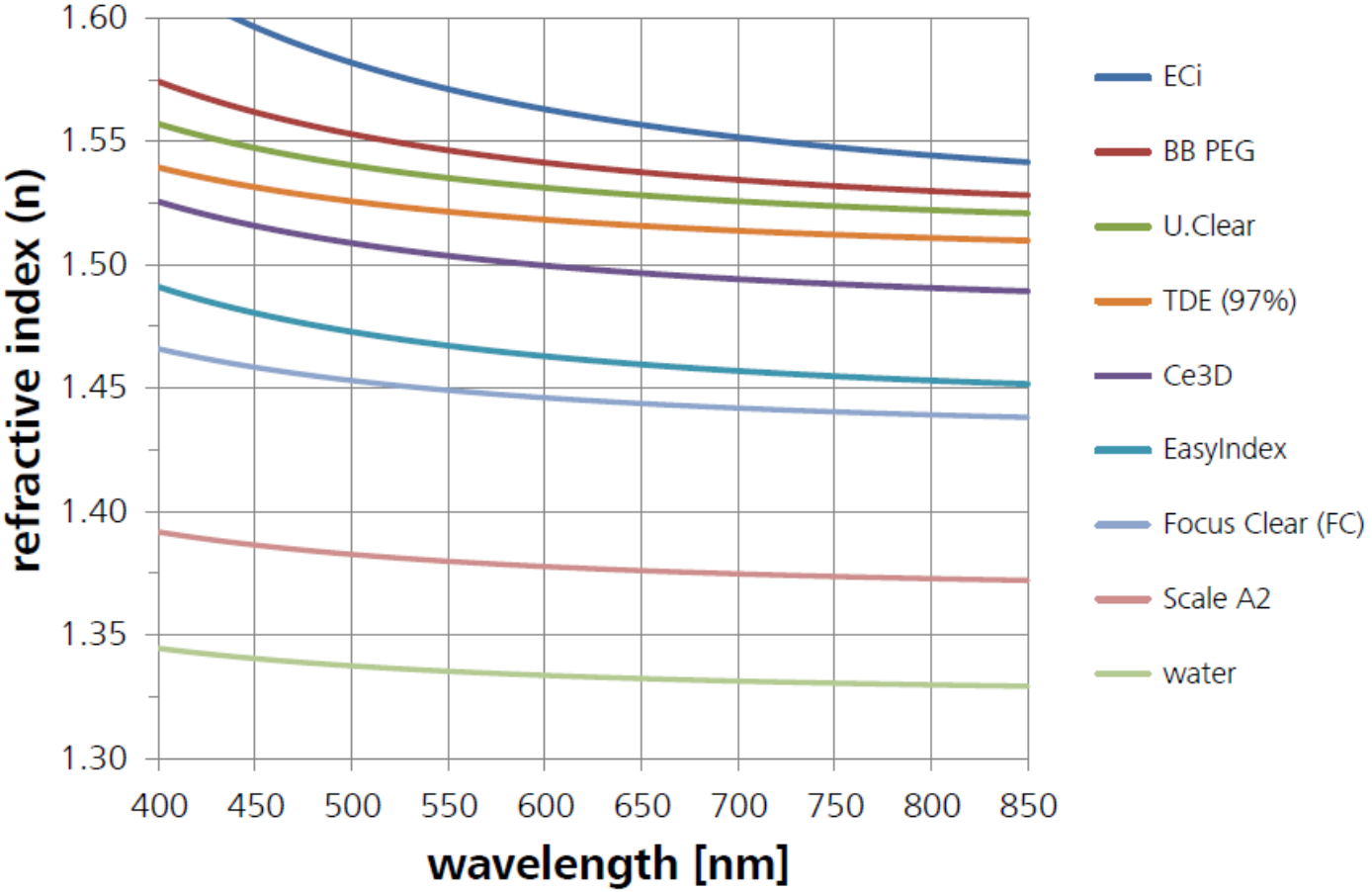
RI mismatch and longitudinal chromatic aberration



$$Abbe - number: v_{546} = \frac{n_{546} - 1}{n_{488} - n_{640}}$$

$$RI\ variation: 1.33 < n < 1.57$$

$$Dispersion\ \text{var.}: 19 < \nu < 56$$

The design of the detection objective 5×/0.16 foc achieves minimal wavelength-dependent focus shifts (longitudinal chromatic aberrations) for a multitude of important imaging media (marked in green). However, extreme cases cannot be covered completely. For water (low dispersion) the objective selection for water should be used. For multi-color imaging in ethyl cinnamate appropriate image acquisition strategies or post processing channel alignment to compensate for chromatic effects is recommended.

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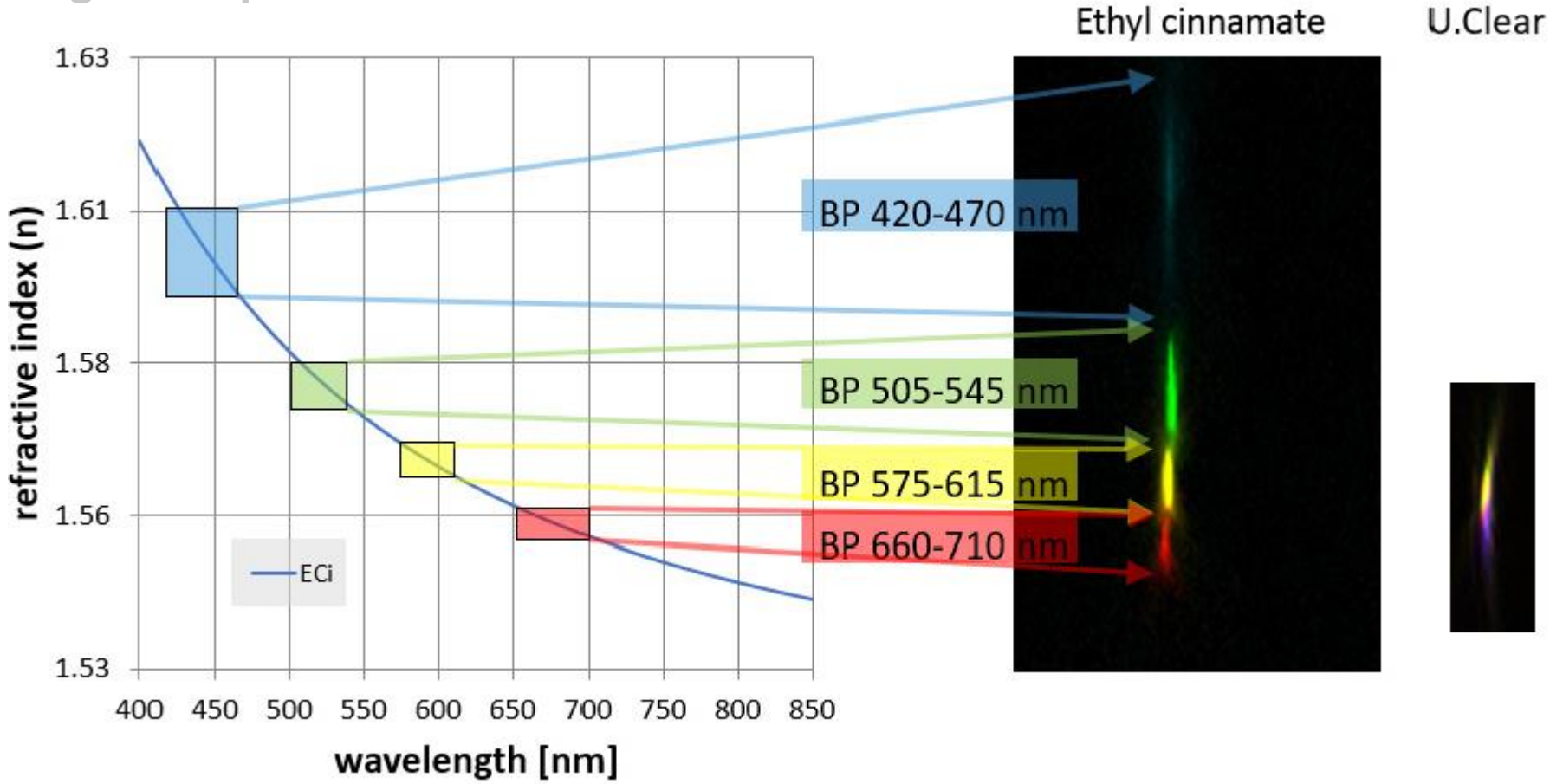
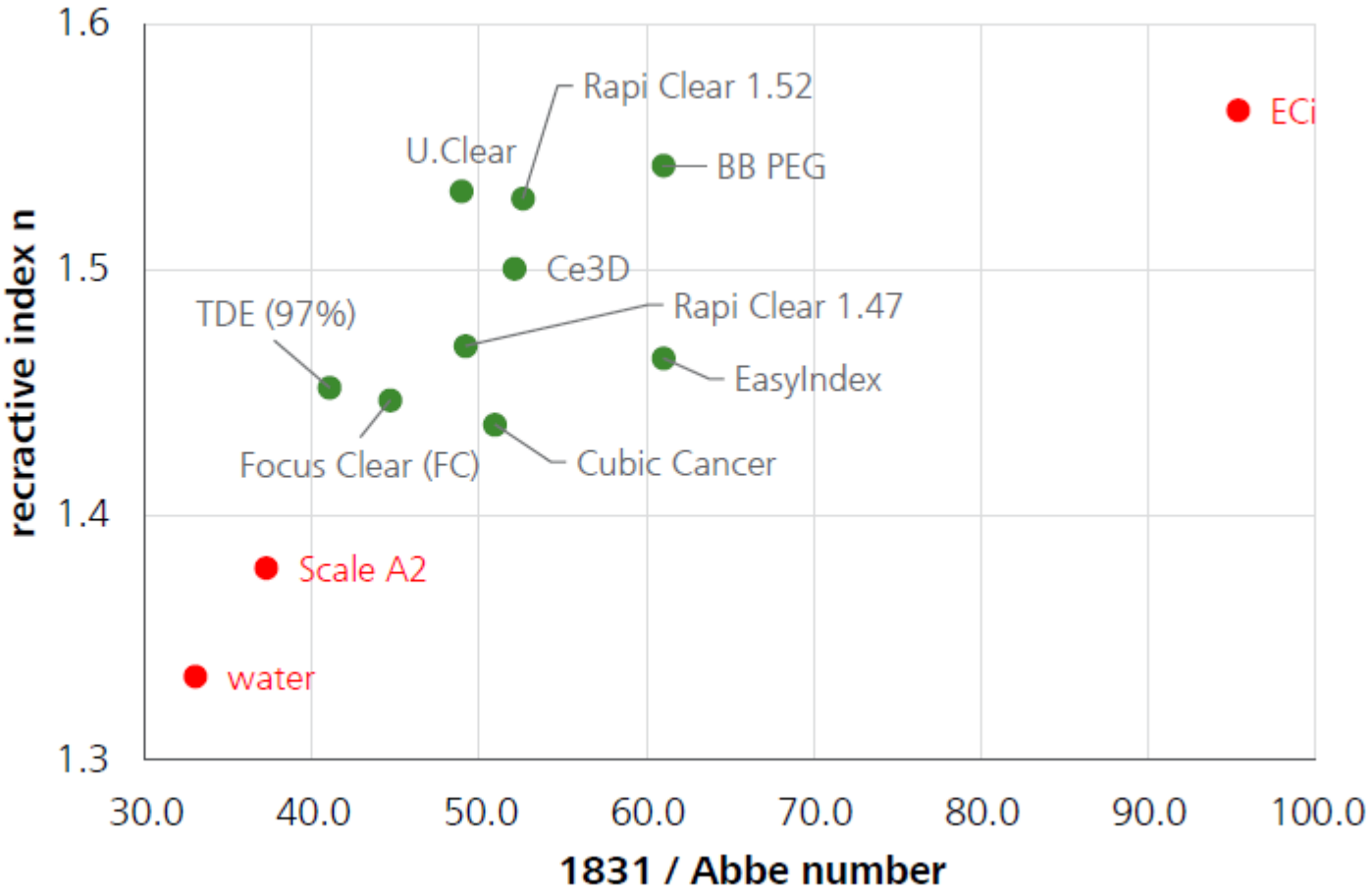
Compensating for dispersion



The strong dispersion of ethyl cinnamate induces a focal shift in the PSFs between the four wavelength channels. Furthermore, each channel shows a broadened extension along the optical (z) axis. This is most prominent for short wavelengths, where dispersion is strongest. The examples are given for the Clr Plan-NEOFLUAR 20×/1.0 nd=1,53, which was designed for U.Clear and shows excellent color correction for this and similar imaging media (see the small PSF on the right).

Light sheet Cleared Tissue Imaging

Compensating for dispersion



Refractive indices for various common imaging media. The broad range of main refractive Indices as well as the range of dispersions are clearly visible.

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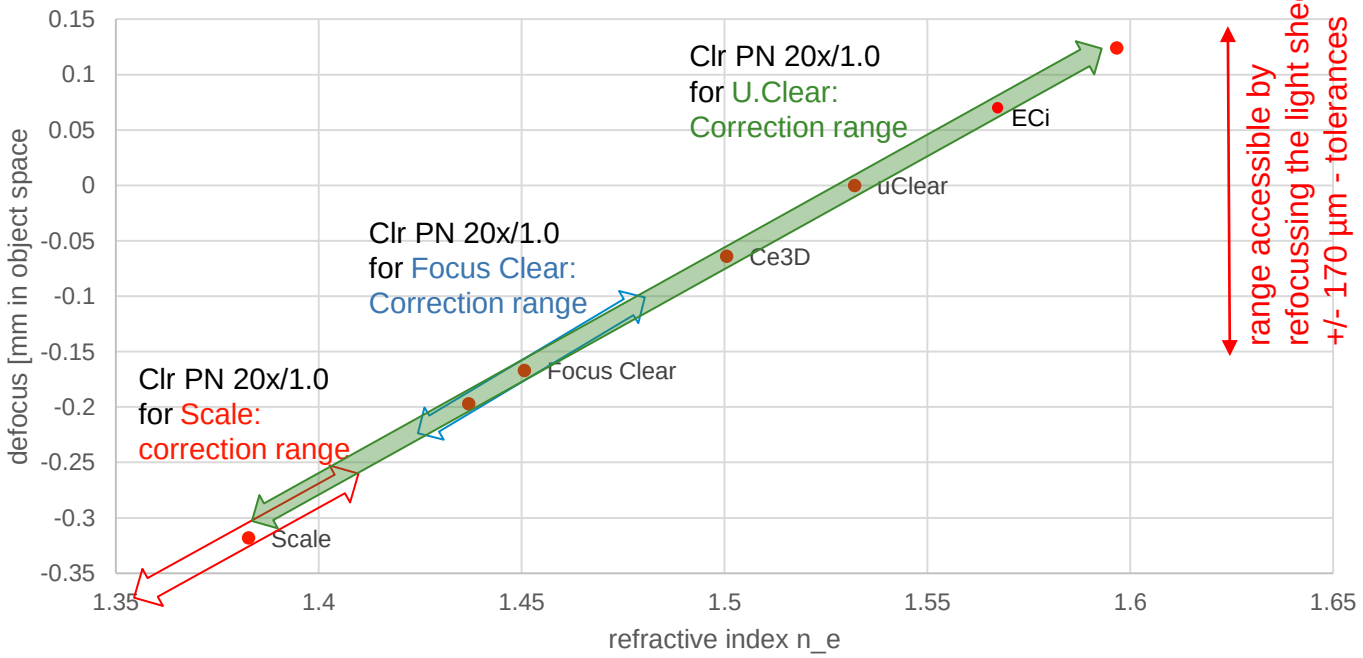
Compensating for dispersion



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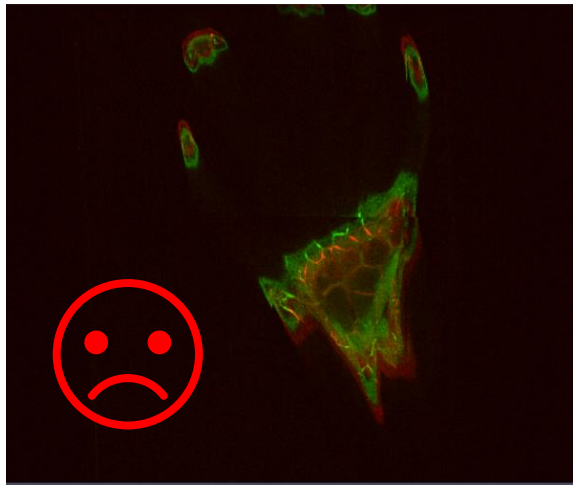
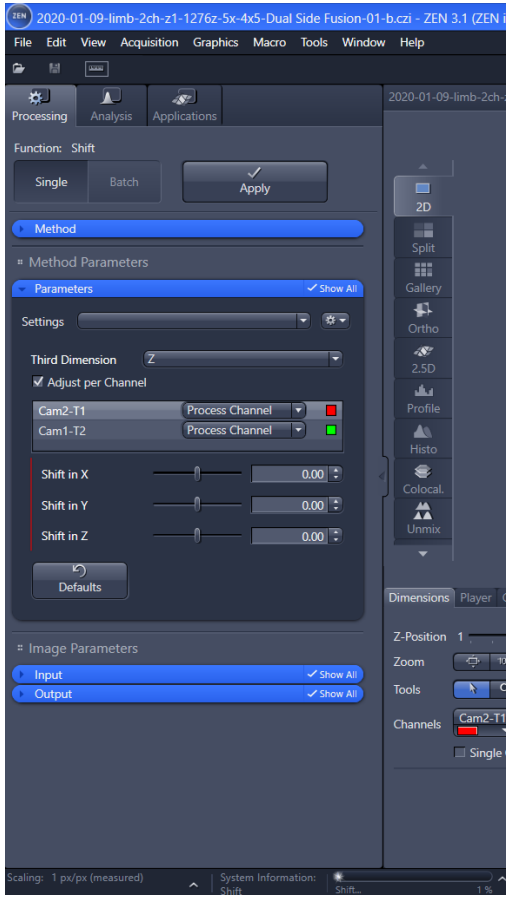
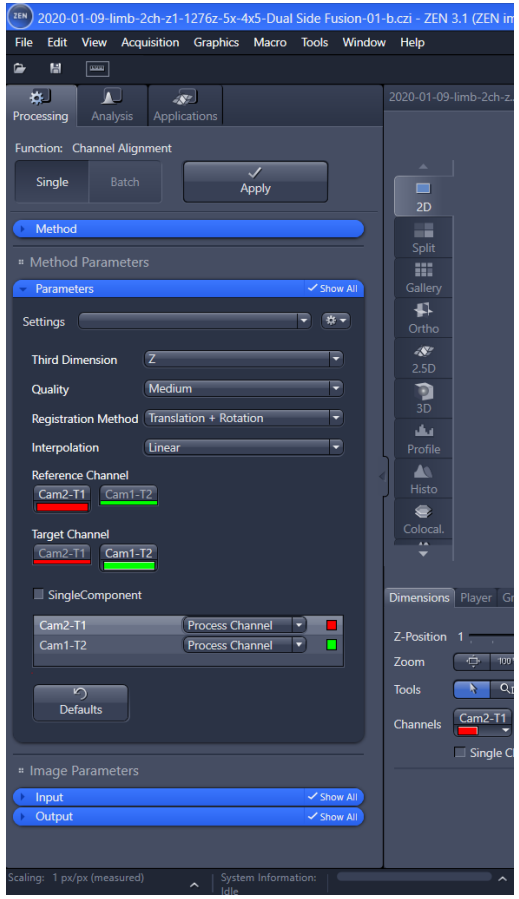
Compensating for dispersion

raw data:
red and green channels
Set off due to ECi dispersion

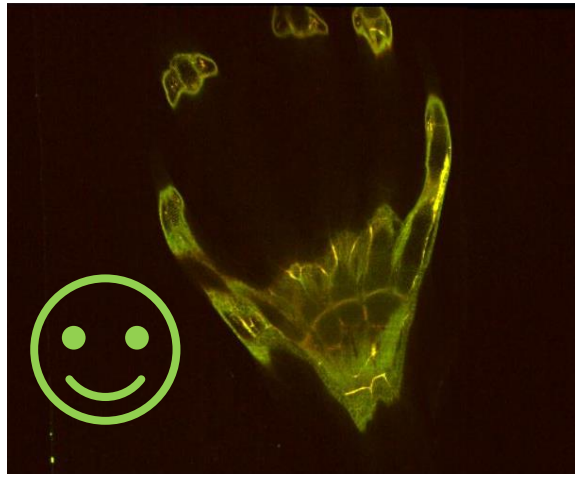


Axolotl limb cleared and imaged in Ethyl Cinnamate at RI=1.57
Courtesy of Tanaka lab, IMP, Vienna, Austria





after Channel Aligment in ZEN



ZEN functions: channel shift & channel aligment

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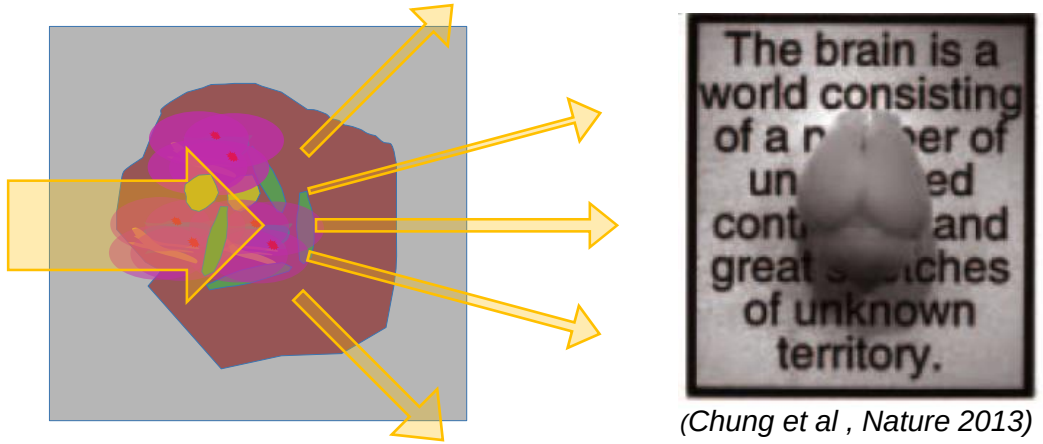
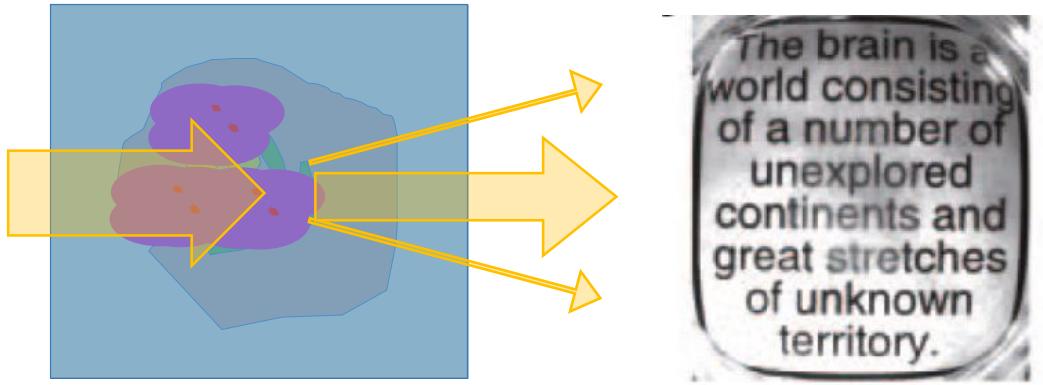
Enable transparency of larger specimen



(Chung et al , Nature 2013)

Absorption and scattering in opaque tissue



Transparent tissue after optical clearing

- Clearing solutions are used to match the refractive index within the tissue while maintaining 3D structure, fluorescent activity
- Light-scattering structures (*e.g.* – lipid membranes, water) within the sample are removed/exchanged
- Image deeper, non-invasively, without need for physical sectioning
- Typical Range: RI = 1.38 – 1.57

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Challenges for clearing

Experimental factors:

- There is no single method for all tissue types and/or experiments:
  - Antibody staining
  - Lipid dyes
  - Dye tracing experiments
- Limited preservation of fluorescence / quenching possible
- Sample mounting
- No living sample experiments

Economical factors:

- Price vs. effort vs. time
- Large file sizes -> big data storage

Safety:

- Toxic and irritating chemicals

Light sheet Cleared Tissue Imaging

A variety of approaches for animal tissue clearing

Cell

Leading Edge
Review

Clarifying Tissue Clearing

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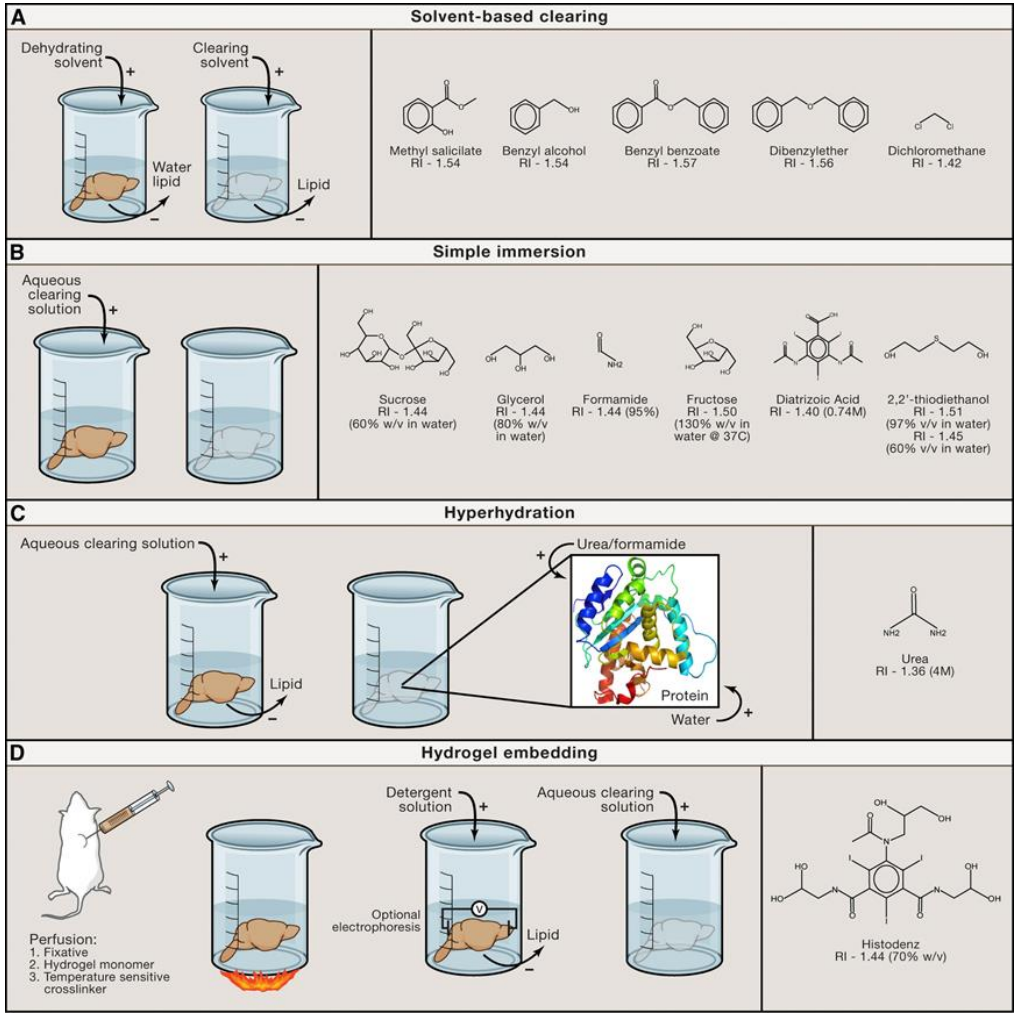
Biological specimens are intrinsically three dimensional; however, because of the obscuring effects of light scatter, imaging deep into a tissue volume is problematic. Although efforts to eliminate the scatter by "clearing" the tissue have been ongoing for over a century, there have been a large number of recent innovations. This Review introduces the physical basis for light scatter in tissue, describes the mechanisms underlying various clearing techniques, and discusses several of the major advances in light microscopy for imaging cleared tissue.



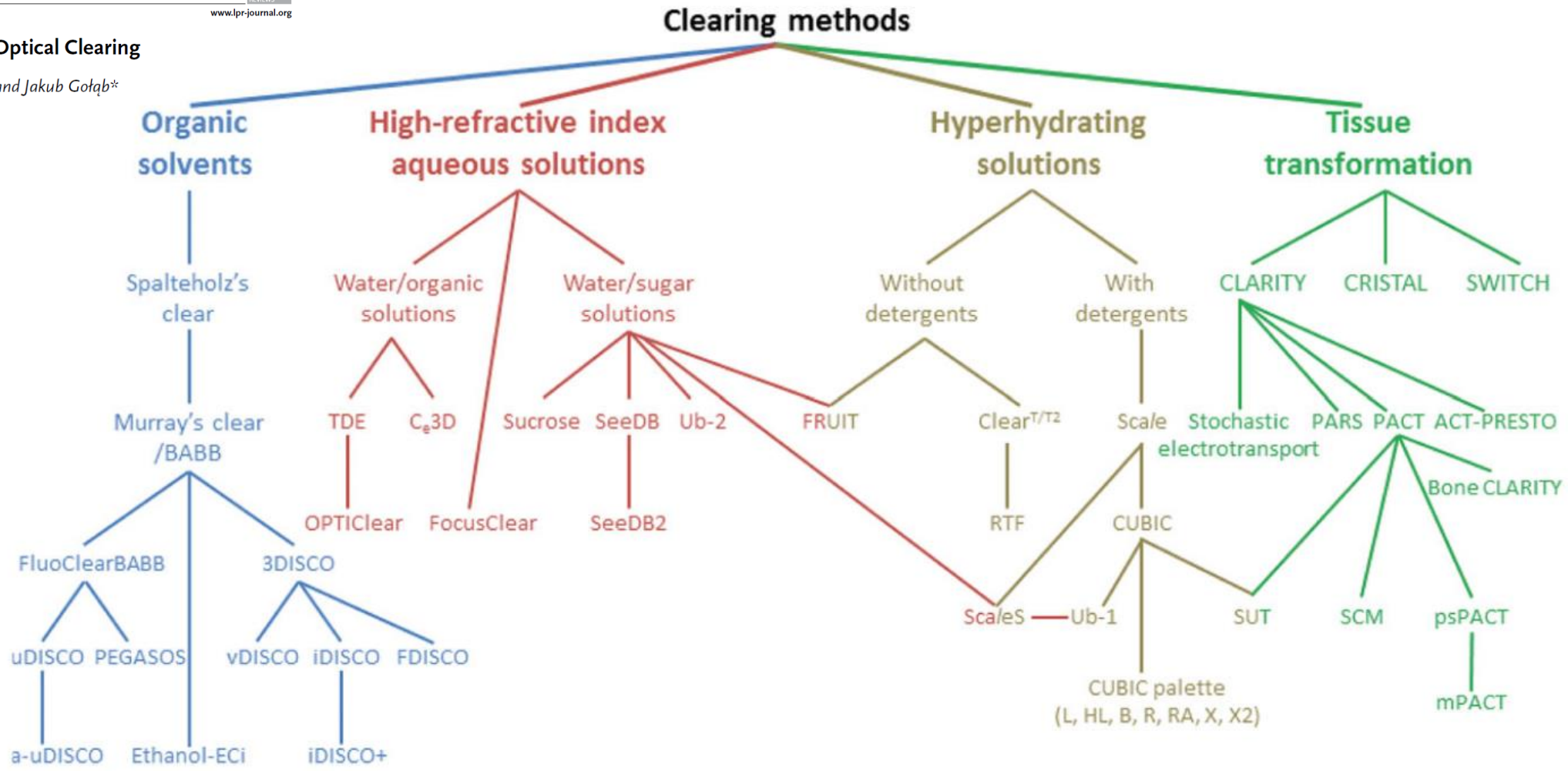
Light sheet Cleared Tissue Imaging

A variety of media for animal tissue clearing

REVIEW
Tissue Optical Clearing
LASER & PHOTONICS REVIEWS
www.lpr-journal.org

Advances in Ex Situ Tissue Optical Clearing

Paweł Matryba, Leszek Kaczmarek, and Jakub Gołąb**



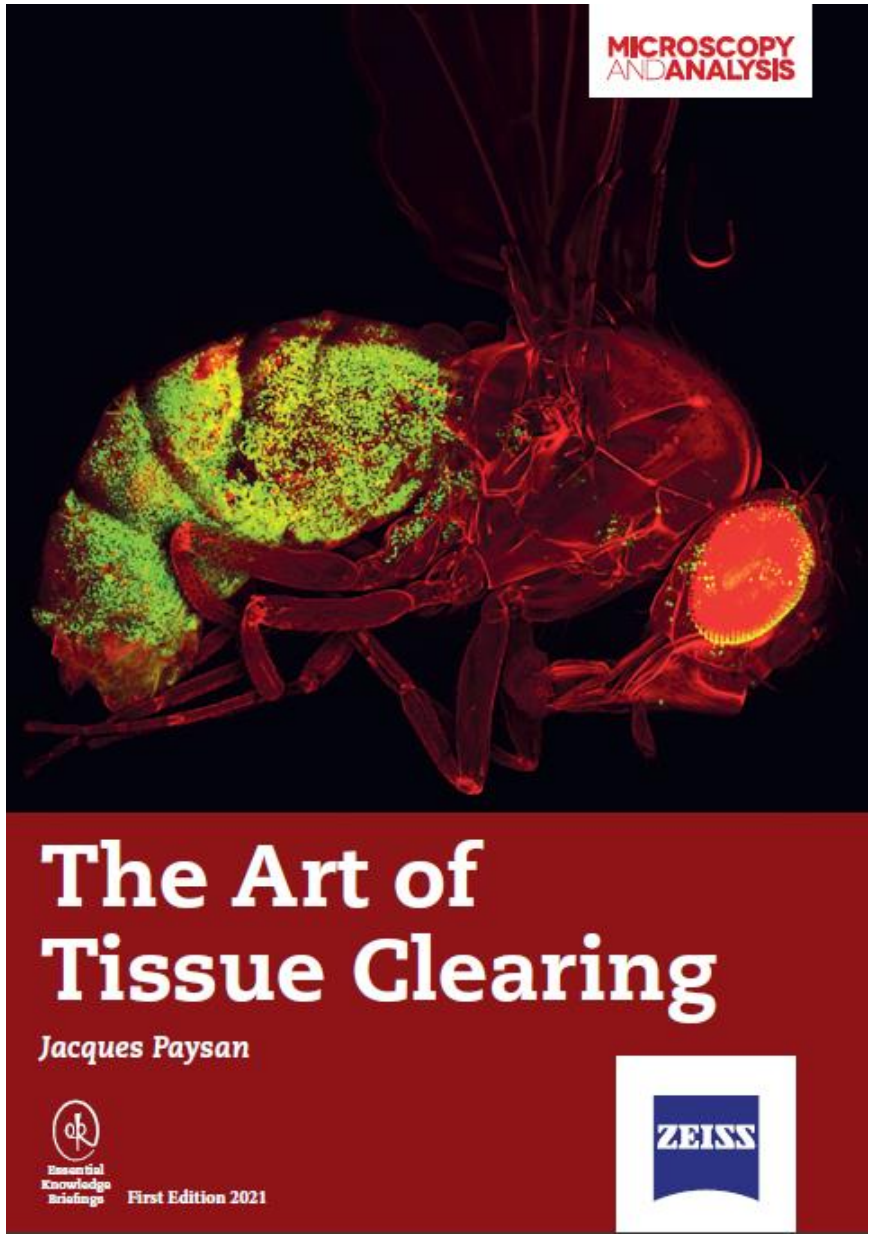
Light sheet Cleared Tissue Imaging

Menu of clearing methods

Organic solvents	Aequous solutions		Hyperhydrating solutions		Tissue Transformation
	organic / water	sugar / water	w/o detergent	w/detergent	
BB MS (Spalteholtz)	RTF	SeeDB	$Clear^{T}$	Sca/e	CLARITY
BABB (Murray)	TDE	SeeDB2	$Clear^{T2}$	Sca/eS	Bone CLARITY
3DISCO	C_e3D	FRUIT		CUBIC	PEA-CLARITY
iDISCO				CUBIC X	ACT-PRESTO
vDISCO					CRISTAL
Eci					Stochastic Electrotransport
2Eci					Switch
PEGASOS					SHIELD
					MYOCLEAR
					RIMS
					PACT, PARS
					psPACT, mPACT
					SCM
					FxClear

Light sheet Cleared Tissue Imaging

The Art of Tissue Clearing



Light sheet Cleared Tissue Imaging

BB MS (Spalteholtz) (Werner Spalteholz (1861 – 1940)

Die Arterien der Herzwand

Anatomische Untersuchungen
an Menschen- und Tierherzen

Nebst Erörterung der Voraussetzungen für die Herstellung
eines Kollateralkreislaufes

von

Werner Spalteholz

Mit einer Lichtdrucktafel, 27 Autotypien auf 15 Tafeln und 46 Textfiguren

Verlag von S. Hirzel in Leipzig / 1924

Das von mir zunächst für die Darstellung der Herzgefäße ausgearbeitete Verfahren des Durchsichtigmachens, das sich in der Folge auch für andere Objekte bewährt hat, ist bereits an anderer Stelle ausführlich erörtert worden.

Indem ich im allgemeinen auf diese Veröffentlichung[1]) verweise, will ich an dieser Stelle nur die Methode in ihrer speziellen Anwendungsform für das Herz kurz zusammenstellen:

1. Injizieren der Koronargefäße;
2. Fixieren in 10 %iger Formalinlösung unter Ausdehnung der Herzhöhlen (s. oben), mindestens 48 Stunden;
3. Bleichen in käuflichem Wasserstoffsuperoxyd, dem 1 % Formalin zugesetzt ist, mehrere Tage, eventuell unter Wechseln der Flüssigkeit;
4. Wässern in fließendem Leitungswasser, mindestens 24 Stunden lang;
5. Übertragen in destilliertes Wasser für 48 Stunden unter zweimaligem Wechsel desselben;
6. Entwässern in steigendem Alkohol, bis zu absolutem Alkohol, der zweimal gewechselt werden muß[2]);

7. Übertragen in Benzol (zweimal wechseln);
8. Einlegen in die Endflüssigkeit (s. unten);
9. Evakuieren unter der Luftpumpe zur Entfernung des Benzols und der Luft.

Als Endflüssigkeit verwende ich für menschliche erwachsene Herzen zunächst ein Gemisch von 1 Gewichtsteil künstliches Wintergrünöl (Gaultheriaöl, Salizylsäuremethylester) und 1 Gewichtsteil Benzylbenzoat (bzw. 9 Teilen Wintergrünöl und 5 Teilen Isosafrol), für Herzen von menschlichen Neugeborenen und von Säugetieren von 4 Teilen Wintergrünöl und 3 Teilen Benzylbenzoat (bzw. 9 Teilen Wintergrünöl und 4 Teilen Isosafrol) und korrigiere dann den Brechungsindex, wenn die Durchsichtigkeit nicht maximal ist, durch Hinzufügen entweder von Wintergrünöl oder von Benzylbenzoat (bzw. Isosafrol).

Light sheet Cleared Tissue Imaging

BABB (Murray's Clear)

RESEARCH PAPER: IMAGING

Organogenesis 5:4, 145-148; October/November/December 2009; © 2009 Landes Bioscience

3D-reconstruction of blood vessels by ultramicroscopy

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Key words: 3D-reconstruction, blood vessels, cancer, LEA, lectin, microvasculature, morphology, ultramicroscopy, whole mount

Abbreviations: BABB, 1 part benzyl alcohol + 2 parts benzylbenzoate; Bl6, black six mice; CCD camera, charge-coupled device camera; CT, computer tomography; FITC, fluorescein isothiocyanate; LEA, *Lycopersicon esculentum* agglutinin; MIP, maximum intensity projection; N.A., numerical aperture; OPFOS, orthogonal plane fluorescence optical sectioning; PB, phosphate buffer; PBS, phosphate buffer saline; PFA, paraformaldehyde; PSF, point spread function; SPIM, single plane illumination microscopy; UM, ultramicroscopy; XFP, fluorescent protein

As recently shown, ultramicroscopy (UM) allows 3D-visualization of even large microscopic structures with µm resolution. Thus, it can be applied to anatomical studies of numerous biological and medical specimens. We reconstructed the three-dimensional architecture of tomato-lectin (*Lycopersicon esculentum*) stained vascular networks by UM in whole mouse organs. The topology of filigree branches of the microvasculature was visualized. Since tumors require an extensive growth of blood vessels to survive, this novel approach may open up new vistas in neurobiology and histology, particularly in cancer research.

Introduction

Various novel microscopical techniques have given new impulses to biological sciences in the last decades. One of these new imaging technique is ultramicroscopy (UM), which recently has been shown to allow three-dimensional reconstructions of even cm-sized specimen with micrometer resolution.[1-3] Thus, UM bridges a gap between confocal microscopy and macroscopic imaging techniques like computer tomography (CT), making it a versatile tool for anatomical studies of numerous biological and medical specimens.

In UM, the specimen is illuminated perpendicular to the observation pathway by a very thin sheet of laser light, formed by optical elements (**Fig. 1**). Because illumination of out of focus layers is avoided in UM, no light has to be excluded by a pinhole later on, like in confocal microscopy. By stepping the specimen chamber vertically through the laser light sheet optical sectioning is obtained. Since in UM the light has to travel horizontally along the whole width of a specimen, it is necessary that specimens are sufficiently transparent. This is achieved by a chemical clearing procedure, which is based on replacing the water in the specimen by a liquid having approximately the same refractive index as protein. As a consequence, light scattering is strongly reduced, and the specimen becomes translucent.[4]

Lectins are proteins that highly specifically bind to sugar complexes, attached to proteins and lipids.[5] Presently, lectins are widely used in research, particular in serology, drug targeting and histopathology. In the recent decade, lectins became especially important as markers for microvascular labeling.[6-9]

Using UM, we three-dimensionally reconstructed several millimeter sized microvascular networks in whole mouse organs. The endothelial cells were labeled using tomato lectin from *Lycopersicon esculentum* (LEA), conjugated with a fluorescent dye (FITC).

Results and Discussion

We imaged lectin labeled mouse brains by UM and computed three-dimensional reconstructions from the obtained image stacks according to Becker, Jährling et al.[2] **Figure 2A** shows a top view of the brain hemispheres and the cerebellum. As can be seen from the figure, even the finest branches of the microvascular network are visualized. The major vessels generally show less staining. **Figure 2B** depicts a single cortical blood vessel, surrounded by complexly branched capillaries.

We also obtained three-dimensional images from the spinal cord (**Fig. 3A**), and of the auricles of the heart (**Fig. 3B**). In the 3D-reconstructions of the spinal cord some of the thin capillaries supporting the spinal nerves are apparent. The spinal canal (*Canalis spinalis*) is visible. In its center any vascularization is clearly completely lacking. The reconstructions of the heart (**Fig. 3B**) illustrate the microvascular architecture in the cardiac auricle (*Auricula atrii*), formed by close loops of capillaries.

UM of lectin-FITC labeled tissue enables three-dimensional imaging of vast vascular networks in whole mouse organs like

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Previously published online: www.landesbioscience.com/journals/organogenesis/article/10403

Claim:
Replacing the water in the specimen by a liquid having approximately the same refractive index as protein. As a consequence, light scattering is strongly reduced, and the specimen becomes translucent.

Method:
We dehydrated the organs using an ascending ethanol series (50%, 80%, 96%, 100%). Then, the organs were cleared in a solution containing 2 part benzyl benzoate and 1 part benzyl alcohol (BABB).

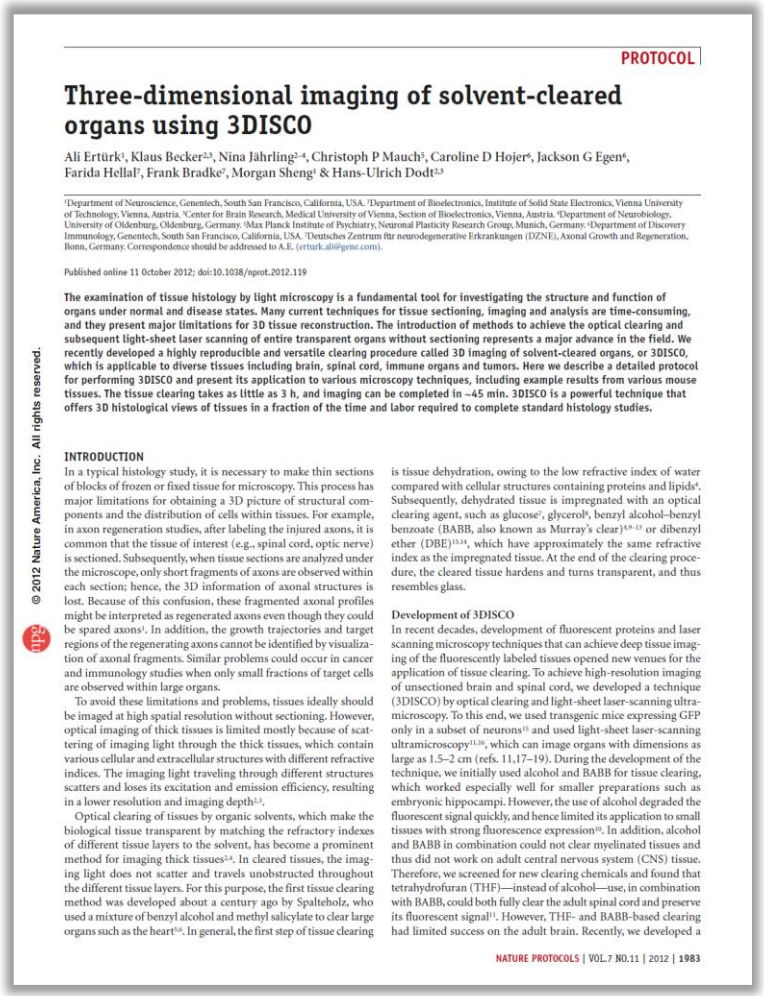
Imaging solution:
BABB. Ethyl Cinnamate

Refractive index: 1,55

Sample tissues:
Brain

Light sheet Cleared Tissue Imaging

3DISCO



PROTOCOL

Three-dimensional imaging of solvent-cleared organs using 3DISCO

Ali Ertürk[1], Klaus Becker[2,3], Nina Jährling[2–4], Christoph P Mauch[5], Caroline D Hojer[6], Jackson G Egen[6], Farida Hellal[7], Frank Bradke[7], Morgan Sheng[1] & Hans-Ulrich Dodt[2,3]

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The examination of tissue histology by light microscopy is a fundamental tool for investigating the structure and function of organs under normal and disease states. Many current techniques for tissue sectioning, imaging and analysis are time-consuming, and they present major limitations for 3D tissue reconstruction. The introduction of methods to achieve the optical clearing and subsequent light-sheet laser scanning of entire transparent organs without sectioning represents a major advance in the field. We recently developed a highly reproducible and versatile clearing procedure called 3D imaging of solvent-cleared organs, or 3DISCO, which is applicable to diverse tissues including brain, spinal cord, immune organs and tumors. Here we describe a detailed protocol for performing 3DISCO and present its application to various microscopy techniques, including example results from various mouse tissues. The tissue clearing takes as little as 3 h, and imaging can be completed in ~45 min. 3DISCO is a powerful technique that offers 3D histological views of tissues in a fraction of the time and labor required to complete standard histology studies.

INTRODUCTION

In a typical histology study, it is necessary to make thin sections of blocks of frozen or fixed tissue for microscopy. This process has major limitations for obtaining a 3D picture of structural components and the distribution of cells within tissues. For example, in axon regeneration studies, after labeling the injured axons, it is common that the tissue of interest (e.g., spinal cord, optic nerve) is sectioned. Subsequently, when tissue sections are analyzed under the microscope, only short fragments of axons are observed within each section; hence, the 3D information of axonal structures is lost. Because of this confusion, these fragmented axonal profiles might be interpreted as regenerated axons even though they could be spared axons[1]. In addition, the growth trajectories and target regions of the regenerating axons cannot be identified by visualization of axonal fragments. Similar problems could occur in cancer and immunology studies when only small fractions of target cells are observed within large organs.

To avoid these limitations and problems, tissues ideally should be imaged at high spatial resolution without sectioning. However, optical imaging of thick tissues is limited mostly because of scattering of imaging light through the thick tissues, which contain various cellular and extracellular structures with different refractive indices. The imaging light traveling through different structures scatters and loses its excitation and emission efficiency, resulting in a lower resolution and imaging depth[2,3].

Optical clearing of tissues by organic solvents, which make the biological tissue transparent by matching the refractory indexes of different tissue layers to the solvent, has become a prominent method for imaging thick tissues[2,4]. In cleared tissues, the imaging light does not scatter and travels unobstructed throughout the different tissue layers. For this purpose, the first tissue clearing method was developed about a century ago by Spalteholz, who used a mixture of benzyl alcohol and methyl salicylate to clear large organs such as the heart[5,6]. In general, the first step of tissue clearing is tissue dehydration, owing to the low refractive index of water compared with cellular structures containing proteins and lipids[4]. Subsequently, dehydrated tissue is impregnated with an optical clearing agent, such as glucose[7], glycerol[8], benzyl alcohol–benzyl benzoate (BABB, also known as Murray's clear)[4,9–12] or dibenzyl ether (DBE)[13,14], which have approximately the same refractive index as the impregnated tissue. At the end of the clearing procedure, the cleared tissue hardens and turns transparent, and thus resembles glass.

Development of 3DISCO

In recent decades, development of fluorescent proteins and laser scanning microscopy techniques that can achieve deep tissue imaging of the fluorescently labeled tissues opened new venues for the application of tissue clearing. To achieve high-resolution imaging of unsectioned brain and spinal cord, we developed a technique (3DISCO) by optical clearing and light-sheet laser-scanning ultramicroscopy. To this end, we used transgenic mice expressing GFP only in a subset of neurons[15] and used light-sheet laser-scanning ultramicroscopy[11,16], which can image organs with dimensions as large as 1.5–2 cm (refs. 11,17–19). During the development of the technique, we initially used alcohol and BABB for tissue clearing, which worked especially well for smaller preparations such as embryonic hippocampi. However, the use of alcohol degraded the fluorescent signal quickly, and hence limited its application to small tissues with strong fluorescence expression[10]. In addition, alcohol and BABB in combination could not clear myelinated tissues and thus did not work on adult central nervous system (CNS) tissue. Therefore, we screened for new clearing chemicals and found that tetrahydrofuran (THF)—instead of alcohol—use, in combination with BABB, could both fully clear the adult spinal cord and preserve its fluorescent signal[11]. However, THF- and BABB-based clearing had limited success on the adult brain. Recently, we developed a

Claim:
Highly reproducible and versatile clearing procedure

Method:
Dehydration in THF and clearing in organic clearing reagents.

Imaging solution: DBE

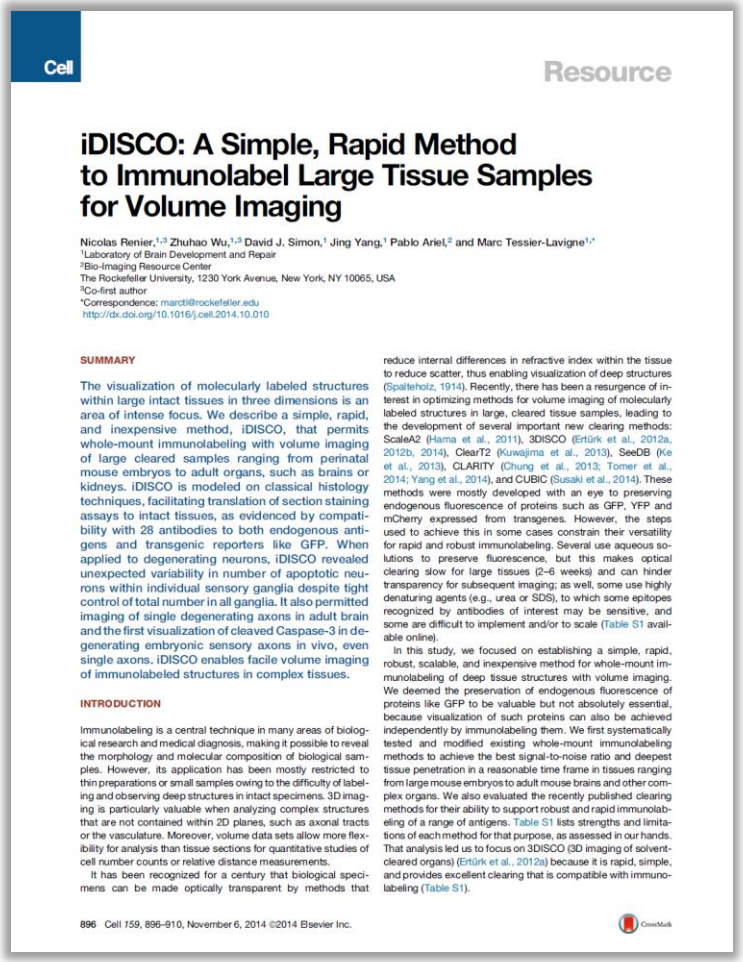
Refractive Index: **1,56**

Sample tissues:
Brain, spinal cord, immune organs and tumors.

Chemical name	Toxic
Tetrahydrofuran	Yes
Dichloromethane	Yes
Dibenzyl ether	Yes
Benzyl alcohol	Yes
Benzyl benzoate	Yes
Paraformaldehyde	Yes

Light sheet Cleared Tissue Imaging

iDISCO



Cell

Resource

iDISCO: A Simple, Rapid Method to Immunolabel Large Tissue Samples for Volume Imaging

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SUMMARY

The visualization of molecularly labeled structures within large intact tissues in three dimensions is an area of intense focus. We describe a simple, rapid, and inexpensive method, iDISCO, that permits whole-mount immunolabeling with volume imaging of large cleared samples ranging from perinatal mouse embryos to adult organs, such as brains or kidneys. iDISCO is modeled on classical histology techniques, facilitating translation of section staining assays to intact tissues, as evidenced by compatibility with 28 antibodies to both endogenous antigens and transgenic reporters like GFP. When applied to degenerating neurons, iDISCO revealed unexpected variability in number of apoptotic neurons within individual sensory ganglia despite tight control of total number in all ganglia. It also permitted imaging of single degenerating axons in adult brain and the first visualization of cleaved Caspase-3 in degenerating embryonic sensory axons in vivo, even single axons. iDISCO enables facile volume imaging of immunolabeled structures in complex tissues.

INTRODUCTION

Immunolabeling is a central technique in many areas of biological research and medical diagnosis, making it possible to reveal the morphology and molecular composition of biological samples. However, its application has been mostly restricted to thin preparations or small samples owing to the difficulty of labeling and observing deep structures in intact specimens. 3D imaging is particularly valuable when analyzing complex structures that are not contained within 2D planes, such as axonal tracts or the vasculature. Moreover, volume data sets allow more flexibility for analysis than tissue sections for quantitative studies of cell number counts or relative distance measurements.

It has been recognized for a century that biological specimens can be made optically transparent by methods that reduce internal differences in refractive index within the tissue to reduce scatter, thus enabling visualization of deep structures (Spalteholz, 1914). Recently, there has been a resurgence of interest in optimizing methods for volume imaging of molecularly labeled structures in large, cleared tissue samples, leading to the development of several important new clearing methods: ScaleA2 (Hama et al., 2011), 3DISCO (Ertürk et al., 2012a, 2012b, 2014), ClearT2 (Kuwajima et al., 2013), SeeDB (Ke et al., 2013), CLARITY (Chung et al., 2013; Tomer et al., 2014; Yang et al., 2014), and CUBIC (Susaki et al., 2014). These methods were mostly developed with an eye to preserving endogenous fluorescence of proteins such as GFP, YFP and mCherry expressed from transgenes. However, the steps used to achieve this in some cases constrain their versatility for rapid and robust immunolabeling. Several use aqueous solutions to preserve fluorescence, but this makes optical clearing slow for large tissues (2–6 weeks) and can hinder transparency for subsequent imaging; as well, some use highly denaturing agents (e.g., urea or SDS), to which some epitopes recognized by antibodies of interest may be sensitive, and some are difficult to implement and/or to scale (Table S1 available online).

In this study, we focused on establishing a simple, rapid, robust, scalable, and inexpensive method for whole-mount immunolabeling of deep tissue structures with volume imaging. We deemed the preservation of endogenous fluorescence of proteins like GFP to be valuable but not absolutely essential, because visualization of such proteins can also be achieved independently by immunolabeling them. We first systematically tested and modified existing whole-mount immunolabeling methods to achieve the best signal-to-noise ratio and deepest tissue penetration in a reasonable time frame in tissues ranging from large mouse embryos to adult mouse brains and other complex organs. We also evaluated the recently published clearing methods for their ability to support robust and rapid immunolabeling of a range of antigens. Table S1 lists strengths and limitations of each method for that purpose, as assessed in our hands. That analysis led us to focus on 3DISCO (3D imaging of solvent-cleared organs) (Ertürk et al., 2012a) because it is rapid, simple, and provides excellent clearing that is compatible with immunolabeling (Table S1).

Claim:
Simple, rapid,and inexpensive method, that permits whole-mount immunolabeling with volume imaging of large cleared samples.

Method:
Dehydrate in methanol series, followed by dichloromethane (DCM) and dibenzylether (DBE).

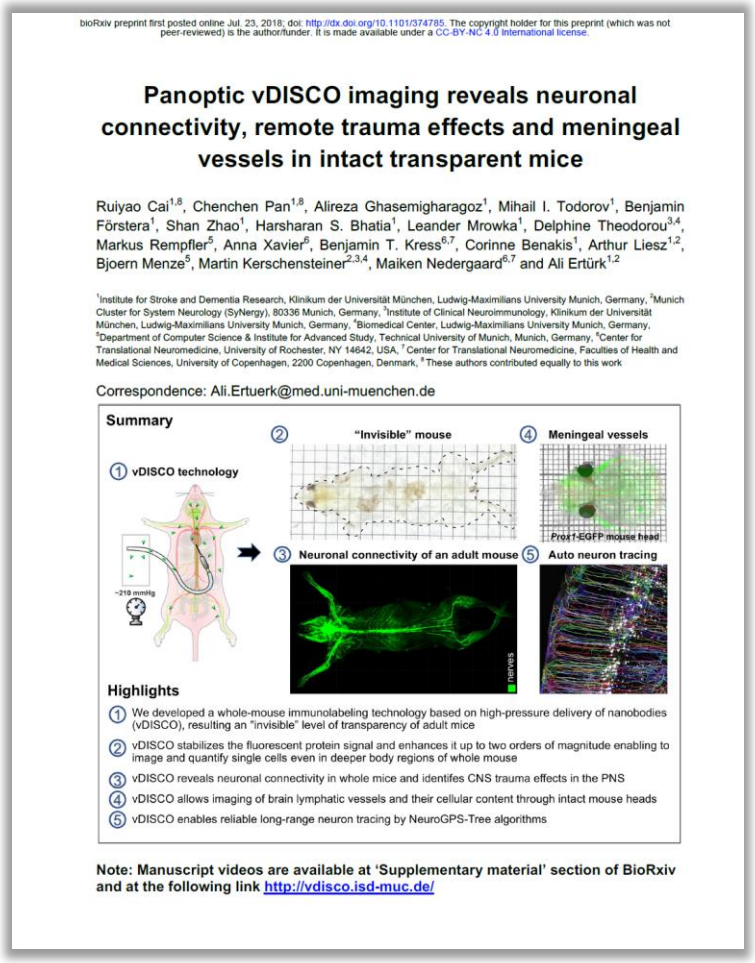
Imaging solution: DBE

Refractive Index: **1,56**

Sample tissues:
Ranging from perinatal mouse embryos to adult organs, such as brains or kidneys.

Light sheet Cleared Tissue Imaging

vDISCO (Ruiyao Cai et al and Ali Ertürk, Bioxiv (2018))



Panoptic vDISCO imaging reveals neuronal connectivity, remote trauma effects and meningeal vessels in intact transparent mice

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Summary

Highlights

① We developed a whole-mouse immunolabeling technology based on high-pressure delivery of nanobodies (vDISCO), resulting an "invisible" level of transparency of adult mice

② vDISCO stabilizes the fluorescent protein signal and enhances it up to two orders of magnitude enabling to image and quantify single cells even in deeper body regions of whole mouse

③ vDISCO reveals neuronal connectivity in whole mice and identifes CNS trauma effects in the PNS

④ vDISCO allows imaging of brain lymphatic vessels and their cellular content through intact mouse heads

⑤ vDISCO enables reliable long-range neuron tracing by NeuroGPS-Tree algorithms

Note: Manuscript videos are available at 'Supplementary material' section of BioRxiv and at the following link http://vdisco.isd-muc.de/

Claim:
A pressure driven, nanobody based whole-body immunolabeling technology to enhance the signal of fluorescent proteins by up to two orders of magnitude.

Method:
After the staining, animals are cleared using a 3DISCO based passive whole-body clearing protocol optimized for big samples. The samples are dehydrated in a tetrahydrofuran dilution series, followed by 3 hours in dichloromethane and finally in BABB.

Imaging Solution: BABB

Refractive Index: 1,559

Sample Tissues:
Whole-body neuronal connectivity of an entire adult mouse; brain trauma induced degeneration of peripheral axons; meningeal lymphatic vessels and immune cells through the intact skull and vertebra in naive animals and trauma models.

Light sheet Cleared Tissue Imaging

Eci (Ethyl cinnamate)

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Fully Automated Evaluation of Total Glomerular Number and Capillary Tuft Size in Nephritic Kidneys Using Lightsheet Microscopy

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Associated Data

• Supplementary Materials

Abstract

Go to:

The total number of glomeruli is a fundamental parameter of kidney function but very difficult to determine using standard methodology. Here, we counted all individual glomeruli in murine kidneys and sized the capillary tufts by combining *in vivo* fluorescence labeling of endothelial cells, a novel tissue–clearing technique, lightsheet microscopy, and automated registration by image analysis. Total hands–on time per organ was <1 hour, and automated counting/sizing was finished in <3 hours. We also investigated the novel use of ethyl-3-phenylprop-2-enoate (ethyl cinnamate) as a nontoxic solvent–based clearing reagent that can be handled without specific safety measures. Ethyl cinnamate rapidly cleared all tested organs, including calcified bone, but the fluorescence of proteins and immunohistochemical labels was maintained over weeks. Using ethyl cinnamate–cleared kidneys, we also quantified the average creatinine clearance rate per glomerulus. This parameter decreased in the first week of experimental nephrotoxic nephritis, whereas reduction in glomerular numbers occurred much later. Our approach delivers fundamental parameters of renal function, and because of its ease of use and speed, it is suitable for high-throughput analysis and could greatly facilitate studies of the effect of kidney diseases on whole-organ physiology.

Keywords: glomerular endothelial cells, glomerular filtration rate, glomerulonephritis, immune complexes, Immunology and pathology, kidney anatomy

A highly organized vascular system is essential for kidney function, and impaired circulatory performance in nephrons results in organ failure. Every nephron contains a tuft of capillaries within the glomerulus that performs blood ultrafiltration.[1] During chronic renal diseases, the numbers and function of nephrons are considered to decline. Until now, enumerating nephrons/glomeruli and validating the integrity of their tuft structures were only possible either by laborious tissue embedding/sectioning and manual stereologic counting[2,3] or after kidney maceration.[4] Therefore, many studies extrapolate glomerular numbers on sample counts, which is prone to errors. Furthermore, precise morphologic information relating to the structure and position of individual glomeruli within the whole organ is lost. The latter is amenable by high–field magnetic resonance imaging, which however, requires advanced laboratory equipment and suffers from relatively poor resolution that precludes distinction between Bowman's capsule and tuft.[5,6]

High–resolution optical three–dimensional reconstructions of kidneys on the basis of physical sectioning and serial imaging can, in principle, distinguish between glomerular walls and tufts. This procedure, however, is highly labor intensive, slow, and prone to errors, and it requires extensive experience for satisfactory results.[7] Lightsheet fluorescence microscopy (LSFM) has revolutionized the field by enabling the reconstruction of intact organs or whole animals with cellular resolution in three dimensions within

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Light-sheet fluorescence microscopy for quantitative biology. [Nat Methods. 2015]
Reconstruction of zebrafish early embryonic development by

Claim:
Ethyl cinnamate rapidly cleared all tested organs, including calcified bone, but the fluorescence of proteins and immunohistochemical labels was maintained over weeks.

Clearing Method:
Passive clearing in EtCi following ethanol dehydration.

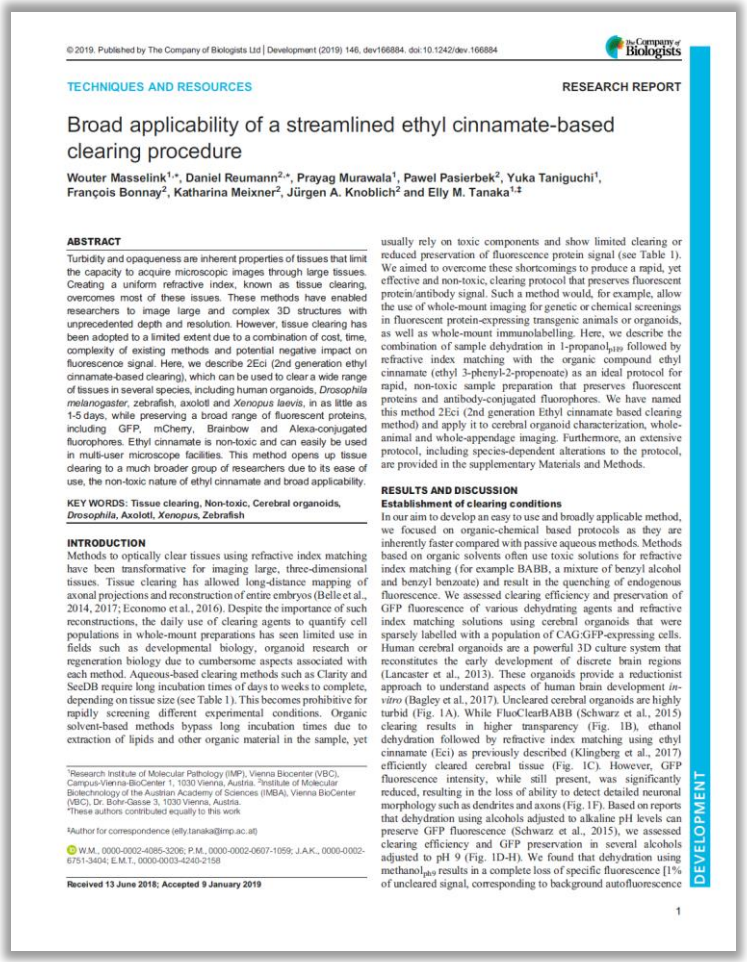
Imaging Solution:
EtCi

Refractive Index: **1,56**

Sample tissues:
Kidney

Light sheet Cleared Tissue Imaging

2Eci (2nd generation ethyl cinnamate-based clearing)



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TECHNIQUES AND RESOURCES

RESEARCH REPORT

Broad applicability of a streamlined ethyl cinnamate-based clearing procedure

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ABSTRACT

Turbidity and opaqueness are inherent properties of tissues that limit the capacity to acquire microscopic images through large tissues. Creating a uniform refractive index, known as tissue clearing, overcomes most of these issues. These methods have enabled researchers to image large and complex 3D structures with unprecedented depth and resolution. However, tissue clearing has been adopted to a limited extent due to a combination of cost, time, complexity of existing methods and potential negative impact on fluorescence signal. Here, we describe 2Eci (2nd generation ethyl cinnamate-based clearing), which can be used to clear a wide range of tissues in several species, including human organoids, *Drosophila melanogaster*, zebrafish, axolotl and *Xenopus laevis*, in as little as 1-5 days, while preserving a broad range of fluorescent proteins, including GFP, mCherry, Brainbow and Alexa-conjugated fluorophores. Ethyl cinnamate is non-toxic and can easily be used in multi-user microscope facilities. This method opens up tissue clearing to a much broader group of researchers due to its ease of use, the non-toxic nature of ethyl cinnamate and broad applicability.

KEY WORDS: Tissue clearing, Non-toxic, Cerebral organoids, *Drosophila*, Axolotl, *Xenopus*, Zebrafish

INTRODUCTION

Methods to optically clear tissues using refractive index matching have been transformative for imaging large, three-dimensional tissues. Tissue clearing has allowed long-distance mapping of axonal projections and reconstruction of entire embryos (Belle et al., 2014, 2017; Economo et al., 2016). Despite the importance of such reconstructions, the daily use of clearing agents to quantify cell populations in whole-mount preparations has seen limited use in fields such as developmental biology, organoid research or regeneration biology due to cumbersome aspects associated with each method. Aqueous-based clearing methods such as Clarity and SeeDB require long incubation times of days to weeks to complete, depending on tissue size (see Table 1). This becomes prohibitive for rapidly screening different experimental conditions. Organic solvent-based methods bypass long incubation times due to extraction of lipids and other organic material in the sample, yet usually rely on toxic components and show limited clearing or reduced preservation of fluorescence protein signal (see Table 1). We aimed to overcome these shortcomings to produce a rapid, yet effective and non-toxic, clearing protocol that preserves fluorescent protein/antibody signal. Such a method would, for example, allow the use of whole-mount imaging for genetic or chemical screenings in fluorescent protein-expressing transgenic animals or organoids, as well as whole-mount immunolabelling. Here, we describe the combination of sample dehydration in 1-propanol$_{ph9}$ followed by refractive index matching with the organic compound ethyl cinnamate (ethyl 3-phenyl-2-propenoate) as an ideal protocol for rapid, non-toxic sample preparation that preserves fluorescent proteins and antibody-conjugated fluorophores. We have named this method 2Eci (2nd generation Ethyl cinnamate based clearing method) and apply it to cerebral organoid characterization, whole-animal and whole-appendage imaging. Furthermore, an extensive protocol, including species-dependent alterations to the protocol, are provided in the supplementary Materials and Methods.

RESULTS AND DISCUSSION

Establishment of clearing conditions

In our aim to develop an easy to use and broadly applicable method, we focused on organic-chemical based protocols as they are inherently faster compared with passive aqueous methods. Methods based on organic solvents often use toxic solutions for refractive index matching (for example BABB, a mixture of benzyl alcohol and benzyl benzoate) and result in the quenching of endogenous fluorescence. We assessed clearing efficiency and preservation of GFP fluorescence of various dehydrating agents and refractive index matching solutions using cerebral organoids that were sparsely labelled with a population of CAG:GFP-expressing cells. Human cerebral organoids are a powerful 3D culture system that reconstitutes the early development of discrete brain regions (Lancaster et al., 2013). These organoids provide a reductionist approach to understand aspects of human brain development *in-vitro* (Bagley et al., 2017). Uncleared cerebral organoids are highly turbid (Fig. 1A). While FluoClearBABB (Schwarz et al., 2015) clearing results in higher transparency (Fig. 1B), ethanol dehydration followed by refractive index matching using ethyl cinnamate (Eci) as previously described (Klingberg et al., 2017) efficiently cleared cerebral tissue (Fig. 1C). However, GFP fluorescence intensity, while still present, was significantly reduced, resulting in the loss of ability to detect detailed neuronal morphology such as dendrites and axons (Fig. 1F). Based on reports that dehydration using alcohols adjusted to alkaline pH levels can preserve GFP fluorescence (Schwarz et al., 2015), we assessed clearing efficiency and GFP preservation in several alcohols adjusted to pH 9 (Fig. 1D-H). We found that dehydration using methanol$_{ph9}$ results in a complete loss of specific fluorescence [1% of uncleared signal, corresponding to background autofluorescence

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DEVELOPMENT

Claim:
Here, we describe 2Eci, which can be used to clear a wide range of tissues in as little as 1-5 days, while preserving a broad range of fluorescent proteins, including GFP, mCherry, Brainbow and Alexa-conjugated fluorophores.

Clearing Method:
Dehydration in 4-butanol$_{ph\ 9}$ or 1-propanol$_{ph9}$ followed by passive clearing in EtCi.

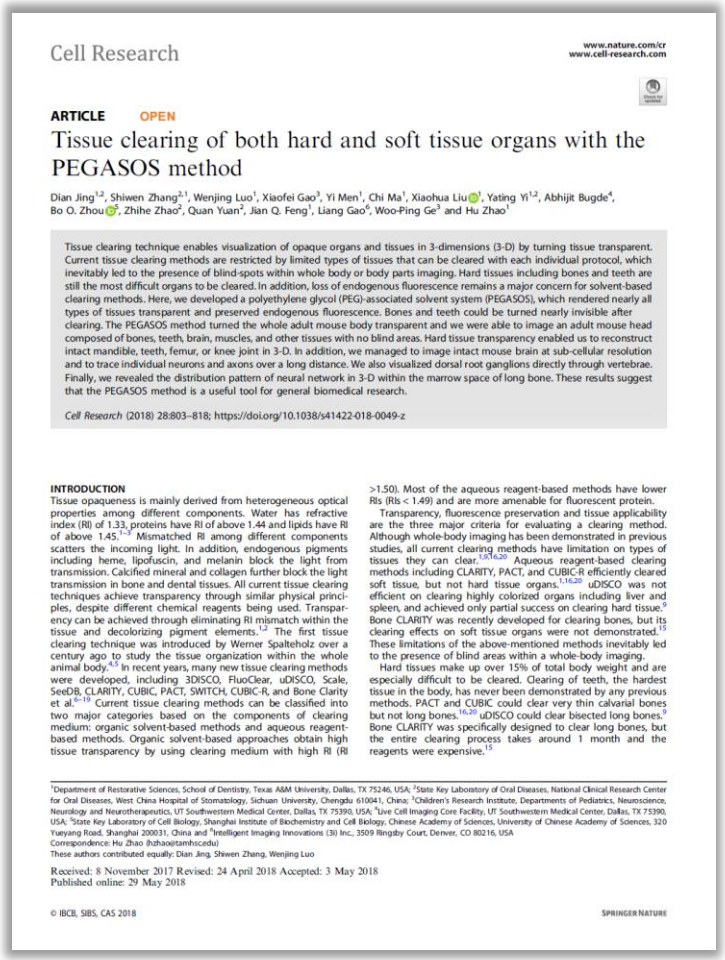
Imaging Solution:
EtCi

Refractive Index: 1,56

Sample tissues:
Human organoids, Drosophila melanogaster, zebrafish, axolotl and Xenopus laevis.

Light sheet Cleared Tissue Imaging

PEGASOS (Polyethylene glycol (PEG) associated solvent system)



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ARTICLE OPEN

Tissue clearing of both hard and soft tissue organs with the PEGASOS method

Dian Jing[1,2], Shiwen Zhang[2,1], Wenjing Luo[1], Xiaofei Gao[3], Yi Men[1], Chi Ma[1], Xiaohua Liu[1], Yating Yi[1,2], Abhijit Bugde[4], Bo O. Zhou[5], Zhihe Zhao[2], Quan Yuan[2], Jian Q. Feng[1], Liang Gao[6], Woo-Ping Ge[3] and Hu Zhao[1]

Tissue clearing technique enables visualization of opaque organs and tissues in 3-dimensions (3-D) by turning tissue transparent. Current tissue clearing methods are restricted by limited types of tissues that can be cleared with each individual protocol, which inevitably led to the presence of blind-spots within whole body or body parts imaging. Hard tissues including bones and teeth are still the most difficult organs to be cleared. In addition, loss of endogenous fluorescence remains a major concern for solvent-based clearing methods. Here, we developed a polyethylene glycol (PEG)-associated solvent system (PEGASOS), which rendered nearly all types of tissues transparent and preserved endogenous fluorescence. Bones and teeth could be turned nearly invisible after clearing. The PEGASOS method turned the whole adult mouse body transparent and we were able to image an adult mouse head composed of bones, teeth, brain, muscles, and other tissues with no blind areas. Hard tissue transparency enabled us to reconstruct intact mandible, teeth, femur, or knee joint in 3-D. In addition, we managed to image intact mouse brain at sub-cellular resolution and to trace individual neurons and axons over a long distance. We also visualized dorsal root ganglions directly through vertebrae. Finally, we revealed the distribution pattern of neural network in 3-D within the marrow space of long bone. These results suggest that the PEGASOS method is a useful tool for general biomedical research.

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INTRODUCTION

Tissue opaqueness is mainly derived from heterogeneous optical properties among different components. Water has refractive index (RI) of 1.33, proteins have RI of above 1.44 and lipids have RI of above 1.45.[1–3] Mismatched RI among different components scatters the incoming light. In addition, endogenous pigments including heme, lipofuscin, and melanin block the light from transmission. Calcified mineral and collagen further block the light transmission in bone and dental tissues. All current tissue clearing techniques achieve transparency through similar physical principles, despite different chemical reagents being used. Transparency can be achieved through eliminating RI mismatch within the tissue and decolorizing pigment elements.[1,2] The first tissue clearing technique was introduced by Werner Spalteholz over a century ago to study the tissue organization within the whole animal body.[4,5] In recent years, many new tissue clearing methods were developed, including 3DISCO, FluoClear, uDISCO, Scale, SeeDB, CLARITY, CUBIC, PACT, SWITCH, CUBIC-R, and Bone Clarity et al.[6–19] Current tissue clearing methods can be classified into two major categories based on the components of clearing medium: organic solvent-based methods and aqueous reagent-based methods. Organic solvent-based approaches obtain high tissue transparency by using clearing medium with high RI (RI >1.50). Most of the aqueous reagent-based methods have lower RIs (RIs < 1.49) and are more amenable for fluorescent protein.

Transparency, fluorescence preservation and tissue applicability are the three major criteria for evaluating a clearing method. Although whole-body imaging has been demonstrated in previous studies, all current clearing methods have limitation on types of tissues they can clear.[1,6,7,16,20] Aqueous reagent-based clearing methods including CLARITY, PACT, and CUBIC-R efficiently cleared soft tissue, but not hard tissue organs.[1,16,20] uDISCO was not efficient on clearing highly colorized organs including liver and spleen, and achieved only partial success on clearing hard tissue.[9] Bone CLARITY was recently developed for clearing bones, but its clearing effects on soft tissue organs were not demonstrated.[15] These limitations of the above-mentioned methods inevitably led to the presence of blind areas within a whole-body imaging.

Hard tissues make up over 15% of total body weight and are especially difficult to be cleared. Clearing of teeth, the hardest tissue in the body, has never been demonstrated by any previous methods. PACT and CUBIC could clear very thin calvarial bones but not long bones.[16,20] uDISCO could clear bisected long bones.[9] Bone CLARITY was specifically designed to clear long bones, but the entire clearing process takes around 1 month and the reagents were expensive.[15]

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Claim:
PEGASOS rendered nearly all types of tissues transparent and preserved endogenous fluorescence. Bones and teeth could be turned nearly invisible after clearing. The PEGASOS method turned the whole adult mouse body transparent and we were able to image an adult mouse head composed of bones, teeth, brain, muscles, and other tissues with no blind areas.

Method:
Tissue samples are fixed in PFA, and immersed in 20% EDTA for 4 days. Samples are then decolorized with Quadrol decolorization and ammonium solution, followed by delipidation in gradient tB delipidation solutions and tB-PEG for dehydration. Finally, samples are immersed in BB PEG medium for clearing.

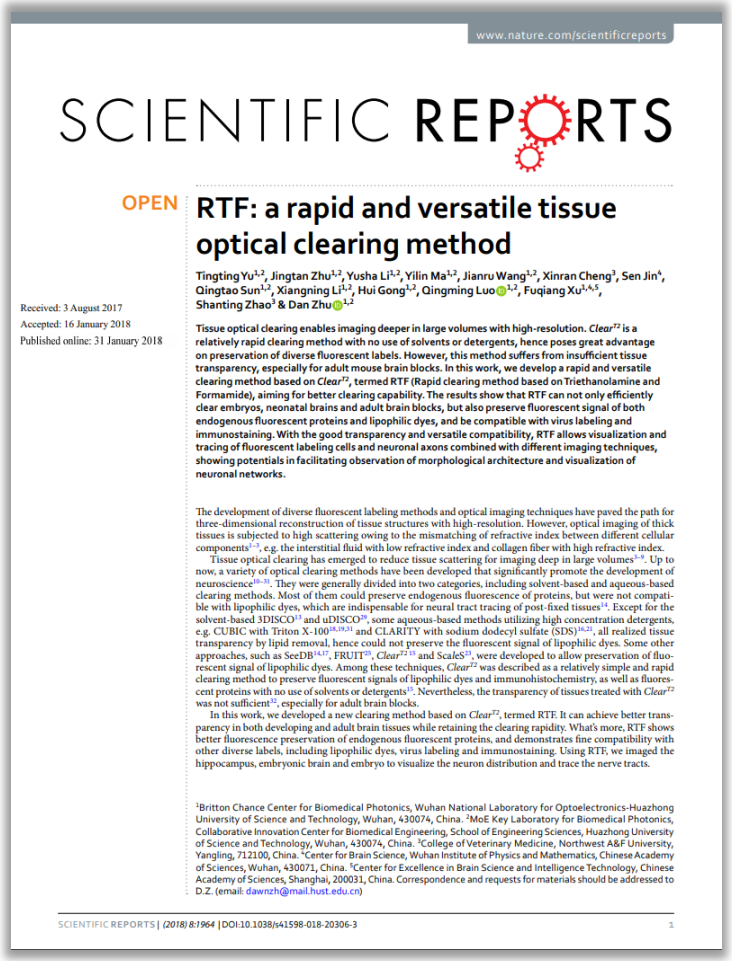
Imaging Solution: BB PEG Clearing Medium

Refractive Index: 1,543

Sample Tissues:
See claims above

Light sheet Cleared Tissue Imaging

RTF (Rapid clearing method based on triethanolamine and formamide)



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SCIENTIFIC REPORTS

OPEN

RTF: a rapid and versatile tissue optical clearing method

Tingting Yu[1,2], Jingtan Zhu[1,2], Yusha Li[1,2], Yilin Ma[1,2], Jianru Wang[1,2], Xinran Cheng[3], Sen Jin[4], Qingtao Sun[1,2], Xiangning Li[1,2], Hui Gong[1,2], Qingming Luo[1,2], Fuqiang Xu[1,4,5], Shanting Zhao[3] & Dan Zhu[1,2]

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Tissue optical clearing enables imaging deeper in large volumes with high-resolution. *Clear*^{T2} is a relatively rapid clearing method with no use of solvents or detergents, hence poses great advantage on preservation of diverse fluorescent labels. However, this method suffers from insufficient tissue transparency, especially for adult mouse brain blocks. In this work, we develop a rapid and versatile clearing method based on *Clear*^{T2}, termed RTF (Rapid clearing method based on Triethanolamine and Formamide), aiming for better clearing capability. The results show that RTF can not only efficiently clear embryos, neonatal brains and adult brain blocks, but also preserve fluorescent signal of both endogenous fluorescent proteins and lipophilic dyes, and be compatible with virus labeling and immunostaining. With the good transparency and versatile compatibility, RTF allows visualization and tracing of fluorescent labeling cells and neuronal axons combined with different imaging techniques, showing potentials in facilitating observation of morphological architecture and visualization of neuronal networks.

The development of diverse fluorescent labeling methods and optical imaging techniques have paved the path for three-dimensional reconstruction of tissue structures with high-resolution. However, optical imaging of thick tissues is subjected to high scattering owing to the mismatching of refractive index between different cellular components[1–3], e.g. the interstitial fluid with low refractive index and collagen fiber with high refractive index.

Tissue optical clearing has emerged to reduce tissue scattering for imaging deep in large volumes[4–9]. Up to now, a variety of optical clearing methods have been developed that significantly promote the development of neuroscience[10–31]. They were generally divided into two categories, including solvent-based and aqueous-based clearing methods. Most of them could preserve endogenous fluorescence of proteins, but were not compatible with lipophilic dyes, which are indispensable for neural tract tracing of post-fixed tissues[14]. Except for the solvent-based 3DISCO[13] and uDISCO[28], some aqueous-based methods utilizing high concentration detergents, e.g. CUBIC with Triton X-100[16,29,31] and CLARITY with sodium dodecyl sulfate (SDS)[16–21], all realized tissue transparency by lipid removal, hence could not preserve the fluorescent signal of lipophilic dyes. Some other approaches, such as SeeDB[14,17], FRUIT[20], *Clear*^{T2}[15] and ScaleS[23], were developed to allow preservation of fluorescent signal of lipophilic dyes. Among these techniques, *Clear*^{T2} was described as a relatively simple and rapid clearing method to preserve fluorescent signals of lipophilic dyes and immunohistochemistry, as well as fluorescent proteins with no use of solvents or detergents[15]. Nevertheless, the transparency of tissues treated with *Clear*^{T2} was not sufficient[15], especially for adult brain blocks.

In this work, we developed a new clearing method based on *Clear*^{T2}, termed RTF. It can achieve better transparency in both developing and adult brain tissues while retaining the clearing rapidity. What's more, RTF shows better fluorescence preservation of endogenous fluorescent proteins, and demonstrates fine compatibility with other diverse labels, including lipophilic dyes, virus labeling and immunostaining. Using RTF, we imaged the hippocampus, embryonic brain and embryo to visualize the neuron distribution and trace the nerve tracts.

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Claim:
"RTF can not only efficiently clear embryos, neonatal brains and adult brain blocks, but also preserve fluorescent signal of both endogenous fluorescent proteins and lipophilic dyes, and be compatible with virus labeling and immunostaining".

Method:
For RTF, the samples were incubated in three solutions of triethanolamine and formamide in distilled water sequentially.

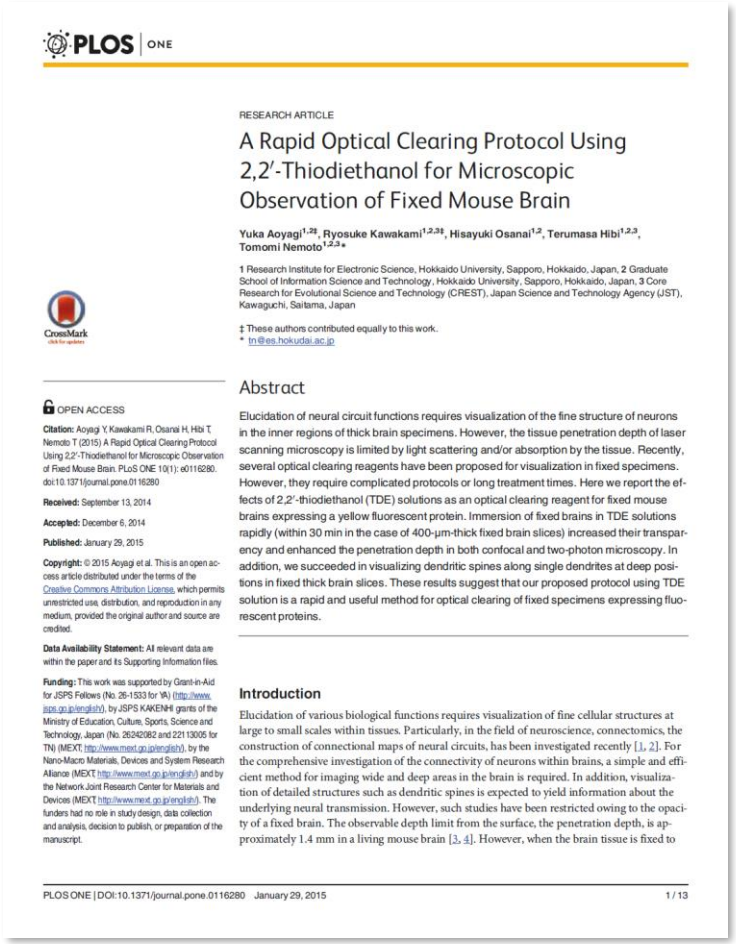
Imaging Solution: unknown

Refractive Index: unknown

Sample Tissues:
Embryos, neonatal and adult brains

Light sheet Cleared Tissue Imaging

TDE



PLOS ONE

RESEARCH ARTICLE

A Rapid Optical Clearing Protocol Using 2,2′-Thiodiethanol for Microscopic Observation of Fixed Mouse Brain

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Abstract

Elucidation of neural circuit functions requires visualization of the fine structure of neurons in the inner regions of thick brain specimens. However, the tissue penetration depth of laser scanning microscopy is limited by light scattering and/or absorption by the tissue. Recently, several optical clearing reagents have been proposed for visualization in fixed specimens. However, they require complicated protocols or long treatment times. Here we report the effects of 2,2′-thiodiethanol (TDE) solutions as an optical clearing reagent for fixed mouse brains expressing a yellow fluorescent protein. Immersion of fixed brains in TDE solutions rapidly (within 30 min in the case of 400-μm-thick fixed brain slices) increased their transparency and enhanced the penetration depth in both confocal and two-photon microscopy. In addition, we succeeded in visualizing dendritic spines along single dendrites at deep positions in fixed thick brain slices. These results suggest that our proposed protocol using TDE solution is a rapid and useful method for optical clearing of fixed specimens expressing fluorescent proteins.

Introduction

Elucidation of various biological functions requires visualization of fine cellular structures at large to small scales within tissues. Particularly, in the field of neuroscience, connectomics, the construction of connectional maps of neural circuits, has been investigated recently [1, 2]. For the comprehensive investigation of the connectivity of neurons within brains, a simple and efficient method for imaging wide and deep areas in the brain is required. In addition, visualization of detailed structures such as dendritic spines is expected to yield information about the underlying neural transmission. However, such studies have been restricted owing to the opacity of a fixed brain. The observable depth limit from the surface, the penetration depth, is approximately 1.4 mm in a living mouse brain [3, 4]. However, when the brain tissue is fixed to

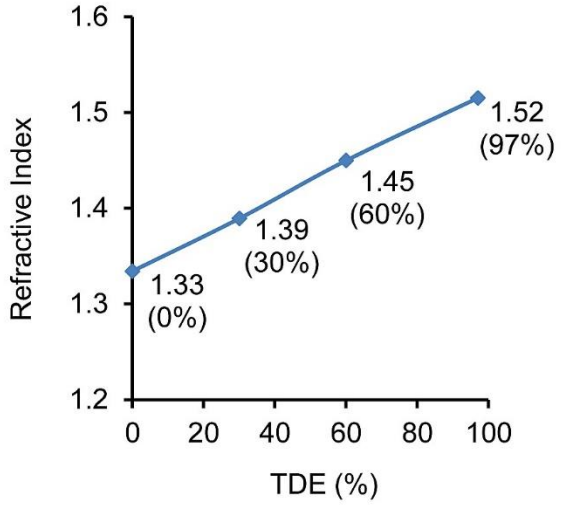
Claim
TDE solution is a rapid and useful method for optical clearing of fixed specimens expressing fluorescent proteins

Method:
Samples are immersed in various concentrations of TDE in PBS (0%, 30%, 60%, and 97%) for 6 h to 7 days.

Imaging Solution:
TDE Solution

Refractive Index: see fig.

Sample Tissues:
Brains, brain slices



Light sheet Cleared Tissue Imaging

C_e3D

Multiplex, quantitative cellular analysis in large tissue volumes with clearing-enhanced 3D microscopy (C_e3D)

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Contributed by Ronald N. Germain, July 19, 2017 (sent for review May 31, 2017; reviewed by Marc K. Jenkins and Mark J. Miller)

Organ homeostasis, cellular differentiation, signal relay, and in situ function all depend on the spatial organization of cells in complex tissues. For this reason, comprehensive, high-resolution mapping of cell positioning, phenotypic identity, and functional state in the context of macroscale tissue structure is critical to a deeper understanding of diverse biological processes. Here we report an easy to use method, clearing-enhanced 3D (C_e3D), which generates excellent tissue transparency for most organs, preserves cellular morphology and protein fluorescence, and is robustly compatible with antibody-based immunolabeling. This enhanced signal quality and capacity for extensive probe multiplexing permits quantitative analysis of distinct, highly intermixed cell populations in intact C_e3D-treated tissues via 3D histo-cytometry. We use this technology to demonstrate large-volume, high-resolution microscopy of diverse cell types in lymphoid and nonlymphoid organs, as well as to perform quantitative analysis of the composition and tissue distribution of multiple cell populations in lymphoid tissues. Combined with histo-cytometry, C_e3D provides a comprehensive strategy for volumetric quantitative imaging and analysis that bridges the gap between conventional section imaging and disassociation-based techniques.

tissue clearing | quantitative microscopy | histo-cytometry | immune system

Major physiological processes rely on the precise positioning of diverse cell types in specific anatomical locations. Such organization allows exposure of cells to appropriate tissue microenvironments that shape their differentiation, promote appropriate cell–cell communication events, and collectively define the global properties of the whole organ. Understanding these structure–function relationships requires a detailed mapping of both the large-scale organization and fine-grained molecular and cellular composition of complex tissues.

The majority of information on such processes comes from microscopic imaging of relatively thin (5–20 μm) "2D" tissue cross-sections, examining several markers of interest to visualize a limited number of cell populations with respect to a tissue's representative structural elements. Although providing an excellent framework for understanding general features and the respective positioning of well-represented cell types, such data lack information on 3D organization, being particularly limiting for irregular structures such as the vasculature, airways, nervous tissue, inflamed sites, tumors, or reactive lymph nodes. Furthermore, detection and analysis of rare cellular events requires imaging of a large number of disconnected sections, which introduces possible image selection bias and suffers from the potential omission of key physiological landmarks located just outside of the sampled area. Finally, many cell types require simultaneous visualization of multiple phenotypic markers for correct subset identification, making interpretation of cell composition within tissues problematic without the use of highly multiplexed imaging panels.

Recently, several tissue clearing methodologies have been developed that render organs transparent and allow section-free imaging of significant volumes, thereby improving our capacity to study the relationships between cell positioning and 3D tissue architecture (1–11). These techniques work by reducing light scattering in tissues through minimizing refractive index mismatches between the immersion medium and the various protein, aqueous, and lipid tissue constituents (12). However, each of the currently reported clearing techniques suffers to a greater or lesser extent from various method-specific limitations. For example, techniques that excel at brain tissue clearing through lipid extraction suffer from limited compatibility with immunolabeling, while solvent-based methods that permit useful antibody-based immunofluorescence microscopy induce substantial tissue shrinkage and are associated with suboptimal signal quality (1, 2, 6, 7, 9). These limitations prevent assessment of a tissue's large-scale structural organization in concert with high-fidelity information on single cell morphology and complex molecular phenotype, while also limiting the combined use of fluorescent genetic markers and antibody-based staining.

One application that requires highly optimized acquisition of such information is histo-cytometry (13). This imaging/analysis pipeline allows multiplex phenotypic identification and quantification of cells directly in tissues, akin to in situ flow cytometry. This technique has been used to study the composition, distribution, and function of densely packed cells with complex phenotypes and morphology in tissue sections (13–21). Even for cell types with relatively simple morphology, such as T and B lymphocytes, histo-cytometry requires a high degree of spatial signal resolution, achieved with high numerical aperture (N.A.) objectives and deconvolution. The ability to simultaneously detect

Significance

Major biological processes rely on the precise positioning of diverse cell types in specific anatomical locations. Existing techniques for studying cellular spatial positioning in tissues, especially with robust identification of densely packed cells, have substantial time, cost, resolution, and multiplexing limitations. Here, we describe an easy-to-use and inexpensive tissue clearing technique for attaining high-quality images of cells and diverse molecules of interest in substantial tissue volumes, enabling simultaneous quantitative analysis of 3D organ structure and fine-grained cellular composition. This technology will enhance our capacity for acquiring a quantitative understanding of the relationships between cells and their microenvironments in the context of broader tissue organization and is directly applicable to diverse biological disciplines as well as diagnostic medicine.

Author contributions: W.L., R.N.G., and M.Y.G. designed research; W.L. and M.Y.G. performed research; W.L. and M.Y.G. contributed new reagents/analytic tools; W.L., R.N.G., and M.Y.G. analyzed data; and W.L., R.N.G., and M.Y.G. wrote the paper.

Reviewers: M.K.J., University of Minnesota; and M.J.M., Washington University in St. Louis.

Conflict of interest statement: The initial patent for the methodology described in this paper was filed with the US Patent Office, E-Numbers: E-168-2016/0-US-01 Appl. Serial No.: 62/380,593, Title: "Clearing-enhanced 3d (ce3d): a novel tissue clearing method preserving cellular morphology, reporter fluorescence, epitope."

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Claim:
An easy to use method, clearing-enhanced 3D (Ce3D), which generates excellent tissue transparency for most organs, preserves cellular morphology and protein fluorescence, and is robustly compatible with antibody-based immunolabeling.

Method:
Stained and washed tissues are placed in Histodenz-based Ce3D medium inside a chemical fume hood and incubated at room temperature on a rotor for 12-72 hours.

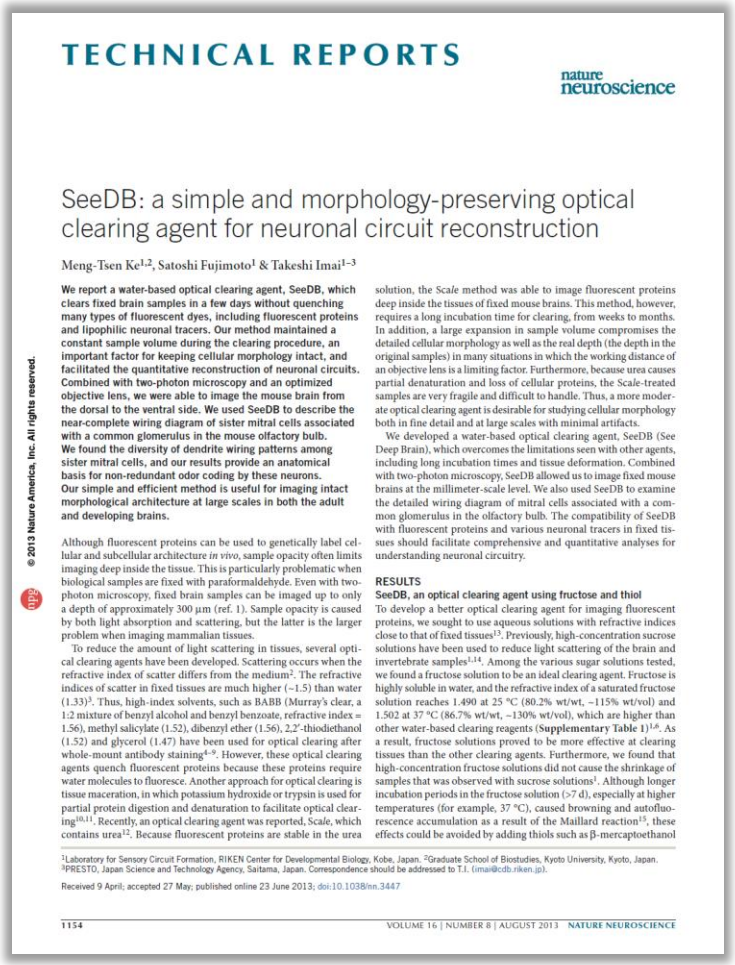
Imaging Solution: Ce3D medium

Refractive Index: 1,49 – 1,5

Sample Tissues:
Mouse brain, muscle, lung, intestine, liver, thymus, bone

Light sheet Cleared Tissue Imaging

SeeDB



TECHNICAL REPORTS

nature neuroscience

SeeDB: a simple and morphology-preserving optical clearing agent for neuronal circuit reconstruction

Meng-Tsen Ke[1,2], Satoshi Fujimoto[1] & Takeshi Imai[1–3]

We report a water-based optical clearing agent, SeeDB, which clears fixed brain samples in a few days without quenching many types of fluorescent dyes, including fluorescent proteins and lipophilic neuronal tracers. Our method maintained a constant sample volume during the clearing procedure, an important factor for keeping cellular morphology intact, and facilitated the quantitative reconstruction of neuronal circuits. Combined with two-photon microscopy and an optimized objective lens, we were able to image the mouse brain from the dorsal to the ventral side. We used SeeDB to describe the near-complete wiring diagram of sister mitral cells associated with a common glomerulus in the mouse olfactory bulb. We found the diversity of dendrite wiring patterns among sister mitral cells, and our results provide an anatomical basis for non-redundant odor coding by these neurons. Our simple and efficient method is useful for imaging intact morphological architecture at large scales in both the adult and developing brains.

Although fluorescent proteins can be used to genetically label cellular and subcellular architecture *in vivo*, sample opacity often limits imaging deep inside the tissue. This is particularly problematic when biological samples are fixed with paraformaldehyde. Even with two-photon microscopy, fixed brain samples can be imaged up to only a depth of approximately 300 μm (ref. 1). Sample opacity is caused by both light absorption and scattering, but the latter is the larger problem when imaging mammalian tissues.

To reduce the amount of light scattering in tissues, several optical clearing agents have been developed. Scattering occurs when the refractive index of scatter differs from the medium[2]. The refractive indices of scatter in fixed tissues are much higher (~1.5) than water (1.33)[3]. Thus, high-index solvents, such as BABB (Murray's clear, a 1:2 mixture of benzyl alcohol and benzyl benzoate, refractive index = 1.56), methyl salicylate (1.52), dibenzyl ether (1.56), 2,2′-thiodiethanol (1.52) and glycerol (1.47) have been used for optical clearing after whole-mount antibody staining[4–9]. However, these optical clearing agents quench fluorescent proteins because these proteins require water molecules to fluoresce. Another approach for optical clearing is tissue maceration, in which potassium hydroxide or trypsin is used for partial protein digestion and denaturation to facilitate optical clearing[10,11]. Recently, an optical clearing agent was reported, Scale, which contains urea[12]. Because fluorescent proteins are stable in the urea solution, the Scale method was able to image fluorescent proteins deep inside the tissues of fixed mouse brains. This method, however, requires a long incubation time for clearing, from weeks to months. In addition, a large expansion in sample volume compromises the detailed cellular morphology as well as the real depth (the depth in the original samples) in many situations in which the working distance of an objective lens is a limiting factor. Furthermore, because urea causes partial denaturation and loss of cellular proteins, the Scale-treated samples are very fragile and difficult to handle. Thus, a more moderate optical clearing agent is desirable for studying cellular morphology both in fine detail and at large scales with minimal artifacts.

We developed a water-based optical clearing agent, SeeDB (See Deep Brain), which overcomes the limitations seen with other agents, including long incubation times and tissue deformation. Combined with two-photon microscopy, SeeDB allowed us to image fixed mouse brains at the millimeter-scale level. We also used SeeDB to examine the detailed wiring diagram of mitral cells associated with a common glomerulus in the olfactory bulb. The compatibility of SeeDB with fluorescent proteins and various neuronal tracers in fixed tissues should facilitate comprehensive and quantitative analyses for understanding neuronal circuitry.

RESULTS

SeeDB, an optical clearing agent using fructose and thiol

To develop a better optical clearing agent for imaging fluorescent proteins, we sought to use aqueous solutions with refractive indices close to that of fixed tissues[13]. Previously, high-concentration sucrose solutions have been used to reduce light scattering of the brain and invertebrate samples[1,14]. Among the various sugar solutions tested, we found a fructose solution to be an ideal clearing agent. Fructose is highly soluble in water, and the refractive index of a saturated fructose solution reaches 1.490 at 25 °C (80.2% wt/wt, ~115% wt/vol) and 1.502 at 37 °C (86.7% wt/wt, ~130% wt/vol), which are higher than other water-based clearing reagents (**Supplementary Table 1**)[1,4]. As a result, fructose solutions proved to be more effective at clearing tissues than the other clearing agents. Furthermore, we found that high-concentration fructose solutions did not cause the shrinkage of samples that was observed with sucrose solutions[1]. Although longer incubation periods in the fructose solution (>7 d), especially at higher temperatures (for example, 37 °C), caused browning and autofluorescence accumulation as a result of the Maillard reaction[15], these effects could be avoided by adding thiols such as β-mercaptoethanol

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Claim:
A water-based optical clearing agent, SeeDB, clears fixed brain samples in a few days without quenching many types of fluorescent dyes, including fluorescent proteins and lipophilic neuronal tracers.

Method:
Fixed (agarose embedded) samples are serially incubated in 20%, 40% and 60% fructose. Samples are then incubated in 80% fructose for 12 h, 100 % fructose for 12 h and finally in SeeDB (80.2% fructose) for 24h. The concentration of fructose could be increased up to 86.7% (SeeDB37) when clearing was performed at 37 °C. All fructose solutions contained 0.5% α-thioglycerol

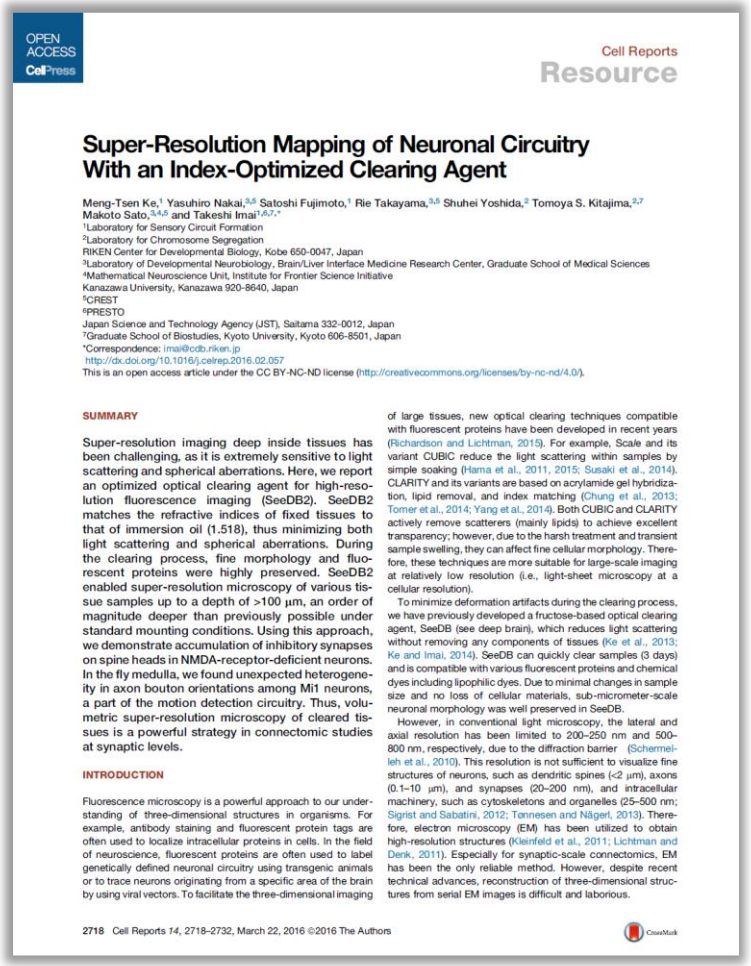
Imaging solution:
80% 2,2′-thiodiethanol (SeeDB). 90% 2,2′-thiodiethanol (SeeDB37).

Refractive index: 1,49 – 1,5

Sample tissues:
Mouse brain, olfactory bulb.

Light sheet Cleared Tissue Imaging

SeeDB2



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Cell Reports
Resource

Super-Resolution Mapping of Neuronal Circuitry With an Index-Optimized Clearing Agent

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SUMMARY

Super-resolution imaging deep inside tissues has been challenging, as it is extremely sensitive to light scattering and spherical aberrations. Here, we report an optimized optical clearing agent for high-resolution fluorescence imaging (SeeDB2). SeeDB2 matches the refractive indices of fixed tissues to that of immersion oil (1.518), thus minimizing both light scattering and spherical aberrations. During the clearing process, fine morphology and fluorescent proteins were highly preserved. SeeDB2 enabled super-resolution microscopy of various tissue samples up to a depth of >100 μm, an order of magnitude deeper than previously possible under standard mounting conditions. Using this approach, we demonstrate accumulation of inhibitory synapses on spine heads in NMDA-receptor-deficient neurons. In the fly medulla, we found unexpected heterogeneity in axon bouton orientations among Mi1 neurons, a part of the motion detection circuitry. Thus, volumetric super-resolution microscopy of cleared tissues is a powerful strategy in connectomic studies at synaptic levels.

INTRODUCTION

Fluorescence microscopy is a powerful approach to our understanding of three-dimensional structures in organisms. For example, antibody staining and fluorescent protein tags are often used to localize intracellular proteins in cells. In the field of neuroscience, fluorescent proteins are often used to label genetically defined neuronal circuitry using transgenic animals or to trace neurons originating from a specific area of the brain by using viral vectors. To facilitate the three-dimensional imaging of large tissues, new optical clearing techniques compatible with fluorescent proteins have been developed in recent years (Richardson and Lichtman, 2015). For example, Scale and its variant CUBIC reduce the light scattering within samples by simple soaking (Hama et al., 2011, 2015; Susaki et al., 2014). CLARITY and its variants are based on acrylamide gel hybridization, lipid removal, and index matching (Chung et al., 2013; Tomer et al., 2014; Yang et al., 2014). Both CUBIC and CLARITY actively remove scatterers (mainly lipids) to achieve excellent transparency; however, due to the harsh treatment and transient sample swelling, they can affect fine cellular morphology. Therefore, these techniques are more suitable for large-scale imaging at relatively low resolution (i.e., light-sheet microscopy at a cellular resolution).

To minimize deformation artifacts during the clearing process, we have previously developed a fructose-based optical clearing agent, SeeDB (see deep brain), which reduces light scattering without removing any components of tissues (Ke et al., 2013; Ke and Imai, 2014). SeeDB can quickly clear samples (3 days) and is compatible with various fluorescent proteins and chemical dyes including lipophilic dyes. Due to minimal changes in sample size and no loss of cellular materials, sub-micrometer-scale neuronal morphology was well preserved in SeeDB.

However, in conventional light microscopy, the lateral and axial resolution has been limited to 200–250 nm and 500–800 nm, respectively, due to the diffraction barrier (Schermelleh et al., 2010). This resolution is not sufficient to visualize fine structures of neurons, such as dendritic spines (<2 μm), axons (0.1–10 μm), and synapses (20–200 nm), and intracellular machinery, such as cytoskeletons and organelles (25–500 nm; Sigrist and Sabatini, 2012; Tønnesen and Nägerl, 2013). Therefore, electron microscopy (EM) has been utilized to obtain high-resolution structures (Kleinfeld et al., 2011; Lichtman and Denk, 2011). Especially for synaptic-scale connectomics, EM has been the only reliable method. However, despite recent technical advances, reconstruction of three-dimensional structures from serial EM images is difficult and laborious.

Claim:
An optimized optical clearing agent for high-reso-lution fluorescence imaging (SeeDB2). SeeDB2 matches the refractive indices of fixed tissues to that of immersion oil (1.518), thus minimizing both light scattering and spherical aberrations.

Method:
Fixed tissue samples are incubated 2% saponin overnight, followed by incubation in different concentrations of Omnipaque 350 in water with 2% saponin.

Imaging solution:
RIMS (Histodenz)

Refractive index: 1,518

Sample tissues:
Mouse embryos, brains, and brain sections, fly brain (for high and superresolution microscopy)

Light sheet Cleared Tissue Imaging
FRUIT

frontiers in NEUROANATOMY

METHODS ARTICLE
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Scalable and Dil-compatible optical clearance of the mammalian brain

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Efficient optical clearance is fundamental for whole brain imaging. In particular, clearance of the brain without membrane damage is required for the imaging of lipophilic tracer-labeled neural tracts. Relying on an ascending gradient of fructose solutions, SeeDB can achieve sufficient transparency of the mouse brain while ensuring that the plasma membrane remains intact. However, it is challenging to extend this method to larger mammalian brains due to the extremely high viscosity of the saturated fructose solution. Here we report a SeeDB-derived optical clearing method, termed FRUIT, which utilizes a cocktail of fructose and urea. As demonstrated in the adult mouse brain, combination of these two highly water-soluble clearing agents exerts a synergistic effect on clearance. More importantly, the final FRUIT solution has low viscosity so as to produce transparency of the whole adult rabbit brain via arterial perfusion, which is impossible to achieve with a saturated fructose solution. In addition to good compatibility with enhanced yellow fluorescent protein, the cocktail also preserves the fluorescence of the lipophilic tracer DiI. This work provides a volume-independent optical clearing method which retains the advantages of SeeDB, particularly compatibility with lipophilic tracers.

Keywords: optical tissue clearing, SeeDB, Urea, tract tracing, whole brain imaging, CLARITY, CUBIC, 3DISCO

INTRODUCTION

Comprehensive depiction of structural layout and neuronal connectivity is indispensible for understanding how the mammalian brain functions. Due to high-throughput mechanical sectioning platforms and powerful automated three-dimensional imaging technologies, mesoscale atlases or connectomes of the entire mouse brain have been successfully reconstructed (Li et al., 2010; Oh et al., 2014). Despite these remarkable advances, the system-level relationship between brain structure and function often needs to be investigated, from a global perspective, in a whole brain rather than by reconstruction across preparations, in order to avoid loss of the detailed structure between sections (DeFelipe, 2010). However, sample opacity seriously limits the depth of imaging through the whole brain, particularly in adult animals. Recently, tissue optical clearing methods have opened a new avenue to extract cellular resolution information from unsectioned mammalian brains (Dodt et al., 2007; Hama et al., 2011; Becker et al., 2012; Erturk et al., 2012a; Chung et al., 2013; Ke et al., 2013; Kim et al., 2013; Kuwajima et al., 2013; Yushchenko and Schultz, 2013; Renier et al., 2014; Susaki et al., 2014; Tomer et al., 2014; Yang et al., 2014; Zhang et al., 2014).

Because tissue opacity results mainly from the scattering of light which occurs when the refractive index (RI) of the scatter differs from that of the medium (Helmchen and Denk, 2005), optical clearing methods usually yield tissue transparency via RI matching. A simple strategy is to merely utilize a clearing agent with a high RI approximating to that of fixed tissue (~1.5) (Dodt et al., 2007; Becker et al., 2012; Erturk et al., 2012a,b; Ke et al., 2013; Susaki et al., 2014). The alternative is to intentionally remove hydrophobic lipids which are the main source of light scattering in the fixed brain (Chung et al., 2013; Susaki et al., 2014). Among these techniques, fructose-based SeeDB does not disrupt the plasma membrane, and can therefore clear brain samples labeled with lipophilic dyes that are indispensible for neural tract tracing of post-fixed brains (Ke et al., 2013). However, the viscosity of saturated fructose (e.g., 130% wt/vol at 37°C) is extremely high, which limits its ability to permeate into brain samples as well as making solution preparation and manipulation difficult. Although incubation at a higher temperature could improve these permeability and fluidity issues, this would cause partial quenching of fluorescent proteins (Ke et al., 2013). Arterial perfusion-assisted delivery is also efficient for rapid diffusion of

Claim
A SeeDB derived volume independent optical clearing method which retains the advantages of SeeDB, particularly compatibility with lipophilic tracers.

Clearing procedure:
Fructose / urea / α-thioglycerol -based passive clearing

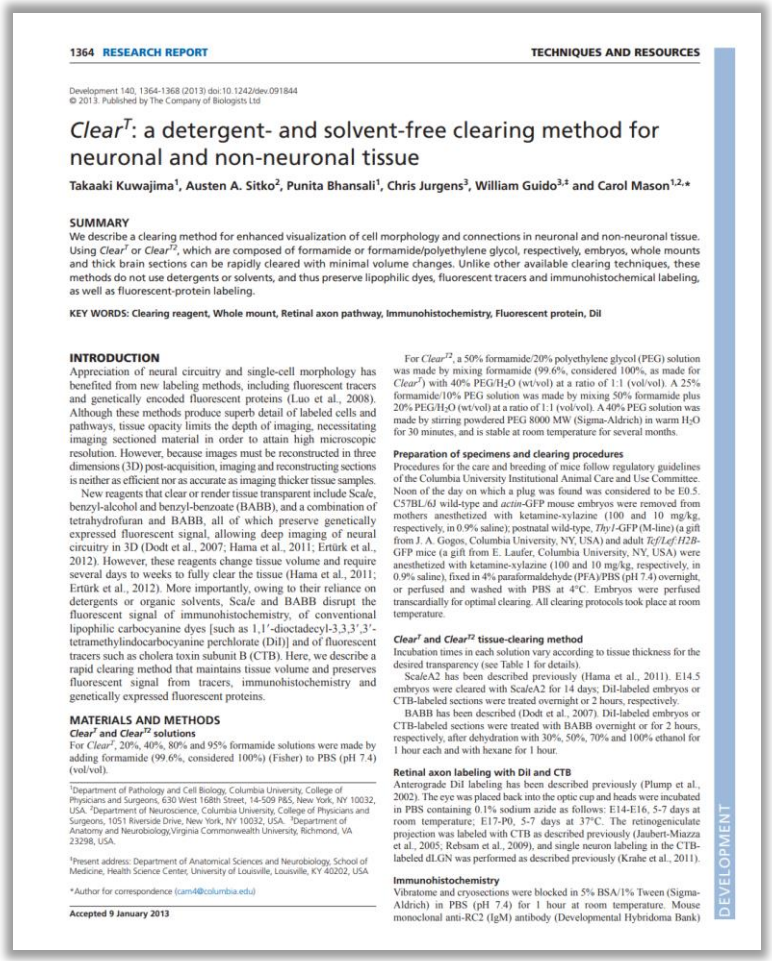
Imaging Solution:
Fructose / urea / α-thioglycerol cocktail

Refractive Index: ~1,48

Sample tissues:
Brain

Light sheet Cleared Tissue Imaging

Clear^T



1364 RESEARCH REPORT TECHNIQUES AND RESOURCES

Development 140, 1364-1368 (2013) doi:10.1242/dev.091844

Clear^T: a detergent- and solvent-free clearing method for neuronal and non-neuronal tissue

Takaaki Kuwajima[1], Austen A. Sitko[2], Punita Bhansali[1], Chris Jurgens[3], William Guido[3,‡] and Carol Mason[1,2,*]

SUMMARY

We describe a clearing method for enhanced visualization of cell morphology and connections in neuronal and non-neuronal tissue. Using *Clear^T* or *Clear^{T2}*, which are composed of formamide or formamide/polyethylene glycol, respectively, embryos, whole mounts and thick brain sections can be rapidly cleared with minimal volume changes. Unlike other available clearing techniques, these methods do not use detergents or solvents, and thus preserve lipophilic dyes, fluorescent tracers and immunohistochemical labeling, as well as fluorescent-protein labeling.

KEY WORDS: Clearing reagent, Whole mount, Retinal axon pathway, Immunohistochemistry, Fluorescent protein, DiI

INTRODUCTION

Appreciation of neural circuitry and single-cell morphology has benefited from new labeling methods, including fluorescent tracers and genetically encoded fluorescent proteins (Luo et al., 2008). Although these methods produce superb detail of labeled cells and pathways, tissue opacity limits the depth of imaging, necessitating imaging sectioned material in order to attain high microscopic resolution. However, because images must be reconstructed in three dimensions (3D) post-acquisition, imaging and reconstructing sections is neither as efficient nor as accurate as imaging thicker tissue samples.

New reagents that clear or render tissue transparent include Sca/e, benzyl-alcohol and benzyl-benzoate (BABB), and a combination of tetrahydrofuran and BABB, all of which preserve genetically expressed fluorescent signal, allowing deep imaging of neural circuitry in 3D (Dodt et al., 2007; Hama et al., 2011; Ertürk et al., 2012). However, these reagents change tissue volume and require several days to weeks to fully clear the tissue (Hama et al., 2011; Ertürk et al., 2012). More importantly, owing to their reliance on detergents or organic solvents, Sca/e and BABB disrupt the fluorescent signal of immunohistochemistry, of conventional lipophilic carbocyanine dyes [such as 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)] and of fluorescent tracers such as cholera toxin subunit B (CTB). Here, we describe a rapid clearing method that maintains tissue volume and preserves fluorescent signal from tracers, immunohistochemistry and genetically expressed fluorescent proteins.

MATERIALS AND METHODS

***Clear^T* and *Clear^{T2}* solutions**

For *Clear^T*, 20%, 40%, 80% and 95% formamide solutions were made by adding formamide (99.6%, considered 100%) (Fisher) to PBS (pH 7.4) (vol/vol).

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Accepted 9 January 2013

For *Clear^{T2}*, a 50% formamide/20% polyethylene glycol (PEG) solution was made by mixing formamide (99.6%, considered 100%, as made for *Clear^T*) with 40% PEG/H_2O (wt/vol) at a ratio of 1:1 (vol/vol). A 25% formamide/10% PEG solution was made by mixing 50% formamide plus 20% PEG/H_2O (wt/vol) at a ratio of 1:1 (vol/vol). A 40% PEG solution was made by stirring powdered PEG 8000 MW (Sigma-Aldrich) in warm H_2O for 30 minutes, and is stable at room temperature for several months.

Preparation of specimens and clearing procedures

Procedures for the care and breeding of mice follow regulatory guidelines of the Columbia University Institutional Animal Care and Use Committee. Noon of the day on which a plug was found was considered to be E0.5. C57BL/6J wild-type and *actin*-GFP mouse embryos were removed from mothers anesthetized with ketamine-xylazine (100 and 10 mg/kg, respectively, in 0.9% saline); postnatal wild-type, *Thy1*-GFP (M-line) (a gift from J. A. Gogos, Columbia University, NY, USA) and adult *Tcf/Lef:H2B*-GFP mice (a gift from E. Laufer, Columbia University, NY, USA) were anesthetized with ketamine-xylazine (100 and 10 mg/kg, respectively, in 0.9% saline), fixed in 4% paraformaldehyde (PFA)/PBS (pH 7.4) overnight, or perfused and washed with PBS at 4°C. Embryos were perfused transcardially for optimal clearing. All clearing protocols took place at room temperature.

***Clear^T* and *Clear^{T2}* tissue-clearing method**

Incubation times in each solution vary according to tissue thickness for the desired transparency (see Table 1 for details).

Sca/eA2 has been described previously (Hama et al., 2011). E14.5 embryos were cleared with Sca/eA2 for 14 days; DiI-labeled embryos or CTB-labeled sections were treated overnight or 2 hours, respectively.

BABB has been described (Dodt et al., 2007). DiI-labeled embryos or CTB-labeled sections were treated with BABB overnight or for 2 hours, respectively, after dehydration with 30%, 50%, 70% and 100% ethanol for 1 hour each and with hexane for 1 hour.

Retinal axon labeling with DiI and CTB

Anterograde DiI labeling has been described previously (Plump et al., 2002). The eye was placed back into the optic cup and heads were incubated in PBS containing 0.1% sodium azide as follows: E14-E16, 5-7 days at room temperature; E17-P0, 5-7 days at 37°C. The retinogeniculate projection was labeled with CTB as described previously (Jaubert-Miazza et al., 2005; Rebsam et al., 2009), and single neuron labeling in the CTB-labeled dLGN was performed as described previously (Krahe et al., 2011).

Immunohistochemistry

Vibratome and cryosections were blocked in 5% BSA/1% Tween (Sigma-Aldrich) in PBS (pH 7.4) for 1 hour at room temperature. Mouse monoclonal anti-RC2 (IgM) antibody (Developmental Hybridoma Bank)

DEVELOPMENT

Claim

Clearing method for enhanced visualization of cell morphology and connections in neuronal and non-neuronal tissue. Embryos, whole mounts and thick brain sections can be rapidly cleared with minimal volume changes. They preserve lipophilic dyes, fluorescent tracers and immunohistochemical labeling, and GFP.

Method:

20%, 40%, 80% and 95% formamide solutions

Imaging Solution:

Formamide solution

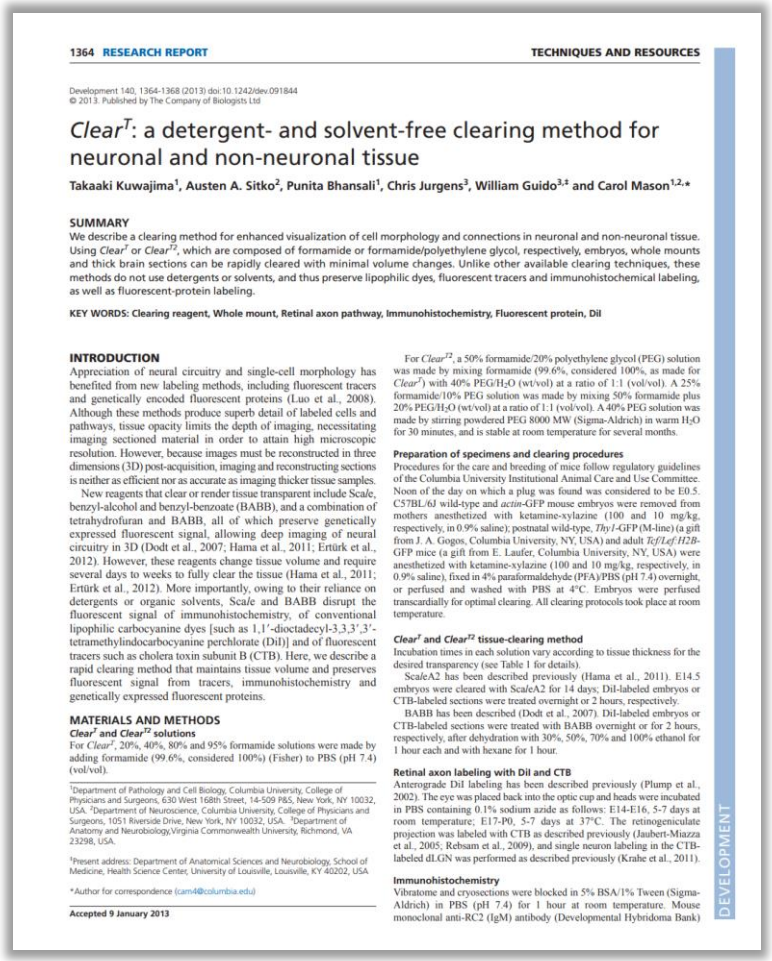
Refractive Index: 1,44

Sample Tissues:

Brain, mouse embryos

Light sheet Cleared Tissue Imaging

Clear^{T2}



1364 RESEARCH REPORT TECHNIQUES AND RESOURCES

Development 140, 1364-1368 (2013) doi:10.1242/dev.091844

Clear^{T}: a detergent- and solvent-free clearing method for neuronal and non-neuronal tissue

Takaaki Kuwajima[1], Austen A. Sitko[2], Punita Bhansali[1], Chris Jurgens[3], William Guido[3,‡] and Carol Mason[1,2,*]

SUMMARY

We describe a clearing method for enhanced visualization of cell morphology and connections in neuronal and non-neuronal tissue. Using *Clear^{T}* or *Clear^{T2}*, which are composed of formamide or formamide/polyethylene glycol, respectively, embryos, whole mounts and thick brain sections can be rapidly cleared with minimal volume changes. Unlike other available clearing techniques, these methods do not use detergents or solvents, and thus preserve lipophilic dyes, fluorescent tracers and immunohistochemical labeling, as well as fluorescent-protein labeling.

KEY WORDS: Clearing reagent, Whole mount, Retinal axon pathway, Immunohistochemistry, Fluorescent protein, DiI

INTRODUCTION

Appreciation of neural circuitry and single-cell morphology has benefited from new labeling methods, including fluorescent tracers and genetically encoded fluorescent proteins (Luo et al., 2008). Although these methods produce superb detail of labeled cells and pathways, tissue opacity limits the depth of imaging, necessitating imaging sectioned material in order to attain high microscopic resolution. However, because images must be reconstructed in three dimensions (3D) post-acquisition, imaging and reconstructing sections is neither as efficient nor as accurate as imaging thicker tissue samples.

New reagents that clear or render tissue transparent include Scale, benzyl-alcohol and benzyl-benzoate (BABB), and a combination of tetrahydrofuran and BABB, all of which preserve genetically expressed fluorescent signal, allowing deep imaging of neural circuitry in 3D (Dodt et al., 2007; Hama et al., 2011; Ertürk et al., 2012). However, these reagents change tissue volume and require several days to weeks to fully clear the tissue (Hama et al., 2011; Ertürk et al., 2012). More importantly, owing to their reliance on detergents or organic solvents, Sca/e and BABB disrupt the fluorescent signal of immunohistochemistry, of conventional lipophilic carbocyanine dyes [such as 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI)] and of fluorescent tracers such as cholera toxin subunit B (CTB). Here, we describe a rapid clearing method that maintains tissue volume and preserves fluorescent signal from tracers, immunohistochemistry and genetically expressed fluorescent proteins.

MATERIALS AND METHODS

***Clear^{T}* and *Clear^{T2}* solutions**

For *Clear^{T}*, 20%, 40%, 80% and 95% formamide solutions were made by adding formamide (99.6%, considered 100%) (Fisher) to PBS (pH 7.4) (vol/vol).

For *Clear^{T2}*, a 50% formamide/20% polyethylene glycol (PEG) solution was made by mixing formamide (99.6%, considered 100%, as made for *Clear^{T}*) with 40% PEG/H_2O (wt/vol) at a ratio of 1:1 (vol/vol). A 25% formamide/10% PEG solution was made by mixing 50% formamide plus 20% PEG/H_2O (wt/vol) at a ratio of 1:1 (vol/vol). A 40% PEG solution was made by stirring powdered PEG 8000 MW (Sigma-Aldrich) in warm H_2O for 30 minutes, and is stable at room temperature for several months.

Preparation of specimens and clearing procedures

Procedures for the care and breeding of mice follow regulatory guidelines of the Columbia University Institutional Animal Care and Use Committee. Noon of the day on which a plug was found was considered to be E0.5. C57BL/6J wild-type and *actin*-GFP mouse embryos were removed from mothers anesthetized with ketamine-xylazine (100 and 10 mg/kg, respectively, in 0.9% saline); postnatal wild-type, *Thy1*-GFP (M-line) (a gift from J. A. Gogos, Columbia University, NY, USA) and adult *Tef/Lef:H2B*-GFP mice (a gift from E. Laufer, Columbia University, NY, USA) were anesthetized with ketamine-xylazine (100 and 10 mg/kg, respectively, in 0.9% saline), fixed in 4% paraformaldehyde (PFA)/PBS (pH 7.4) overnight, or perfused and washed with PBS at 4°C. Embryos were perfused transcardially for optimal clearing. All clearing protocols took place at room temperature.

***Clear^{T}* and *Clear^{T2}* tissue-clearing method**

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BABB has been described (Dodt et al., 2007). DiI-labeled embryos or CTB-labeled sections were treated with BABB overnight or for 2 hours, respectively, after dehydration with 30%, 50%, 70% and 100% ethanol for 1 hour each and with hexane for 1 hour.

Retinal axon labeling with DiI and CTB

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Immunohistochemistry

Vibratome and cryosections were blocked in 5% BSA/1% Tween (Sigma-Aldrich) in PBS (pH 7.4) for 1 hour at room temperature. Mouse monoclonal anti-RC2 (IgM) antibody (Developmental Hybridoma Bank)

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Accepted 9 January 2013

DEVELOPMENT

Claim

Clearing method for enhanced visualization of cell morphology and connections in neuronal and non-neuronal tissue. Embryos, whole mounts and thick brain sections can be rapidly cleared with minimal volume changes. They preserve lipophilic dyes, fluorescent tracers and immunohistochemical labeling, and GFP.

Method:

20%, 40%, 80% and 95% formamide / 20 % PEG solutions

Imaging Solution:

95 % Formamide / 20 % PEG solution

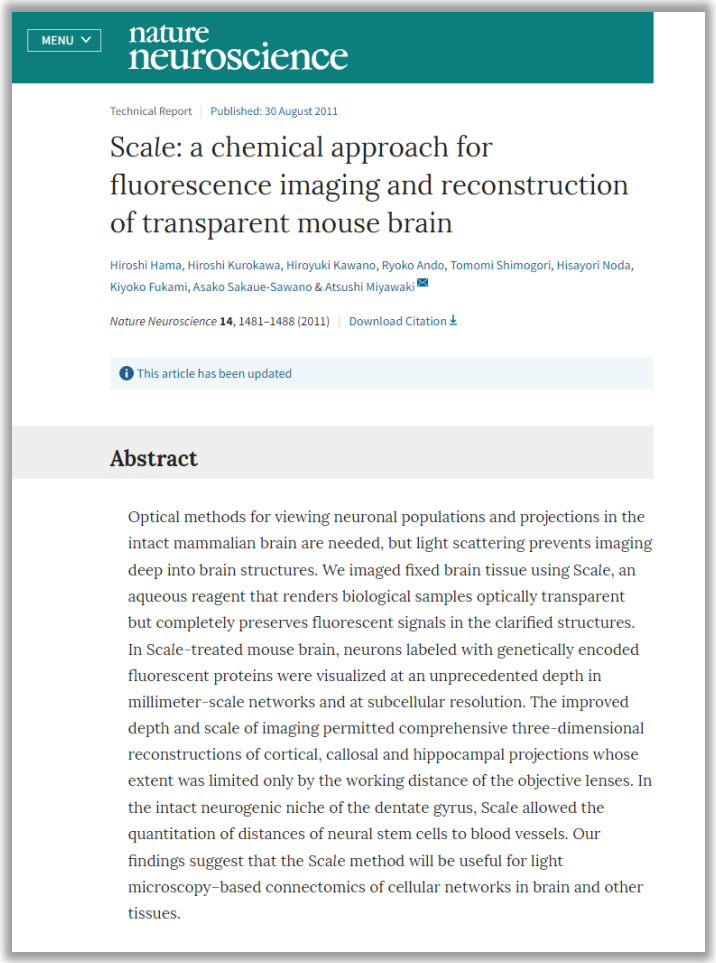
Refractive Index: 1,44

Sample Tissues:

Embryos, whole mounts and thick brain sections

Light sheet Cleared Tissue Imaging

Sca/e



MENU

nature neuroscience

Technical Report | Published: 30 August 2011

Scale: a chemical approach for fluorescence imaging and reconstruction of transparent mouse brain

Hiroshi Hama, Hiroshi Kurokawa, Hiroyuki Kawano, Ryoko Ando, Tomomi Shimogori, Hisayori Noda, Kiyoko Fukami, Asako Sakaue-Sawano & Atsushi Miyawaki

Nature Neuroscience **14**, 1481–1488 (2011) | Download Citation

This article has been updated

Abstract

Optical methods for viewing neuronal populations and projections in the intact mammalian brain are needed, but light scattering prevents imaging deep into brain structures. We imaged fixed brain tissue using Scale, an aqueous reagent that renders biological samples optically transparent but completely preserves fluorescent signals in the clarified structures. In Scale-treated mouse brain, neurons labeled with genetically encoded fluorescent proteins were visualized at an unprecedented depth in millimeter-scale networks and at subcellular resolution. The improved depth and scale of imaging permitted comprehensive three-dimensional reconstructions of cortical, callosal and hippocampal projections whose extent was limited only by the working distance of the objective lenses. In the intact neurogenic niche of the dentate gyrus, Scale allowed the quantitation of distances of neural stem cells to blood vessels. Our findings suggest that the Scale method will be useful for light microscopy–based connectomics of cellular networks in brain and other tissues.

Claim

Sca*l*e, an aqueous reagent that renders biological samples optically transparent but completely preserves fluorescent signals in the clarified structures.

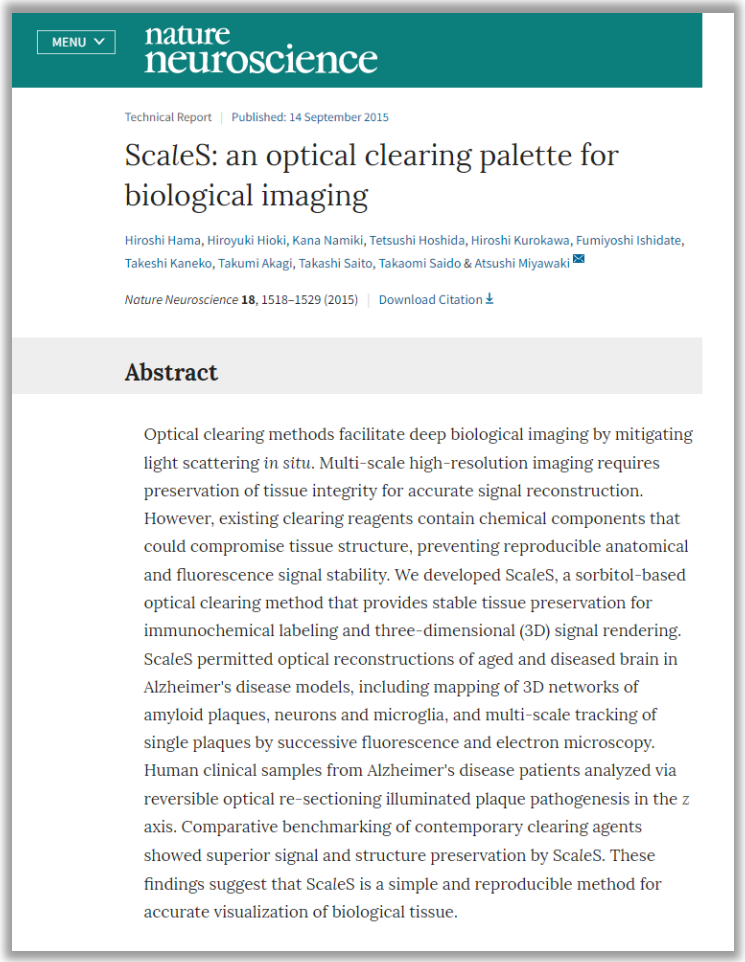
Method:

Imaging Solution: Sca/e A2, Sca/e B4

Refractive Index: 1.38

Sample Tissues: Brain

Light sheet Cleared Tissue Imaging
Sca/eS



MENU

nature neuroscience

Technical Report | Published: 14 September 2015

ScaleS: an optical clearing palette for biological imaging

Hiroshi Hama, Hiroyuki Hioki, Kana Namiki, Tetsushi Hoshida, Hiroshi Kurokawa, Fumiyoshi Ishidate, Takeshi Kaneko, Takumi Akagi, Takashi Saito, Takaomi Saido & Atsushi Miyawaki

Nature Neuroscience **18**, 1518–1529 (2015) | Download Citation

Abstract

Optical clearing methods facilitate deep biological imaging by mitigating light scattering *in situ*. Multi-scale high-resolution imaging requires preservation of tissue integrity for accurate signal reconstruction. However, existing clearing reagents contain chemical components that could compromise tissue structure, preventing reproducible anatomical and fluorescence signal stability. We developed ScaleS, a sorbitol-based optical clearing method that provides stable tissue preservation for immunochemical labeling and three-dimensional (3D) signal rendering. ScaleS permitted optical reconstructions of aged and diseased brain in Alzheimer's disease models, including mapping of 3D networks of amyloid plaques, neurons and microglia, and multi-scale tracking of single plaques by successive fluorescence and electron microscopy. Human clinical samples from Alzheimer's disease patients analyzed via reversible optical re-sectioning illuminated plaque pathogenesis in the z axis. Comparative benchmarking of contemporary clearing agents showed superior signal and structure preservation by ScaleS. These findings suggest that ScaleS is a simple and reproducible method for accurate visualization of biological tissue.

Claim
Sca*l*eS, a sorbitol-based optical clearing method that provides stable tissue preservation for immunochemical labeling and three-dimensional (3D) signal rendering.

Method:
Permeability enhancement; incubation in urea-containing and salt-free Scale solutions that gradually clear the sample; restoration of sample and mounting in imaging solution.

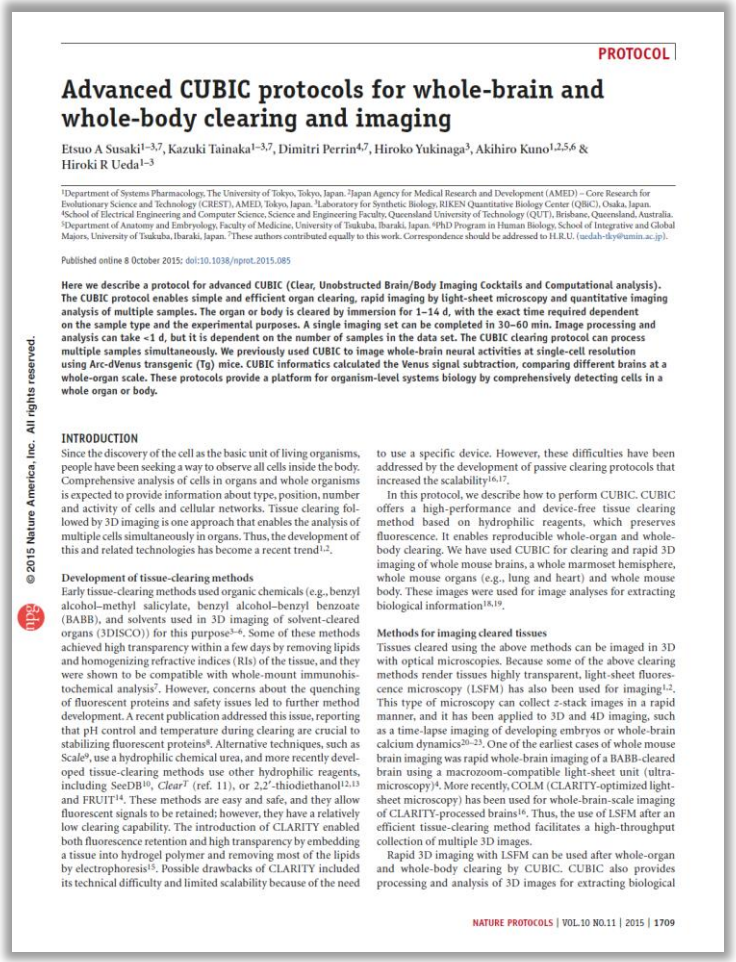
Imaging Solution:
Sca*l*eA4

Refractive Index: 1,38

Sample Tissues:
Brain

Light sheet Cleared Tissue Imaging

CUBIC



PROTOCOL

Advanced CUBIC protocols for whole-brain and whole-body clearing and imaging

Etsuo A Susaki[1–3,7], Kazuki Tainaka[1–3,7], Dimitri Perrin[4,7], Hiroko Yukinaga[3], Akihiro Kuno[1,2,5,6] & Hiroki R Ueda[1–3]

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Here we describe a protocol for advanced CUBIC (Clear, Unobstructed Brain/Body Imaging Cocktails and Computational analysis). The CUBIC protocol enables simple and efficient organ clearing, rapid imaging by light-sheet microscopy and quantitative imaging analysis of multiple samples. The organ or body is cleared by immersion for 1–14 d, with the exact time required dependent on the sample type and the experimental purposes. A single imaging set can be completed in 30–60 min. Image processing and analysis can take <1 d, but it is dependent on the number of samples in the data set. The CUBIC clearing protocol can process multiple samples simultaneously. We previously used CUBIC to image whole-brain neural activities at single-cell resolution using Arc-dVenus transgenic (Tg) mice. CUBIC informatics calculated the Venus signal subtraction, comparing different brains at a whole-organ scale. These protocols provide a platform for organism-level systems biology by comprehensively detecting cells in a whole organ or body.

INTRODUCTION

Since the discovery of the cell as the basic unit of living organisms, people have been seeking a way to observe all cells inside the body. Comprehensive analysis of cells in organs and whole organisms is expected to provide information about type, position, number and activity of cells and cellular networks. Tissue clearing followed by 3D imaging is one approach that enables the analysis of multiple cells simultaneously in organs. Thus, the development of this and related technologies has become a recent trend[1,2].

Development of tissue-clearing methods

Early tissue-clearing methods used organic chemicals (e.g., benzyl alcohol–methyl salicylate, benzyl alcohol–benzyl benzoate (BABB), and solvents used in 3D imaging of solvent-cleared organs (3DISCO)) for this purpose[3–6]. Some of these methods achieved high transparency within a few days by removing lipids and homogenizing refractive indices (RIs) of the tissue, and they were shown to be compatible with whole-mount immunohistochemical analysis[7]. However, concerns about the quenching of fluorescent proteins and safety issues led to further method development. A recent publication addressed this issue, reporting that pH control and temperature during clearing are crucial to stabilizing fluorescent proteins[8]. Alternative techniques, such as Scale[9], use a hydrophilic chemical urea, and more recently developed tissue-clearing methods use other hydrophilic reagents, including SeeDB[10], *Clear*^{I} (ref. 11), or 2,2′-thiodiethanol[12,13] and FRUIT[14]. These methods are easy and safe, and they allow fluorescent signals to be retained; however, they have a relatively low clearing capability. The introduction of CLARITY enabled both fluorescence retention and high transparency by embedding a tissue into hydrogel polymer and removing most of the lipids by electrophoresis[15]. Possible drawbacks of CLARITY included its technical difficulty and limited scalability because of the need to use a specific device. However, these difficulties have been addressed by the development of passive clearing protocols that increased the scalability[16,17].

In this protocol, we describe how to perform CUBIC. CUBIC offers a high-performance and device-free tissue clearing method based on hydrophilic reagents, which preserves fluorescence. It enables reproducible whole-organ and whole-body clearing. We have used CUBIC for clearing and rapid 3D imaging of whole mouse brains, a whole marmoset hemisphere, whole mouse organs (e.g., lung and heart) and whole mouse body. These images were used for image analyses for extracting biological information[18,19].

Methods for imaging cleared tissues

Tissues cleared using the above methods can be imaged in 3D with optical microscopies. Because some of the above clearing methods render tissues highly transparent, light-sheet fluorescence microscopy (LSFM) has also been used for imaging[1,2]. This type of microscopy can collect *z*-stack images in a rapid manner, and it has been applied to 3D and 4D imaging, such as a time-lapse imaging of developing embryos or whole-brain calcium dynamics[20–23]. One of the earliest cases of whole mouse brain imaging was rapid whole-brain imaging of a BABB-cleared brain using a macrozoom-compatible light-sheet unit (ultramicroscopy)[4]. More recently, COLM (CLARITY-optimized light-sheet microscopy) has been used for whole-brain-scale imaging of CLARITY-processed brains[16]. Thus, the use of LSFM after an efficient tissue-clearing method facilitates a high-throughput collection of multiple 3D images.

Rapid 3D imaging with LSFM can be used after whole-organ and whole-body clearing by CUBIC. CUBIC also provides processing and analysis of 3D images for extracting biological

Claim:
CUBIC-perfusion protocol enables rapid whole-body and whole-organ imaging at single-cell resolution by using light-sheet fluorescent microscopy. The CUBIC protocol is also applicable to 3D pathology, anatomy, and immunohistochemistry of various organs.

Method:
Fixed organs were immersed in or perfused with CUBIC-1 and CUBIC-2 reagent.

Imaging Solution: Mineral oil Sigma-Aldrich, M8410

Refractive Index: 1.467

Sample Tissues:
Whole mouse

Light sheet Cleared Tissue Imaging

CUBIC X

nature neuroscience

RESOURCE

https://doi.org/10.1038/s41593-018-0109-1

A three-dimensional single-cell-resolution whole-brain atlas using CUBIC-X expansion microscopy and tissue clearing

Tatsuya C. Murakami[1], Tomoyuki Mano[1,2], Shu Saikawa[3], Shuhei A. Horiguchi[4,5], Daichi Shigeta[1], Kousuke Baba[6,7], Hiroshi Sekiya[8], Yoshihiro Shimizu[9], Kenji F. Tanaka[10], Hiroshi Kiyonari[11], Masamitsu Iino[8,12], Hideki Mochizuki[6], Kazuki Tainaka[1,13] and Hiroki R. Ueda[1,2,4]*

A three-dimensional single-cell-resolution mammalian brain atlas will accelerate systems-level identification and analysis of cellular circuits underlying various brain functions. However, its construction requires efficient subcellular-resolution imaging throughout the entire brain. To address this challenge, we developed a fluorescent-protein-compatible, whole-organ clearing and homogeneous expansion protocol based on an aqueous chemical solution (CUBIC-X). The expanded, well-cleared brain enabled us to construct a point-based mouse brain atlas with single-cell annotation (CUBIC-Atlas). CUBIC-Atlas reflects inhomogeneous whole-brain development, revealing a significant decrease in the cerebral visual and somatosensory cortical areas during postnatal development. Probabilistic activity mapping of pharmacologically stimulated Arc-dVenus reporter mouse brains onto CUBIC-Atlas revealed the existence of distinct functional structures in the hippocampal dentate gyrus. CUBIC-Atlas is shareable by an open-source web-based viewer, providing a new platform for whole-brain cell profiling.

The mammalian brain is composed of various cellular circuits of different physiological functions. Comprehensive analysis of such complex cellular circuits in the entire mammalian brain is one of the fundamental challenges in neuroscience. Toward this goal, a number of mammalian brain atlases, including ones for nonhuman primates, have been constructed[1–4] and provided useful anatomical platforms. These mammalian brain atlases can be used (i) as a platform for mapping different cellular populations over the entire brain when combined with genome-wide expression data by in situ hybridization[5] and microarrays[6,7], (ii) as a platform for mapping cellular activities by measuring gene expression of immediate early genes[12–14] and (iii) as a platform for mapping cellular connections when combined with neural projection analysis based on adeno-associated virus[15] or rabies virus[16,17].

Three-dimensional (3D) reconstitution of imaging data acquired from 2D serial sections has been a common strategy for mammalian brain atlases[1–3,8]. Although the image-based atlases can provide high-resolution information, the enormity of the data prevents flexible editing and update of the atlas. Considering that the cell is the basic unit of life, a cell-based atlas would provide an attractive alternative to image-based atlases. A compact atlas of this type may be editable via an open-source platform and therefore easily updated by overlaying various cellular functions (for example, activity, gene expression, cell type and neural connection).

To construct a single-cell-resolution mouse brain atlas, it is essential to accurately identify whole cells in the whole brain. To this end, an appropriate imaging technique should provide both subcellular resolution and whole-brain-scale coverage. The recent development of potent tissue-clearing methods, including BABB, 3DISCO and uDISCO, CLARITY/PACT-PARS, CUBIC and others[18–24], enables rapid single-cell-resolution imaging of an adult mouse brain when combined with light-sheet fluorescence microscopy (LSFM). However, rapid subcellular-resolution imaging over the entire brain is still challenging because of the limited resolution of current LSFM techniques and insufficient transparency of tissues.

Expansion microscopy, developed by the Boyden group[25], and another expansion protocol developed by the Gradinaru group[26] provide a new approach to improving optical resolution by physically expanding tissues. The Chung group further applied expansion microscopy to whole organs[27]. These polymer-based expansion microscopy methods may allow subcellular-resolution imaging of samples. In addition to the improved resolution, tissue expansion is expected to provide optical advantages in tissue transparency because the refractive indices (RI) inside tissues would become more homogenous. However, since excess expansion makes tissue too fragile[28], moderate tissue expansion is called for.

In this study, we developed an editable, point-based mouse brain atlas with single-cell resolution by an intensive tissue-clearing method combined with tissue expansion. Describing the whole mouse brain as an ensemble of cellular points with less than 3 GB of data was achieved by subcellular-resolution imaging of a nuclear-stained brain and automatic detection of cells. With reference to

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Claim:
A fluorescent-protein-compatible, whole-organ clearing and homogeneous expansion protocol based on an aqueous chemical solution.

Method:
Clearing by expansion.

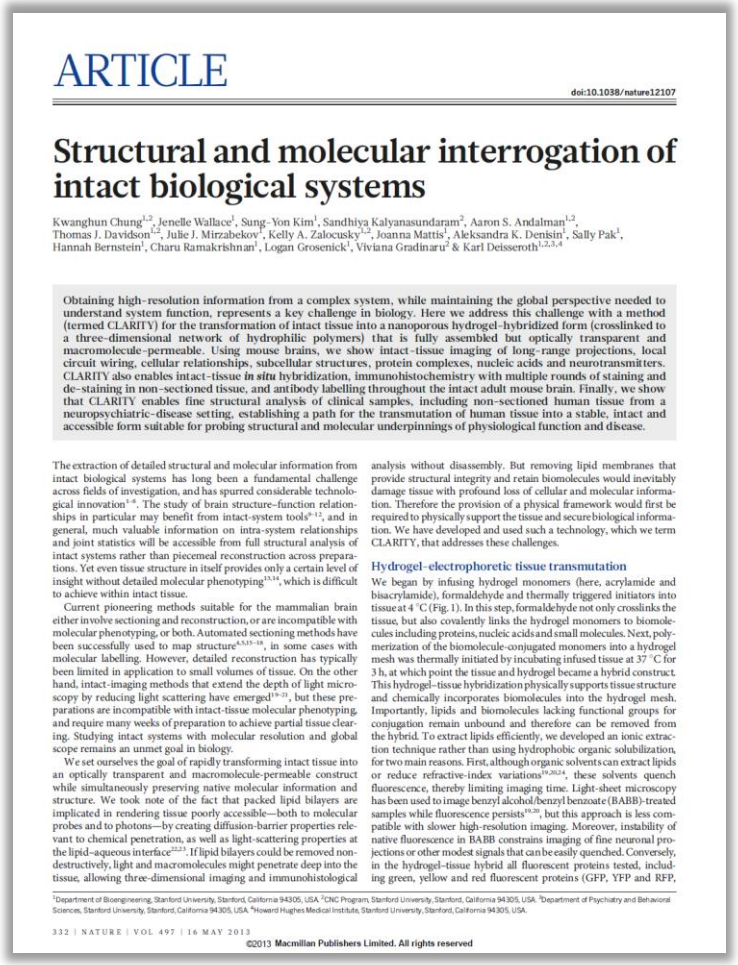
Imaging Solution:

Refractive Index:

Sample Tissues:
Whole mouse brain

Light sheet Cleared Tissue Imaging

CLARITY (Clear Lipid-exchanged Acrylamide-hybridised Rigid Imaging/Immunostaining/In situ-hybridisation-compatible Tissue-hYdrogel)



ARTICLE

doi:10.1038/nature12107

Structural and molecular interrogation of intact biological systems

Kwanghun Chung[1,2], Jenelle Wallace[1], Sung-Yon Kim[1], Sandhiya Kalyanasundaram[2], Aaron S. Andalman[1,2], Thomas J. Davidson[1,2], Julie J. Mirzabekov[1], Kelly A. Zalocusky[1,2], Joanna Mattis[1], Aleksandra K. Denisin[1], Sally Pak[1], Hannah Bernstein[1], Charu Ramakrishnan[1], Logan Grosenick[1], Viviana Gradinaru[2] & Karl Deisseroth[1,2,3,4]

Obtaining high-resolution information from a complex system, while maintaining the global perspective needed to understand system function, represents a key challenge in biology. Here we address this challenge with a method (termed CLARITY) for the transformation of intact tissue into a nanoporous hydrogel–hybridized form (crosslinked to a three-dimensional network of hydrophilic polymers) that is fully assembled but optically transparent and macromolecule-permeable. Using mouse brains, we show intact-tissue imaging of long-range projections, local circuit wiring, cellular relationships, subcellular structures, protein complexes, nucleic acids and neurotransmitters. CLARITY also enables intact-tissue *in situ* hybridization, immunohistochemistry with multiple rounds of staining and de-staining in non-sectioned tissue, and antibody labelling throughout the intact adult mouse brain. Finally, we show that CLARITY enables fine structural analysis of clinical samples, including non-sectioned human tissue from a neuropsychiatric-disease setting, establishing a path for the transmutation of human tissue into a stable, intact and accessible form suitable for probing structural and molecular underpinnings of physiological function and disease.

The extraction of detailed structural and molecular information from intact biological systems has long been a fundamental challenge across fields of investigation, and has spurred considerable technological innovation[1–8]. The study of brain structure–function relationships in particular may benefit from intact-system tools[9–12], and in general, much valuable information on intra-system relationships and joint statistics will be accessible from full structural analysis of intact systems rather than piecemeal reconstruction across preparations. Yet even tissue structure in itself provides only a certain level of insight without detailed molecular phenotyping[13,14], which is difficult to achieve within intact tissue.

Current pioneering methods suitable for the mammalian brain either involve sectioning and reconstruction, or are incompatible with molecular phenotyping, or both. Automated sectioning methods have been successfully used to map structure[9,15–18], in some cases with molecular labelling. However, detailed reconstruction has typically been limited in application to small volumes of tissue. On the other hand, intact-imaging methods that extend the depth of light microscopy by reducing light scattering have emerged[19–21], but these preparations are incompatible with intact-tissue molecular phenotyping, and require many weeks of preparation to achieve partial tissue clearing. Studying intact systems with molecular resolution and global scope remains an unmet goal in biology.

We set ourselves the goal of rapidly transforming intact tissue into an optically transparent and macromolecule-permeable construct while simultaneously preserving native molecular information and structure. We took note of the fact that packed lipid bilayers are implicated in rendering tissue poorly accessible—both to molecular probes and to photons—by creating diffusion-barrier properties relevant to chemical penetration, as well as light-scattering properties at the lipid–aqueous interface[22,23]. If lipid bilayers could be removed non-destructively, light and macromolecules might penetrate deep into the tissue, allowing three-dimensional imaging and immunohistological analysis without disassembly. But removing lipid membranes that provide structural integrity and retain biomolecules would inevitably damage tissue with profound loss of cellular and molecular information. Therefore the provision of a physical framework would first be required to physically support the tissue and secure biological information. We have developed and used such a technology, which we term CLARITY, that addresses these challenges.

Hydrogel–electrophoretic tissue transmutation

We began by infusing hydrogel monomers (here, acrylamide and bisacrylamide), formaldehyde and thermally triggered initiators into tissue at 4 °C (Fig. 1). In this step, formaldehyde not only crosslinks the tissue, but also covalently links the hydrogel monomers to biomolecules including proteins, nucleic acids and small molecules. Next, polymerization of the biomolecule-conjugated monomers into a hydrogel mesh was thermally initiated by incubating infused tissue at 37 °C for 3 h, at which point the tissue and hydrogel became a hybrid construct. This hydrogel–tissue hybridization physically supports tissue structure and chemically incorporates biomolecules into the hydrogel mesh. Importantly, lipids and biomolecules lacking functional groups for conjugation remain unbound and therefore can be removed from the hybrid. To extract lipids efficiently, we developed an ionic extraction technique rather than using hydrophobic organic solubilization, for two main reasons. First, although organic solvents can extract lipids or reduce refractive-index variations[19,20,24], these solvents quench fluorescence, thereby limiting imaging time. Light-sheet microscopy has been used to image benzyl alcohol/benzyl benzoate (BABB)-treated samples while fluorescence persists[19,20], but this approach is less compatible with slower high-resolution imaging. Moreover, instability of native fluorescence in BABB constrains imaging of fine neuronal projections or other modest signals that can be easily quenched. Conversely, in the hydrogel–tissue hybrid all fluorescent proteins tested, including green, yellow and red fluorescent proteins (GFP, YFP and RFP,

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Claim:
CLARITY is a newly developed technology that can be used to transform intact biological tissue into a hybrid form in which tissue components are removed and replaced with exogenous elements for increased accessibility and functionality.

Method:
Electrophoresis of hydrogel embedded tissue in sodium borate buffer (200 mM, pH8.5) containing 4% (wt/vol) SDS.

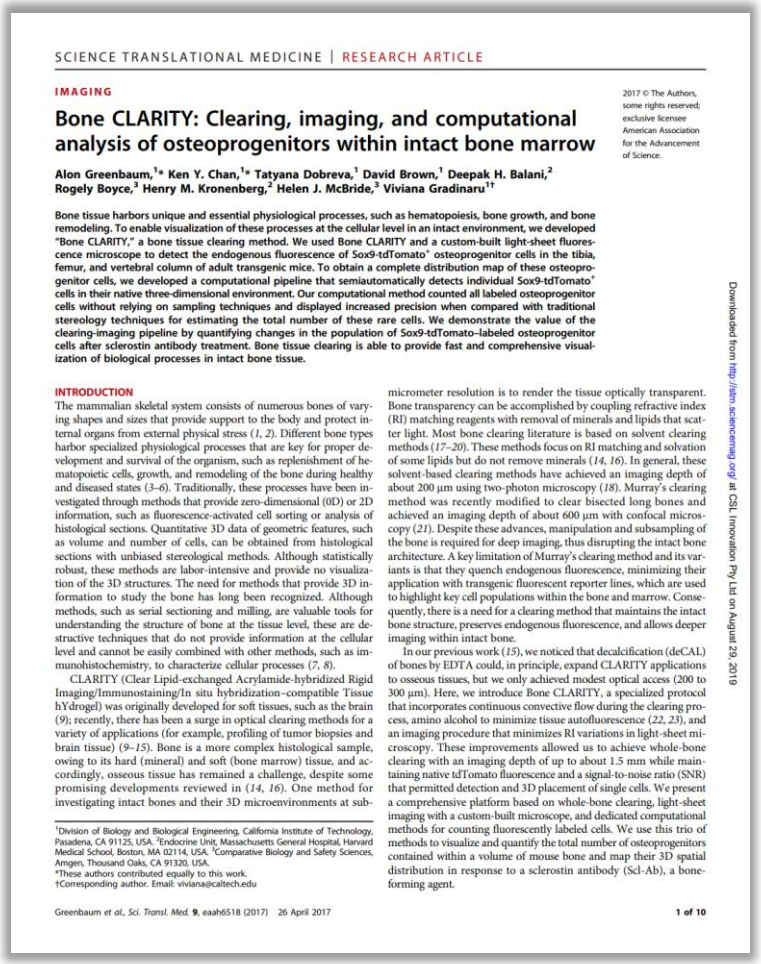
Imaging solution:
FocusClear or 80% glycerol

Refractive Index: 1,45

Sample tissues:
Mouse brain, fish, plant

Light sheet Cleared Tissue Imaging

Bone CLARITY



SCIENCE TRANSLATIONAL MEDICINE | RESEARCH ARTICLE

IMAGING

Bone CLARITY: Clearing, imaging, and computational analysis of osteoprogenitors within intact bone marrow

Alon Greenbaum,[1]* Ken Y. Chan,[1]* Tatyana Dobreva,[1] David Brown,[1] Deepak H. Balani,[2] Rogely Boyce,[3] Henry M. Kronenberg,[2] Helen J. McBride,[3] Viviana Gradinaru[1]†

Bone tissue harbors unique and essential physiological processes, such as hematopoiesis, bone growth, and bone remodeling. To enable visualization of these processes at the cellular level in an intact environment, we developed "Bone CLARITY," a bone tissue clearing method. We used Bone CLARITY and a custom-built light-sheet fluorescence microscope to detect the endogenous fluorescence of Sox9-tdTomato⁺ osteoprogenitor cells in the tibia, femur, and vertebral column of adult transgenic mice. To obtain a complete distribution map of these osteoprogenitor cells, we developed a computational pipeline that semiautomatically detects individual Sox9-tdTomato⁺ cells in their native three-dimensional environment. Our computational method counted all labeled osteoprogenitor cells without relying on sampling techniques and displayed increased precision when compared with traditional stereology techniques for estimating the total number of these rare cells. We demonstrate the value of the clearing-imaging pipeline by quantifying changes in the population of Sox9-tdTomato–labeled osteoprogenitor cells after sclerostin antibody treatment. Bone tissue clearing is able to provide fast and comprehensive visualization of biological processes in intact bone tissue.

INTRODUCTION

The mammalian skeletal system consists of numerous bones of varying shapes and sizes that provide support to the body and protect internal organs from external physical stress (*1*, *2*). Different bone types harbor specialized physiological processes that are key for proper development and survival of the organism, such as replenishment of hematopoietic cells, growth, and remodeling of the bone during healthy and diseased states (*3*–*6*). Traditionally, these processes have been investigated through methods that provide zero-dimensional (0D) or 2D information, such as fluorescence-activated cell sorting or analysis of histological sections. Quantitative 3D data of geometric features, such as volume and number of cells, can be obtained from histological sections with unbiased stereological methods. Although statistically robust, these methods are labor-intensive and provide no visualization of the 3D structures. The need for methods that provide 3D information to study the bone has long been recognized. Although methods, such as serial sectioning and milling, are valuable tools for understanding the structure of bone at the tissue level, these are destructive techniques that do not provide information at the cellular level and cannot be easily combined with other methods, such as immunohistochemistry, to characterize cellular processes (*7*, *8*).

CLARITY (Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging/Immunostaining/In situ hybridization–compatible Tissue hYdrogel) was originally developed for soft tissues, such as the brain (*9*); recently, there has been a surge in optical clearing methods for a variety of applications (for example, profiling of tumor biopsies and brain tissue) (*9*–*15*). Bone is a more complex histological sample, owing to its hard (mineral) and soft (bone marrow) tissue, and accordingly, osseous tissue has remained a challenge, despite some promising developments reviewed in (*14*, *16*). One method for investigating intact bones and their 3D microenvironments at submicrometer resolution is to render the tissue optically transparent. Bone transparency can be accomplished by coupling refractive index (RI) matching reagents with removal of minerals and lipids that scatter light. Most bone clearing literature is based on solvent clearing methods (*17*–*20*). These methods focus on RI matching and solvation of some lipids but do not remove minerals (*14*, *16*). In general, these solvent-based clearing methods have achieved an imaging depth of about 200 μm using two-photon microscopy (*18*). Murray's clearing method was recently modified to clear bisected long bones and achieved an imaging depth of about 600 μm with confocal microscopy (*21*). Despite these advances, manipulation and subsampling of the bone is required for deep imaging, thus disrupting the intact bone architecture. A key limitation of Murray's clearing method and its variants is that they quench endogenous fluorescence, minimizing their application with transgenic fluorescent reporter lines, which are used to highlight key cell populations within the bone and marrow. Consequently, there is a need for a clearing method that maintains the intact bone structure, preserves endogenous fluorescence, and allows deeper imaging within intact bone.

In our previous work (*15*), we noticed that decalcification (deCAL) of bones by EDTA could, in principle, expand CLARITY applications to osseous tissues, but we only achieved modest optical access (200 to 300 μm). Here, we introduce Bone CLARITY, a specialized protocol that incorporates continuous convective flow during the clearing process, amino alcohol to minimize tissue autofluorescence (*22*, *23*), and an imaging procedure that minimizes RI variations in light-sheet microscopy. These improvements allowed us to achieve whole-bone clearing with an imaging depth of up to about 1.5 mm while maintaining native tdTomato fluorescence and a signal-to-noise ratio (SNR) that permitted detection and 3D placement of single cells. We present a comprehensive platform based on whole-bone clearing, light-sheet imaging with a custom-built microscope, and dedicated computational methods for counting fluorescently labeled cells. We use this trio of methods to visualize and quantify the total number of osteoprogenitors contained within a volume of mouse bone and map their 3D spatial distribution in response to a sclerostin antibody (Scl-Ab), a bone-forming agent.

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Claim:
Bone tissue clearing is able to provide fast and comprehensive visualization of biological processes in intact bone tissue.

Method:
The bone is decalcified to increase light and molecular penetration while leaving a framework of bone matrix. Bone CLARITY uses an acrylamide hydrogel to support the tissue structure and minimize protein loss before the delipidation step.

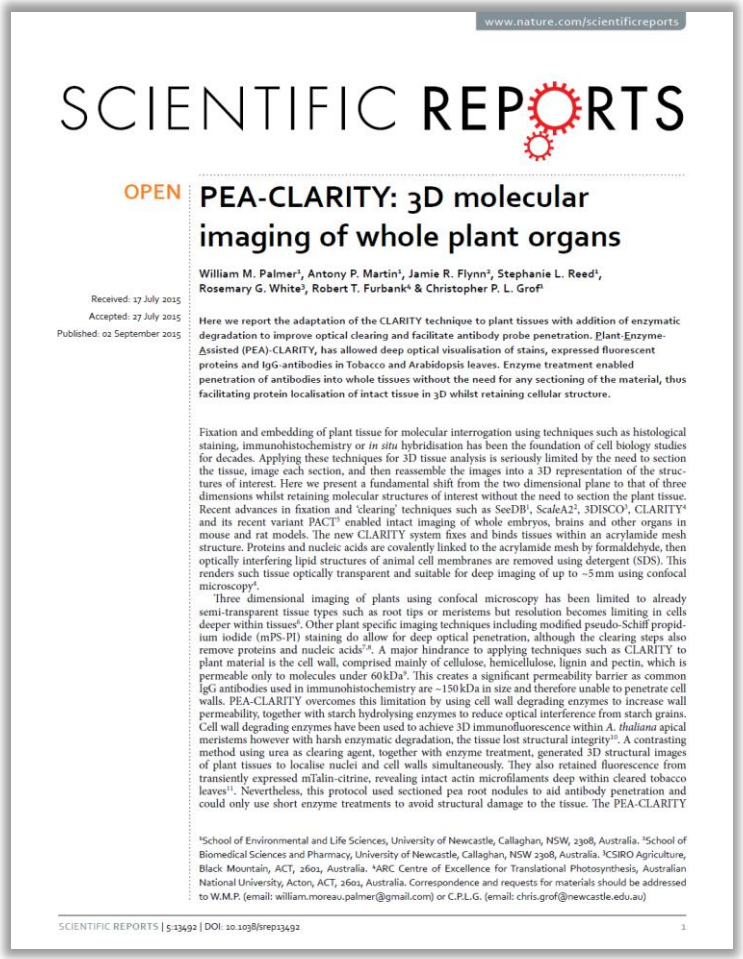
Imaging Solution:
RIMS

Refractive Index: 1,47

Sample Tissues:
Tibia, femur and vertebral column of mouse

Light sheet Cleared Tissue Imaging

PEA-CLARITY (Plant-Enzyme-Assisted-CLARITY)



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PEA-CLARITY: 3D molecular imaging of whole plant organs

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Here we report the adaptation of the CLARITY technique to plant tissues with addition of enzymatic degradation to improve optical clearing and facilitate antibody probe penetration. Plant-Enzyme-Assisted (PEA)-CLARITY, has allowed deep optical visualisation of stains, expressed fluorescent proteins and IgG-antibodies in Tobacco and Arabidopsis leaves. Enzyme treatment enabled penetration of antibodies into whole tissues without the need for any sectioning of the material, thus facilitating protein localisation of intact tissue in 3D whilst retaining cellular structure.

Fixation and embedding of plant tissue for molecular interrogation using techniques such as histological staining, immunohistochemistry or *in situ* hybridisation has been the foundation of cell biology studies for decades. Applying these techniques for 3D tissue analysis is seriously limited by the need to section the tissue, image each section, and then reassemble the images into a 3D representation of the structures of interest. Here we present a fundamental shift from the two dimensional plane to that of three dimensions whilst retaining molecular structures of interest without the need to section the plant tissue. Recent advances in fixation and 'clearing' techniques such as SeeDB[1], ScaleA2[2], 3DISCO[3], CLARITY[4] and its recent variant PACT[5] enabled intact imaging of whole embryos, brains and other organs in mouse and rat models. The new CLARITY system fixes and binds tissues within an acrylamide mesh structure. Proteins and nucleic acids are covalently linked to the acrylamide mesh by formaldehyde, then optically interfering lipid structures of animal cell membranes are removed using detergent (SDS). This renders such tissue optically transparent and suitable for deep imaging of up to ~5 mm using confocal microscopy[6].

Three dimensional imaging of plants using confocal microscopy has been limited to already semi-transparent tissue types such as root tips or meristems but resolution becomes limiting in cells deeper within tissues[6]. Other plant specific imaging techniques including modified pseudo-Schiff propidium iodide (mPS-PI) staining do allow for deep optical penetration, although the clearing steps also remove proteins and nucleic acids[7,8]. A major hindrance to applying techniques such as CLARITY to plant material is the cell wall, comprised mainly of cellulose, hemicellulose, lignin and pectin, which is permeable only to molecules under 60 kDa[9]. This creates a significant permeability barrier as common IgG antibodies used in immunohistochemistry are ~150 kDa in size and therefore unable to penetrate cell walls. PEA-CLARITY overcomes this limitation by using cell wall degrading enzymes to increase wall permeability, together with starch hydrolysing enzymes to reduce optical interference from starch grains. Cell wall degrading enzymes have been used to achieve 3D immunofluorescence within *A. thaliana* apical meristems however with harsh enzymatic degradation, the tissue lost structural integrity[10]. A contrasting method using urea as clearing agent, together with enzyme treatment, generated 3D structural images of plant tissues to localise nuclei and cell walls simultaneously. They also retained fluorescence from transiently expressed mTalin-citrine, revealing intact actin microfilaments deep within cleared tobacco leaves[11]. Nevertheless, this protocol used sectioned pea root nodules to aid antibody penetration and could only use short enzyme treatments to avoid structural damage to the tissue. The PEA-CLARITY

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Claim:
Plant-Enzyme-Assisted (PEA)-CLARITY, has allowed deep optical visualisation of stains, expressed fluorescent proteins and IgG-antibodies in Tobacco and Arabidopsis leaves.

Method:
PEA-Clarity involves hydrogel fixation, tissue clearing and enzymatic degradation, without loss of structural integrity.

Imaging Solution: PBS

Refractive Index: 1,33

Sample Tissues:
Plant, Tobacco and Arabidopsis leaves

PEA-CLARITY

Plant-Enzyme-Assisted (PEA)-CLARITY

Light sheet Cleared Tissue Imaging

ACT-PRESTO (*A*ctive *c*larity *t*echnique–*p*ressure *r*elated *e*fficient and *s*table *t*ransfer of macromolecules into *o*rgans)

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ACT-PRESTO: Rapid and consistent tissue clearing and labeling method for 3-dimensional (3D) imaging

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Understanding the structural organization of organs and organisms at the cellular level is a fundamental challenge in biology. This task has been approached by reconstructing three-dimensional structure from images taken from serially sectioned tissues, which is not only labor-intensive and time-consuming but also error-prone. Recent advances in tissue clearing techniques allow visualization of cellular structures and neural networks inside of unsectioned whole tissues or the entire body. However, currently available protocols require long process times. Here, we present the rapid and highly reproducible ACT-PRESTO (*active clarity technique-pressure related efficient and stable transfer of macromolecules into organs*) method that clears tissues or the whole body within 1 day while preserving tissue architecture and protein-based signals derived from endogenous fluorescent proteins. Moreover, ACT-PRESTO is compatible with conventional immunolabeling methods and expedites antibody penetration into thick specimens by applying pressure. The speed and consistency of this method will allow high-content mapping and analysis of normal and pathological features in intact organs and bodies.

Volume imaging with single-cell resolution should allow molecular and structural analyses of biological systems[1–3] and enable more accurate medical diagnosis[4,5]. Conventional volume imaging requires tissue sectioning, labeling of serially sectioned tissues with probes for specific targets, such as macromolecules (proteins and nucleotides), and reconstructing individual two-dimensional (2D) images into three-dimensional (3D) structures[6–12]. Such processes are not only labor-intensive and time-consuming, but also prone to errors, such as mechanical distortion of tissues caused by sectioning and inaccurate 3D reconstruction due to the complexity of mapping reference points.

Development of methods to transform tissues and organs into optically transparent samples and image cellular structures in unsectioned, intact organs has attracted attention[1–3,7,13–19]. One way to achieve transparency is immersing samples into selective media with a suitable refractive index (approximately 1.45) to minimize light scattering[20,21]. Several hydrophobic reagents have been developed to render tissues nearly transparent, but many of these reagents cause rapid quenching of fluorescent signals during the dehydration step[19,22]. Hydrophilic reagents have been introduced to maintain fluorescent signals[1,13,15], but optical clearing is generally slow, particularly for large tissues.

Substantial progress has been made in tissue (brain in particular) clearing and promoting the penetration of labeling reagents with the advent of hydrogen-based clearing methods[2,3,23]. These methods include a protein and acrylamide crosslinking step that selectively immobilizes proteins and other macromolecules, resulting in a tissue-embedded hydrogel[24,25]. Lipid components are selectively removed either by passive diffusion or actively by electrophoresis. Removing the lipid components markedly increases optical transparency, and the porous nature of the hydrogel allows penetration of labeling reagents deep inside thick tissues[2,3]. However, hydrogel-embedded tissue clearing methods retain a dense extracellular matrix (ECM), which hinders penetration of macromolecules

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Claim
ACT-PRESTO allows for tissue clearance within several hours and enables rapid immunolabeling with conventional methods and accelerates antibody penetration into the deep layer of densely formed, thick specimens by applying pressure or convection flow.

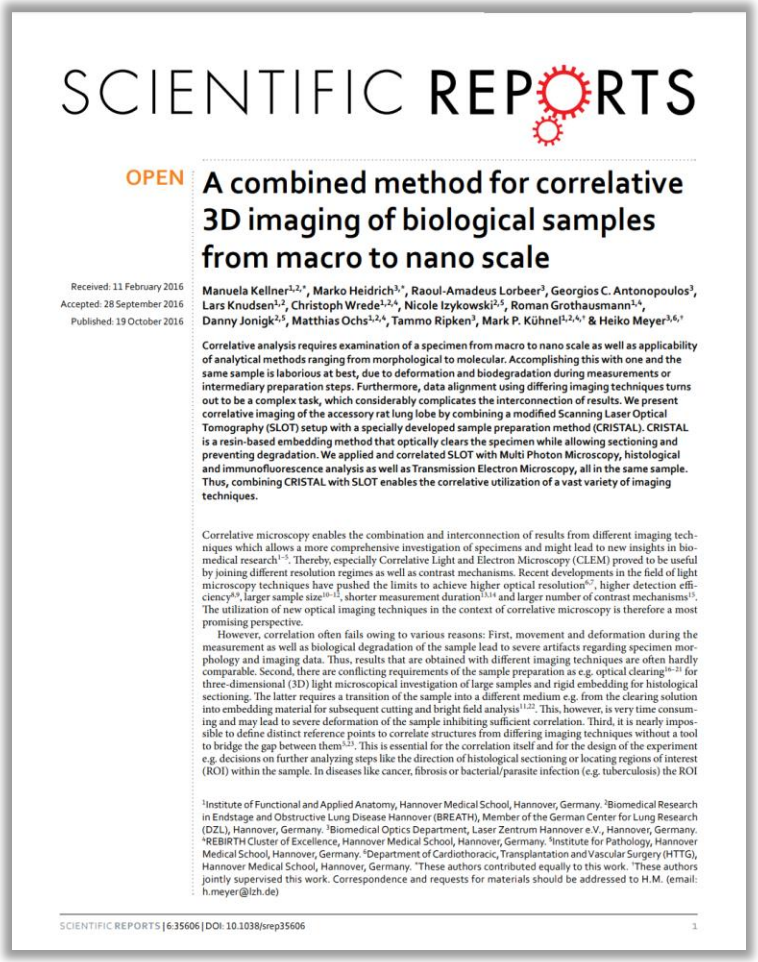
Method:
Electrophoretic tissue clearing (ETC) of polymerized sample.

Imaging Solution:
Cubic Mounting Solution.

Refractive Index: ~1,45

Sample Tissues:
Mouse brain

Light sheet Cleared Tissue Imaging
CRISTAL



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A combined method for correlative 3D imaging of biological samples from macro to nano scale

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Correlative analysis requires examination of a specimen from macro to nano scale as well as applicability of analytical methods ranging from morphological to molecular. Accomplishing this with one and the same sample is laborious at best, due to deformation and biodegradation during measurements or intermediary preparation steps. Furthermore, data alignment using differing imaging techniques turns out to be a complex task, which considerably complicates the interconnection of results. We present correlative imaging of the accessory rat lung lobe by combining a modified Scanning Laser Optical Tomography (SLOT) setup with a specially developed sample preparation method (CRISTAL). CRISTAL is a resin-based embedding method that optically clears the specimen while allowing sectioning and preventing degradation. We applied and correlated SLOT with Multi Photon Microscopy, histological and immunofluorescence analysis as well as Transmission Electron Microscopy, all in the same sample. Thus, combining CRISTAL with SLOT enables the correlative utilization of a vast variety of imaging techniques.

Correlative microscopy enables the combination and interconnection of results from different imaging techniques which allows a more comprehensive investigation of specimens and might lead to new insights in biomedical research[1–5]. Thereby, especially Correlative Light and Electron Microscopy (CLEM) proved to be useful by joining different resolution regimes as well as contrast mechanisms. Recent developments in the field of light microscopy techniques have pushed the limits to achieve higher optical resolution[6,7], higher detection efficiency[8,9], larger sample size[10–12], shorter measurement duration[13,14] and larger number of contrast mechanisms[15]. The utilization of new optical imaging techniques in the context of correlative microscopy is therefore a most promising perspective.

However, correlation often fails owing to various reasons: First, movement and deformation during the measurement as well as biological degradation of the sample lead to severe artifacts regarding specimen morphology and imaging data. Thus, results that are obtained with different imaging techniques are often hardly comparable. Second, there are conflicting requirements of the sample preparation as e.g. optical clearing[16–21] for three-dimensional (3D) light microscopical investigation of large samples and rigid embedding for histological sectioning. The latter requires a transition of the sample into a different medium e.g. from the clearing solution into embedding material for subsequent cutting and bright field analysis[11,22]. This, however, is very time consuming and may lead to severe deformation of the sample inhibiting sufficient correlation. Third, it is nearly impossible to define distinct reference points to correlate structures from differing imaging techniques without a tool to bridge the gap between them[2,23]. This is essential for the correlation itself and for the design of the experiment e.g. decisions on further analyzing steps like the direction of histological sectioning or locating regions of interest (ROI) within the sample. In diseases like cancer, fibrosis or bacterial/parasite infection (e.g. tuberculosis) the ROI

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Claim
CRISTAL is a resin-based embedding method that optically clears the specimen while allowing sectioning and preventing degradation.

Method:
Ethanol dehydration series, Xylene

Imaging Solution:
Resin NOA68 and NOA71 embedded.

Refractive Index: 1,556

Sample Tissues:
Lung

Light sheet Cleared Tissue Imaging

Stochastic Electrotransport

PNAS PNAS PNAS

CrossMark

Stochastic electrotransport selectively enhances the transport of highly electromobile molecules

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Nondestructive chemical processing of porous samples such as fixed biological tissues typically relies on molecular diffusion. Diffusion into a porous structure is a slow process that significantly delays completion of chemical processing. Here, we present a novel electrokinetic method termed stochastic electrotransport for rapid nondestructive processing of porous samples. This method uses a rotational electric field to selectively disperse highly electromobile molecules throughout a porous sample without displacing the low-electromobility molecules that constitute the sample. Using computational models, we show that stochastic electrotransport can rapidly disperse electromobile molecules in a porous medium. We apply this method to completely clear mouse organs within 1–3 days and to stain them with nuclear dyes, proteins, and antibodies within 1 day. Our results demonstrate the potential of stochastic electrotransport to process large and dense tissue samples that were previously infeasible in time when relying on diffusion.

stochastic electrotransport | molecular transport | tissue clearing | tissue labeling | CLARITY

Diffusion is a slow process that governs the overall speed of many biochemical and engineering processes. Diffusion is produced by random molecular motion (a "random walk"), and it leads to complete dispersion of particles but is inherently slow (1). Diffusion is, therefore, effective for small-length-scale applications but becomes impractical for applications requiring larger length scales. This is especially true when the sample contains dense architectures with small and tortuous pores that hinder molecular movement. Diffusion of molecules into and out of such a sample (e.g., fixed biological tissues) can take an impractically long time. For instance, it can take weeks for antibodies to diffuse a few millimeters into fixed tissues (2). The slow nature of diffusive transport has long limited the application of many existing and emerging techniques in biology and medicine to small or thin tissue samples (3–7).

External forces can enhance transport of otherwise slowly diffusive molecules into and out of porous samples, but they have many limitations. For instance, hydrodynamic pressure can generate a convective flow across a porous sample (8), but the high pressure required to generate the flow can deform fragile samples such as soft tissues or polymeric materials (9). An electric field can drive electrophoresis of charged particles through a porous sample (10), but if the sample contains charged molecules, the electric field can also damage the sample. For this reason, electrophoresis may not be suitable for tissues or biomolecule–polymer hybrids containing charged endogenous biomolecules (11, 12). To avoid damaging samples, then, conventional chemical and biomedical methods for biological processing rely on the slow but safe diffusion method.

However, with the development of in situ molecular interrogation methods (6, 13, 14) and tissue clearing techniques (2, 15–25) and an emphasis on studying organ-scale tissue as a whole, a pressing need has arisen for a means of expediting the transportation of various molecules into intact tissues. For example, many emerging tissue clearing techniques use surfactant micelles to directly remove lipids from a tissue and thus eliminate light-scattering boundaries to improve optical penetration for holistic visualization (2, 15–25), but transporting these micelles into the intact tissue via diffusion can take weeks (2, 15). Although electrophoresis can speed up this process, as demonstrated in CLARITY, its application has been limited to low electric fields because using high fields can damage tissue structures (2). The problem is compounded by the fact that different regions of a tissue can have widely different electrical properties (26), leading to regions with concentrated electric fields. Electrophoresis, therefore, is ineffective for hastening transport of surfactant micelles into large, dense samples because only low electric fields can be used without risking damage to the sample.

Faster transportation of molecular probes into intact tissue is also needed to reduce the time required to label large tissues. Diverse methods of tissue labeling are used in many areas of biological research and medical diagnosis for visualizing various biomolecules of interest. However, these techniques have been mostly confined to small samples owing to the difficulty of labeling and examining deep structures in large-scale intact tissues (6, 27–31). CLARITY and other emerging tissue-clearing techniques (2, 15–25) render intact tissues optically transparent and macromolecule-permeable, allowing examination deep inside

Significance

Many chemical and biomedical techniques rely on slow diffusive transport because existing pressure-based methods or electrokinetic methods can incidentally damage the sample. This study introduces a novel transport concept termed stochastic electrotransport that can selectively and nondestructively expedite transport of electromobile molecules into a porous sample, such as fixed biological tissues. We use the method to rapidly transport several classes of molecules into whole mouse brains and other organs and achieve rapid clearing and staining of the entire tissue in record time without damaging the sample. Our new method may facilitate the application of various molecular techniques to large and dense tissues.

Author contributions: K.C. conceived the idea; J.H.C. developed the theoretical models; S.-Y.K., J.H.C., E.M., and K.C. designed research; S.-Y.K., J.H.C., E.M., N.B., H.C., K.O., L.R., A.H., M.M., S.L.V., P.J.K., and K.C. performed research. P.J.K. contributed new reagents/analytic tools; S.-Y.K., J.H.C., and K.C. analyzed data; S.-Y.K., J.H.C., and K.C. wrote the paper; and K.C. supervised all aspects of the work.

Conflict of interest statement: The authors hold intellectual property related to the concept of stochastic transport and its implementation.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1510133112/-/DCSupplemental.

Claim:
Novel electrokinetic method termed for rapid nondestructive processing of porous samples. Completely clears mouse organs within 1–3 days and stains them with nuclear dyes, proteins, and antibodies within 1 day.

Method:
Uses a rotational electric field to selectively disperse highly electromobile molecules throughout
a porous sample without displacing the low-electromobility molecules that constitute the sample.

Imaging Solution: Custom made RIMS

Refractive Index: Unknown

Sample Tissues:
Mouse brain and intestine

Light sheet Cleared Tissue Imaging

SWITCH

HHS Public Access
Author manuscript
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Simple, scalable proteomic imaging for high-dimensional profiling of intact systems

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AUTHOR CONTRIBUTIONS

K.C. conceived the SWITCH idea. E.M., J.H.C, D.G., T.K., J.S., and K.C. designed the experiments. E.M. designed and performed multiplexed staining and imaging experiments. D.G. and H.S.S. developed and implemented the registration algorithm. T.K. performed all analysis related to the human visual cortex sample with input from K.C., M.P.F., and H.S.S. J.H.C. performed all analysis related to DiD datasets. E.M., N.B., and S.-Y.K. performed antibody compatibility testing. E.M., J.H.C, N.B., A.H., and J.S. performed testing of cross-linked protein gels with various fixatives. E.M. performed protein loss and structural preservation experiments. E.M., J.S., and N.B. designed and performed experiments relating to thermal clearing and use of anti-browning agents. E.M. and M.M. performed DiD staining experiments. E.M. and Y.-G.P. performed antibody SWITCH experiments. H.C. designed and built the light-sheet microscope, and J.H.C. developed the software. J.-Y.P. provided spinal cord samples. Y.-G.P. performed mRNA experiments. M.P.F. provided human samples. H.S.S., V.W., and M.P.F. provided helpful discussion regarding the manuscript. S.V. provided experimental support. E.M., J.H.C., D.G., T.K., J.S., S.-Y.K., V.J.W., and K.C. wrote the manuscript. K.C. supervised all aspects of the work.

Claim:
SWITCH uniformly secures tissue architecture, native biomolecules, and antigenicity across an entire system by synchronizing the tissue preservation reaction.

Method:
Tissue preservation by synchronized dialdehyde-tissue-gel formation. Aqueous clearing solution containing SDS, lithium hydroxide, boric acid, and anti-browning agent. Samples were incubated at 60–80°C until clear. The method also comprises specific staining procedures.

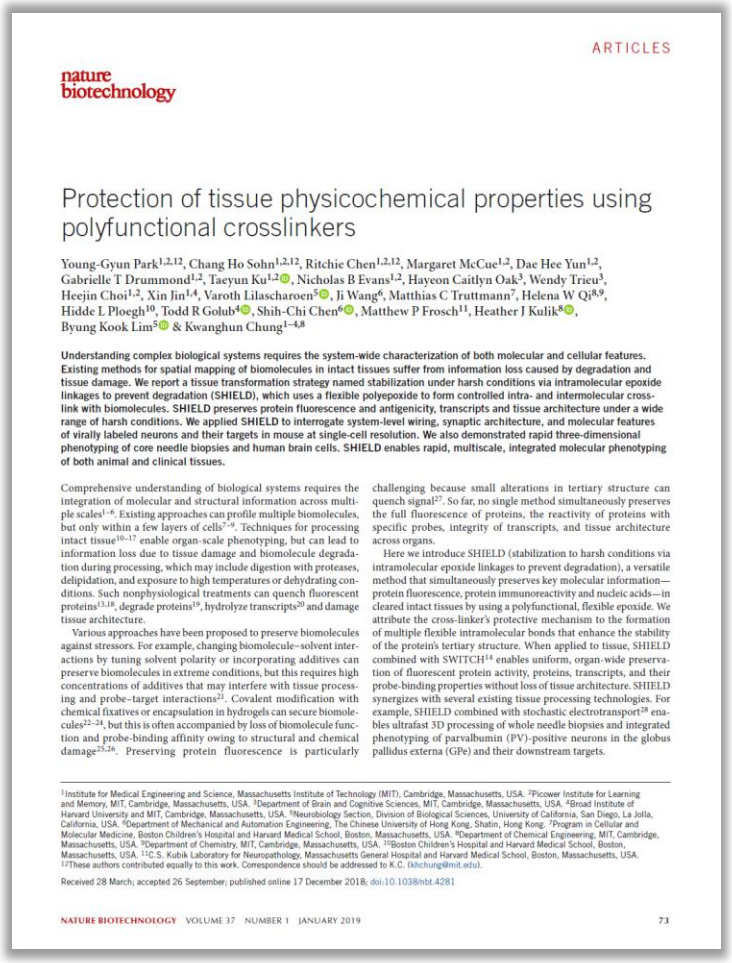
Imaging Solution: PBST

Refractive Index: 1,33

Sample Tissues:
Rodent brain

Light sheet Cleared Tissue Imaging

SHIELD (Stabilization to harsh conditions via intramolecular epoxide linkages to prevent degradation)



ARTICLES

nature biotechnology

Protection of tissue physicochemical properties using polyfunctional crosslinkers

Young-Gyun Park[1,2,12], Chang Ho Sohn[1,2,12], Ritchie Chen[1,2,12], Margaret McCue[1,2], Dae Hee Yun[1,2], Gabrielle T Drummond[1,2], Taeyun Ku[1,2], Nicholas B Evans[1,2], Hayeon Caitlyn Oak[3], Wendy Trieu[3], Heejin Choi[1,2], Xin Jin[1,4], Varoth Lilascharoen[5], Ji Wang[6], Matthias C Truttmann[7], Helena W Qi[8,9], Hidde L Ploegh[10], Todd R Golub[4], Shih-Chi Chen[6], Matthew P Frosch[11], Heather J Kulik[8], Byung Kook Lim[5] & Kwanghun Chung[1–4,8]

Understanding complex biological systems requires the system-wide characterization of both molecular and cellular features. Existing methods for spatial mapping of biomolecules in intact tissues suffer from information loss caused by degradation and tissue damage. We report a tissue transformation strategy named stabilization under harsh conditions via intramolecular epoxide linkages to prevent degradation (SHIELD), which uses a flexible polyepoxide to form controlled intra- and intermolecular cross-link with biomolecules. SHIELD preserves protein fluorescence and antigenicity, transcripts and tissue architecture under a wide range of harsh conditions. We applied SHIELD to interrogate system-level wiring, synaptic architecture, and molecular features of virally labeled neurons and their targets in mouse at single-cell resolution. We also demonstrated rapid three-dimensional phenotyping of core needle biopsies and human brain cells. SHIELD enables rapid, multiscale, integrated molecular phenotyping of both animal and clinical tissues.

Comprehensive understanding of biological systems requires the integration of molecular and structural information across multiple scales[1–6]. Existing approaches can profile multiple biomolecules, but only within a few layers of cells[7–9]. Techniques for processing intact tissue[10–17] enable organ-scale phenotyping, but can lead to information loss due to tissue damage and biomolecule degradation during processing, which may include digestion with proteases, delipidation, and exposure to high temperatures or dehydrating conditions. Such nonphysiological treatments can quench fluorescent proteins[13,18], degrade proteins[19], hydrolyze transcripts[20] and damage tissue architecture.

Various approaches have been proposed to preserve biomolecules against stressors. For example, changing biomolecule–solvent interactions by tuning solvent polarity or incorporating additives can preserve biomolecules in extreme conditions, but this requires high concentrations of additives that may interfere with tissue processing and probe–target interactions[21]. Covalent modification with chemical fixatives or encapsulation in hydrogels can secure biomolecules[22–24], but this is often accompanied by loss of biomolecule function and probe-binding affinity owing to structural and chemical damage[25,26]. Preserving protein fluorescence is particularly challenging because small alterations in tertiary structure can quench signal[27]. So far, no single method simultaneously preserves the full fluorescence of proteins, the reactivity of proteins with specific probes, integrity of transcripts, and tissue architecture across organs.

Here we introduce SHIELD (stabilization to harsh conditions via intramolecular epoxide linkages to prevent degradation), a versatile method that simultaneously preserves key molecular information—protein fluorescence, protein immunoreactivity and nucleic acids—in cleared intact tissues by using a polyfunctional, flexible epoxide. We attribute the cross-linker's protective mechanism to the formation of multiple flexible intramolecular bonds that enhance the stability of the protein's tertiary structure. When applied to tissue, SHIELD combined with SWITCH[14] enables uniform, organ-wide preservation of fluorescent protein activity, proteins, transcripts, and their probe-binding properties without loss of tissue architecture. SHIELD synergizes with several existing tissue processing technologies. For example, SHIELD combined with stochastic electrotransport[28] enables ultrafast 3D processing of whole needle biopsies and integrated phenotyping of parvalbumin (PV)-positive neurons in the globus pallidus externa (GPe) and their downstream targets.

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Claim:
When applied to tissue, SHIELD combined with SWITCH enables uniform, organ-wide preservation of fluorescent protein activity, proteins, transcripts, and their probe-binding properties without loss of tissue architecture.

Method:
*Fixation by p*erfusion with SHIELD solution, followed by incubation in SHIELD-OFF solution for 24 h. Samples are then placed in SHIELD-ON solution and incubated for 24 h. SHIELD-fixed organs are then delipidated, either passively in SDS-based clearing buffer, or rapidly using stochastic electrotransport. Delipidated tissues are then incubated in Protos-based immersion media for optical clearing and imaging.

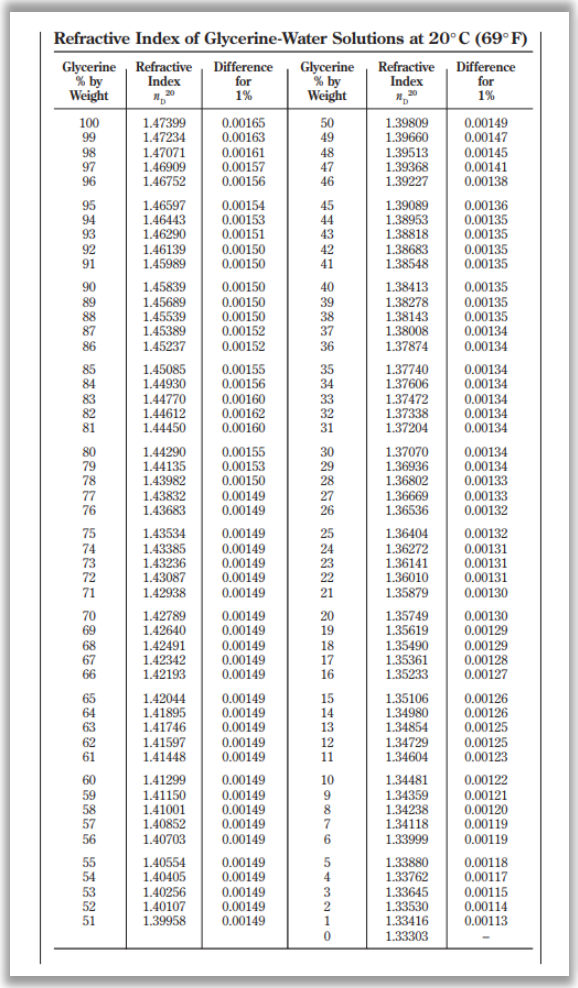
Imaging Solution: Protos-based immersion media (recipe provided in paper)

Refractive Index: 1.458

Sample Tissues: Mouse and rat, biopsies

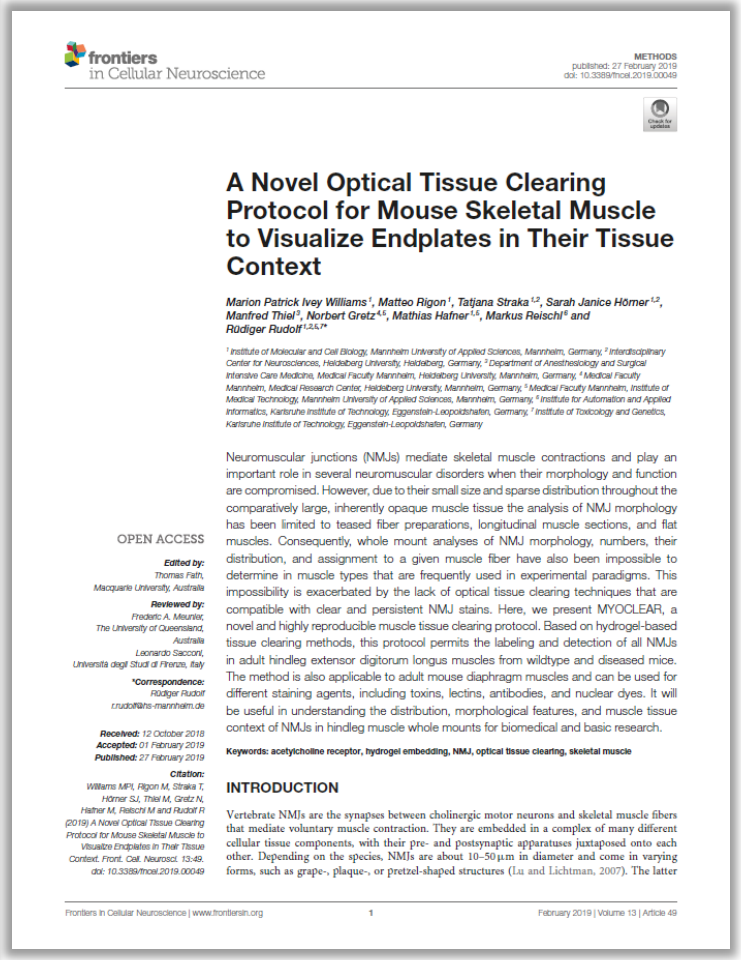
Light sheet Cleared Tissue Imaging

MYOCLEAR



Refractive Index of Glycerine-Water Solutions at 20°C (69°F)

Glycerine % by Weight	Refractive Index n_D^{20}	Difference for 1%	Glycerine % by Weight	Refractive Index n_D^{20}	Difference for 1%
100	1.47399	0.00165	50	1.39809	0.00149
99	1.47234	0.00163	49	1.39660	0.00147
98	1.47071	0.00161	48	1.39513	0.00145
97	1.46909	0.00157	47	1.39368	0.00141
96	1.46752	0.00156	46	1.39227	0.00138
95	1.46597	0.00154	45	1.39089	0.00136
94	1.46443	0.00153	44	1.38953	0.00135
93	1.46290	0.00151	43	1.38818	0.00135
92	1.46139	0.00150	42	1.38683	0.00135
91	1.45989	0.00150	41	1.38548	0.00135
90	1.45839	0.00150	40	1.38413	0.00135
89	1.45689	0.00150	39	1.38278	0.00135
88	1.45539	0.00150	38	1.38143	0.00135
87	1.45389	0.00152	37	1.38008	0.00134
86	1.45237	0.00152	36	1.37874	0.00134
85	1.45085	0.00155	35	1.37740	0.00134
84	1.44930	0.00156	34	1.37606	0.00134
83	1.44770	0.00160	33	1.37472	0.00134
82	1.44612	0.00162	32	1.37338	0.00134
81	1.44450	0.00160	31	1.37204	0.00134
80	1.44290	0.00155	30	1.37070	0.00134
79	1.44135	0.00153	29	1.36936	0.00134
78	1.43982	0.00150	28	1.36802	0.00133
77	1.43832	0.00149	27	1.36669	0.00133
76	1.43683	0.00149	26	1.36536	0.00132
75	1.43534	0.00149	25	1.36404	0.00132
74	1.43385	0.00149	24	1.36272	0.00131
73	1.43236	0.00149	23	1.36141	0.00131
72	1.43087	0.00149	22	1.36010	0.00131
71	1.42938	0.00149	21	1.35879	0.00130
70	1.42789	0.00149	20	1.35749	0.00130
69	1.42640	0.00149	19	1.35619	0.00129
68	1.42491	0.00149	18	1.35490	0.00129
67	1.42342	0.00149	17	1.35361	0.00128
66	1.42193	0.00149	16	1.35233	0.00127
65	1.42044	0.00149	15	1.35106	0.00126
64	1.41895	0.00149	14	1.34980	0.00126
63	1.41746	0.00149	13	1.34854	0.00125
62	1.41597	0.00149	12	1.34729	0.00125
61	1.41448	0.00149	11	1.34604	0.00123
60	1.41299	0.00149	10	1.34481	0.00122
59	1.41150	0.00149	9	1.34359	0.00121
58	1.41001	0.00149	8	1.34238	0.00120
57	1.40852	0.00149	7	1.34118	0.00119
56	1.40703	0.00149	6	1.33999	0.00119
55	1.40554	0.00149	5	1.33880	0.00118
54	1.40405	0.00149	4	1.33762	0.00117
53	1.40256	0.00149	3	1.33645	0.00115
52	1.40107	0.00149	2	1.33530	0.00114
51	1.39958	0.00149	1	1.33416	0.00113
			0	1.33303	–



frontiers in Cellular Neuroscience

METHODS
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A Novel Optical Tissue Clearing Protocol for Mouse Skeletal Muscle to Visualize Endplates in Their Tissue Context

*Marion Patrick Ivey Williams[1], Matteo Rigon[1], Tatjana Straka[1,2], Sarah Janice Hörner[1,2], Manfred Thiel[3], Norbert Gretz[4,5], Mathias Hafner[1,5], Markus Reischl[6] and Rüdiger Rudolf[1,2,5,7]**

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Neuromuscular junctions (NMJs) mediate skeletal muscle contractions and play an important role in several neuromuscular disorders when their morphology and function are compromised. However, due to their small size and sparse distribution throughout the comparatively large, inherently opaque muscle tissue the analysis of NMJ morphology has been limited to teased fiber preparations, longitudinal muscle sections, and flat muscles. Consequently, whole mount analyses of NMJ morphology, numbers, their distribution, and assignment to a given muscle fiber have also been impossible to determine in muscle types that are frequently used in experimental paradigms. This impossibility is exacerbated by the lack of optical tissue clearing techniques that are compatible with clear and persistent NMJ stains. Here, we present MYOCLEAR, a novel and highly reproducible muscle tissue clearing protocol. Based on hydrogel-based tissue clearing methods, this protocol permits the labeling and detection of all NMJs in adult hindleg extensor digitorum longus muscles from wildtype and diseased mice. The method is also applicable to adult mouse diaphragm muscles and can be used for different staining agents, including toxins, lectins, antibodies, and nuclear dyes. It will be useful in understanding the distribution, morphological features, and muscle tissue context of NMJs in hindleg muscle whole mounts for biomedical and basic research.

Keywords: acetylcholine receptor, hydrogel embedding, NMJ, optical tissue clearing, skeletal muscle

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INTRODUCTION

Vertebrate NMJs are the synapses between cholinergic motor neurons and skeletal muscle fibers that mediate voluntary muscle contraction. They are embedded in a complex of many different cellular tissue components, with their pre- and postsynaptic apparatuses juxtaposed onto each other. Depending on the species, NMJs are about 10–50 µm in diameter and come in varying forms, such as grape-, plaque-, or pretzel-shaped structures (Lu and Lichtman, 2007). The latter

Claim:
A novel and highly reproducible muscle tissue clearing protocol.

Method:
Samples are infiltrated with hydrogel monomer solution, polymerized, and stained. Lastly, samples are incubated in 88% glycerol at room temperature for a minimum of 24 h for RI matching and long-term storage

Imaging Solution: 88% glycerol

Refractive Index: 1,45

Sample Tissues:
NMJs in adult hindleg extensor digitorum longus muscles from wildtype and diseased mice; adult mouse diaphragm muscles; Can be used for different staining agents, including toxins, lectins, antibodies, and nuclear dyes.

Light sheet Cleared Tissue Imaging

RIMS (Refractive Index Matching Solution)

eLIFE elifesciences.org

TOOLS AND RESOURCES

A tunable refractive index matching medium for live imaging cells, tissues and model organisms

Tobias Boothe[1,2,3], Lennart Hilbert[1,2,3], Michael Heide[1], Lea Berninger[1], Wieland B Huttner[1], Vasily Zaburdaev[2,3], Nadine L Vastenhouw[1], Eugene W Myers[1,3], David N Drechsel[1†], Jochen C Rink[1*]

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Abstract In light microscopy, refractive index mismatches between media and sample cause spherical aberrations that often limit penetration depth and resolution. Optical clearing techniques can alleviate these mismatches, but they are so far limited to fixed samples. We present Iodixanol as a non-toxic medium supplement that allows refractive index matching in live specimens and thus substantially improves image quality in live-imaged primary cell cultures, planarians, zebrafish and human cerebral organoids.

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Introduction

Live imaging is a key tool in understanding the organization and function of cells, tissues and organisms, since it allows the visualization of dynamic processes within their native environment. However, in practice, the live-imaging of multi-layered tissues with different cell types often poses major challenges. Refractive index mismatches between tissue and surrounding medium result in spherical aberrations that misalign the optical paths and ultimately distort and attenuate the microscopic image. This effect increases with complexity and thickness of the specimen, making imaging in deep tissue layers difficult and technically demanding (***Richardson and Lichtman, 2015***).

Microscope optimization constitutes a first approach to optimize deep imaging. 2-photon microscopy greatly improves depth penetration by excitation with low scattering, near-infrared wavelengths (***Helmchen and Denk, 2005***). However, 2-photon microscopy cannot alleviate spherical aberration effects (***Richardson and Lichtman, 2015***). These can be partially compensated by the recent introduction of adaptive optics microscopes (***Booth, 2014***), yet at the cost of reduced image acquisition rates and the need for intense excitation light. A second approach to improving depth penetration is the direct adjustment of the refractive indexes (RI) of sample and environment (***Richardson and Lichtman, 2015***). Indeed, recently developed optical clearing techniques can render tissues effectively transparent by equilibrating refractive index heterogeneity within biological samples (***Chung et al., 2013***; ***Hama et al., 2015***). Unfortunately, these protocols remain limited to fixed specimens due to their reliance on harsh mounting conditions and/or toxic chemicals (***Richardson and Lichtman, 2015***).

Results

Towards the goal of developing an RI matching medium for live-imaging, we searched for compounds that combine high water solubility as prerequisite for dilution into regular culture media,

Claim
A non-toxic medium supplement that allows refractive index matching in live specimens and thus substantially improves image quality in live-imaging

Method:
Histodenz solutions

Imaging Solution:
RIMS

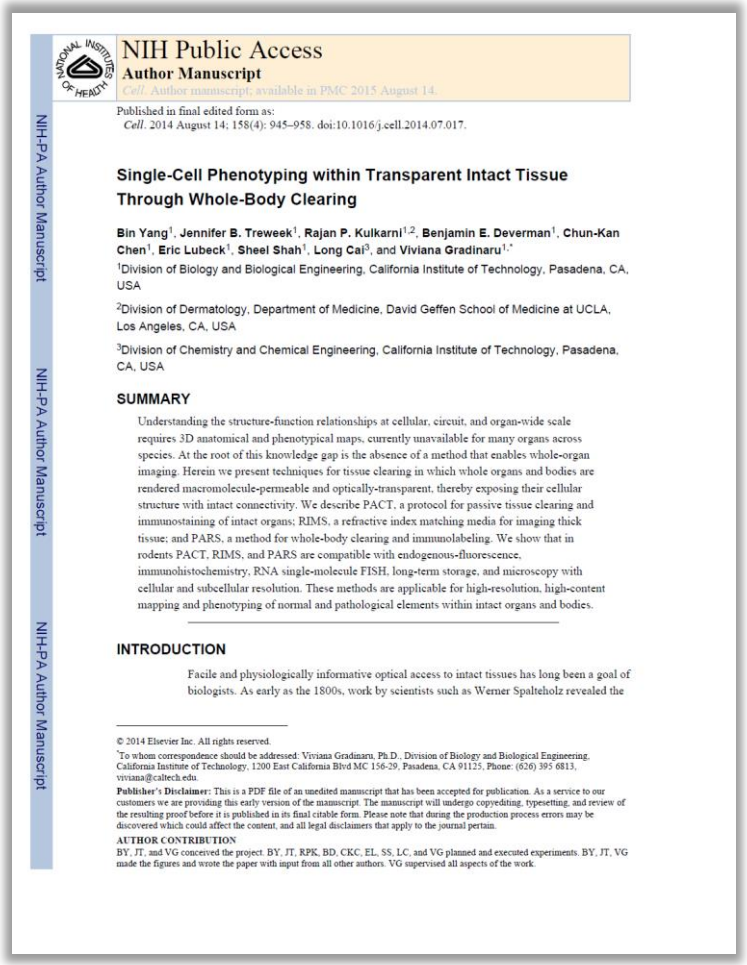
Refractive Index: adjustable

$$c_{\%\text{Iodixanol}} \approx \frac{(RI_{media} - 1.333)}{0.0016}$$

Sample Tissues:
Live primary cell cultures, planarians, zebrafish and human cerebral organoids.

Light sheet Cleared Tissue Imaging

PACT, PARS (Bin Yang, others and Viviana Gradinaru)



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Single-Cell Phenotyping within Transparent Intact Tissue Through Whole-Body Clearing

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SUMMARY

Understanding the structure-function relationships at cellular, circuit, and organ-wide scale requires 3D anatomical and phenotypical maps, currently unavailable for many organs across species. At the root of this knowledge gap is the absence of a method that enables whole-organ imaging. Herein we present techniques for tissue clearing in which whole organs and bodies are rendered macromolecule-permeable and optically-transparent, thereby exposing their cellular structure with intact connectivity. We describe PACT, a protocol for passive tissue clearing and immunostaining of intact organs; RIMS, a refractive index matching media for imaging thick tissue; and PARS, a method for whole-body clearing and immunolabeling. We show that in rodents PACT, RIMS, and PARS are compatible with endogenous-fluorescence, immunohistochemistry, RNA single-molecule FISH, long-term storage, and microscopy with cellular and subcellular resolution. These methods are applicable for high-resolution, high-content mapping and phenotyping of normal and pathological elements within intact organs and bodies.

INTRODUCTION

Facile and physiologically informative optical access to intact tissues has long been a goal of biologists. As early as the 1800s, work by scientists such as Werner Spalteholz revealed the

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AUTHOR CONTRIBUTION

BY, JT, and VG conceived the project. BY, JT, RPK, BD, CKC, EL, SS, LC, and VG planned and executed experiments. BY, JT, VG made the figures and wrote the paper with input from all other authors. VG supervised all aspects of the work.

Claim:
PACT, RIMS, and PARS are compatible with endogenous-fluorescence, immunohistochemistry, RNA single-molecule FISH, long-term storage, and microscopy with subcellular resolution.

Method:
PFA-fixed tissue sections are incubated acrylamide and then brought to 37 °C to initiate tissue-hydrogel hybridization. Tissue-hydrogel matrices were transferred into 8% SDS in PBS, and depending on tissue size, were incubated for 2–5 days at 37 °C with shaking, prior to immunostaining and incubation in imaging media (RIMS). For PARS, all subsequent PACT and immunolabeling reagents are continuously circulated through rodent vasculature via a peristaltic pump.

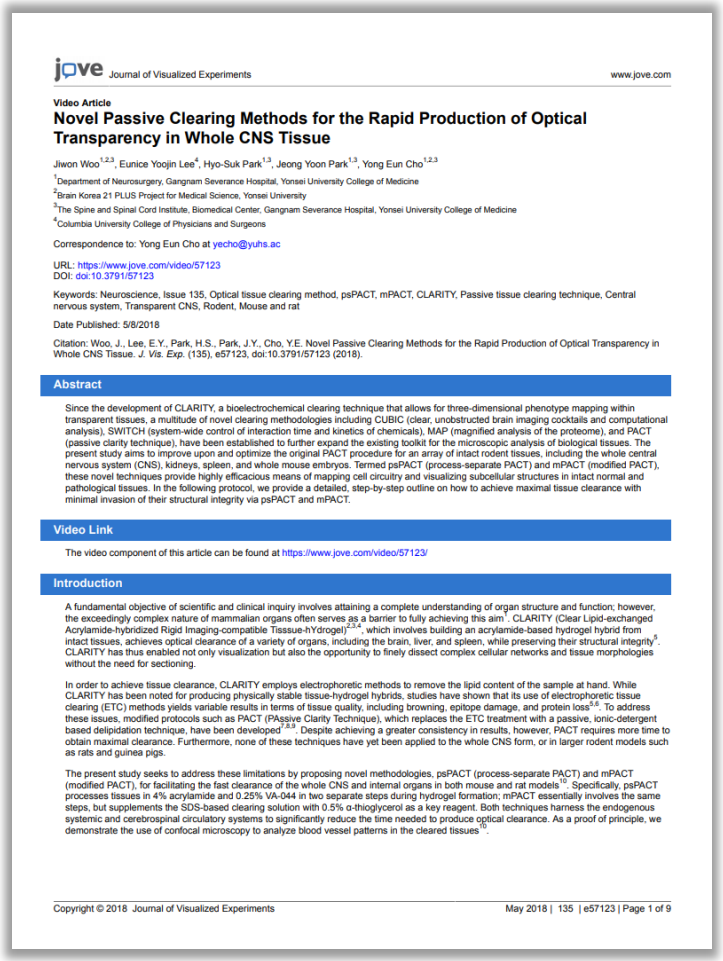
Imaging Solution: RIMS

Refractive Index: 1,46

Sample Tissues:
Rat brain, spinal chord and peripheral organs

Light sheet Cleared Tissue Imaging

psPACT, mPact



jove Journal of Visualized Experiments www.jove.com

Video Article

Novel Passive Clearing Methods for the Rapid Production of Optical Transparency in Whole CNS Tissue

Jiwon Woo[1,2,3], Eunice Yoojin Lee[4], Hyo-Suk Park[1,3], Jeong Yoon Park[1,3], Yong Eun Cho[1,2,3]

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URL: https://www.jove.com/video/57123
DOI: doi:10.3791/57123

Keywords: Neuroscience, Issue 135, Optical tissue clearing method, psPACT, mPACT, CLARITY, Passive tissue clearing technique, Central nervous system, Transparent CNS, Rodent, Mouse and rat

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Abstract

Since the development of CLARITY, a bioelectrochemical clearing technique that allows for three-dimensional phenotype mapping within transparent tissues, a multitude of novel clearing methodologies including CUBIC (clear, unobstructed brain imaging cocktails and computational analysis), SWITCH (system-wide control of interaction time and kinetics of chemicals), MAP (magnified analysis of the proteome), and PACT (passive clarity technique), have been established to further expand the existing toolkit for the microscopic analysis of biological tissues. The present study aims to improve upon and optimize the original PACT procedure for an array of intact rodent tissues, including the whole central nervous system (CNS), kidneys, spleen, and whole mouse embryos. Termed psPACT (process-separate PACT) and mPACT (modified PACT), these novel techniques provide highly efficacious means of mapping cell circuitry and visualizing subcellular structures in intact normal and pathological tissues. In the following protocol, we provide a detailed, step-by-step outline on how to achieve maximal tissue clearance with minimal invasion of their structural integrity via psPACT and mPACT.

Video Link

The video component of this article can be found at https://www.jove.com/video/57123/

Introduction

A fundamental objective of scientific and clinical inquiry involves attaining a complete understanding of organ structure and function; however, the exceedingly complex nature of mammalian organs often serves as a barrier to fully achieving this aim[1]. CLARITY (Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging-compatible Tissue-hYdrogel)[2,3,4], which involves building an acrylamide-based hydrogel hybrid from intact tissues, achieves optical clearance of a variety of organs, including the brain, liver, and spleen, while preserving their structural integrity[5]. CLARITY has thus enabled not only visualization but also the opportunity to finely dissect complex cellular networks and tissue morphologies without the need for sectioning.

In order to achieve tissue clearance, CLARITY employs electrophoretic methods to remove the lipid content of the sample at hand. While CLARITY has been noted for producing physically stable tissue-hydrogel hybrids, studies have shown that its use of electrophoretic tissue clearing (ETC) methods yields variable results in terms of tissue quality, including browning, epitope damage, and protein loss[5,6]. To address these issues, modified protocols such as PACT (PAssive Clarity Technique), which replaces the ETC treatment with a passive, ionic-detergent based delipidation technique, have been developed[7,8,9]. Despite achieving a greater consistency in results, however, PACT requires more time to obtain maximal clearance. Furthermore, none of these techniques have yet been applied to the whole CNS form, or in larger rodent models such as rats and guinea pigs.

The present study seeks to address these limitations by proposing novel methodologies, psPACT (process-separate PACT) and mPACT (modified PACT), for facilitating the fast clearance of the whole CNS and internal organs in both mouse and rat models[10]. Specifically, psPACT processes tissues in 4% acrylamide and 0.25% VA-044 in two separate steps during hydrogel formation; mPACT essentially involves the same steps, but supplements the SDS-based clearing solution with 0.5% α-thioglycerol as a key reagent. Both techniques harness the endogenous systemic and cerebrospinal circulatory systems to significantly reduce the time needed to produce optical clearance. As a proof of principle, we demonstrate the use of confocal microscopy to analyze blood vessel patterns in the cleared tissues[10].

Claim:
The present study aims to improve upon and optimize the original PACT procedure for an array of intact rodent tissues, including the whole central nervous system (CNS), kidneys, spleen, and whole mouse embryos.

Method:
PACT modification as described in paper

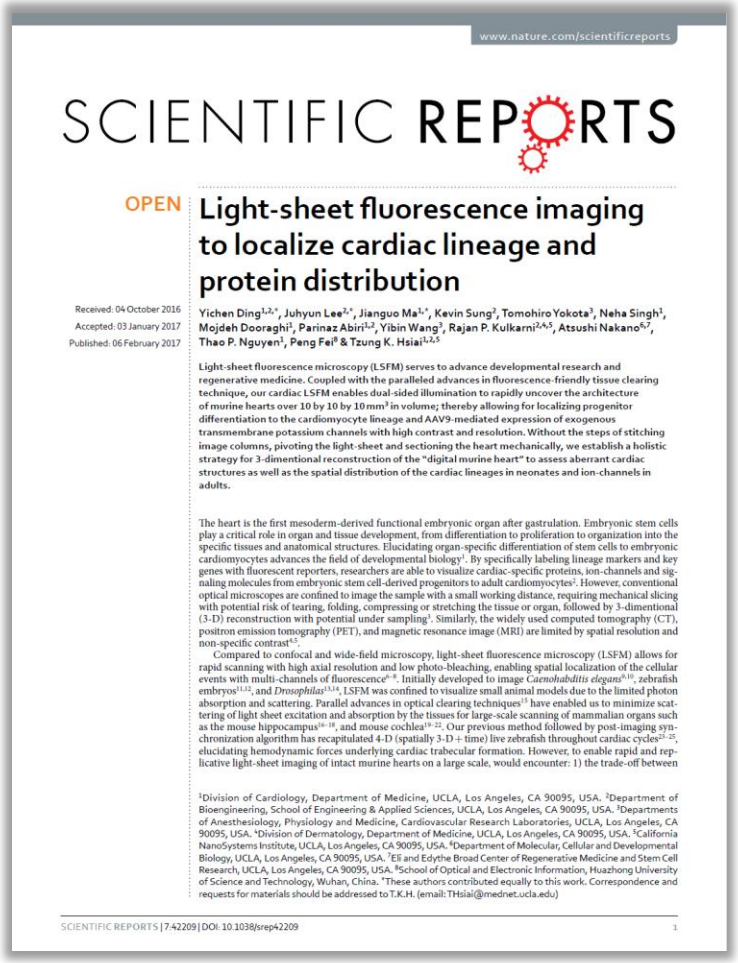
Imaging Solution:
Nycodenz-based Refractive Index Matching Solution

Refractive Index: 1,42 (at 60%)

Sample Tissues:
Mouse brain, kidney, spleen, whole embryos

Light sheet Cleared Tissue Imaging

SCM (Simplified Clarity method)



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SCIENTIFIC REPORTS

OPEN

Light-sheet fluorescence imaging to localize cardiac lineage and protein distribution

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Accepted: 03 January 2017
Published: 06 February 2017

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Light-sheet fluorescence microscopy (LSFM) serves to advance developmental research and regenerative medicine. Coupled with the paralleled advances in fluorescence-friendly tissue clearing technique, our cardiac LSFM enables dual-sided illumination to rapidly uncover the architecture of murine hearts over 10 by 10 by 10 mm^3 in volume; thereby allowing for localizing progenitor differentiation to the cardiomyocyte lineage and AAV9-mediated expression of exogenous transmembrane potassium channels with high contrast and resolution. Without the steps of stitching image columns, pivoting the light-sheet and sectioning the heart mechanically, we establish a holistic strategy for 3-dimentional reconstruction of the "digital murine heart" to assess aberrant cardiac structures as well as the spatial distribution of the cardiac lineages in neonates and ion-channels in adults.

The heart is the first mesoderm-derived functional embryonic organ after gastrulation. Embryonic stem cells play a critical role in organ and tissue development, from differentiation to proliferation to organization into the specific tissues and anatomical structures. Elucidating organ-specific differentiation of stem cells to embryonic cardiomyocytes advances the field of developmental biology[1]. By specifically labeling lineage markers and key genes with fluorescent reporters, researchers are able to visualize cardiac-specific proteins, ion-channels and signaling molecules from embryonic stem cell-derived progenitors to adult cardiomyocytes[2]. However, conventional optical microscopes are confined to image the sample with a small working distance, requiring mechanical slicing with potential risk of tearing, folding, compressing or stretching the tissue or organ, followed by 3-dimentional (3-D) reconstruction with potential under sampling[3]. Similarly, the widely used computed tomography (CT), positron emission tomography (PET), and magnetic resonance image (MRI) are limited by spatial resolution and non-specific contrast[4,5].

Compared to confocal and wide-field microscopy, light-sheet fluorescence microscopy (LSFM) allows for rapid scanning with high axial resolution and low photo-bleaching, enabling spatial localization of the cellular events with multi-channels of fluorescence[6–8]. Initially developed to image *Caenohabditis elegans*[9,10], zebrafish embryos[11,12], and *Drosophilas*[13,14], LSFM was confined to visualize small animal models due to the limited photon absorption and scattering. Parallel advances in optical clearing techniques[15] have enabled us to minimize scattering of light sheet excitation and absorption by the tissues for large-scale scanning of mammalian organs such as the mouse hippocampus[16–18], and mouse cochlea[19–22]. Our previous method followed by post-imaging synchronization algorithm has recapitulated 4-D (spatially 3-D + time) live zebrafish throughout cardiac cycles[23–25], elucidating hemodynamic forces underlying cardiac trabecular formation. However, to enable rapid and replicative light-sheet imaging of intact murine hearts on a large scale, would encounter: 1) the trade-off between

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Claim:
The main differences between our CLARITY method and the previously published protocol involve the reduction in clearing reagent use and the simplification of the protocol.

Method:
Hearts are fixed in paraformaldehyde and polymerized in acrylamide solution. Tissues are then rinsed with PBS, placed in clearing solution (SDS and boric acid), and incubated until clear.

Imaging Solution:
RIMS (Histodenz)

Refractive Index:

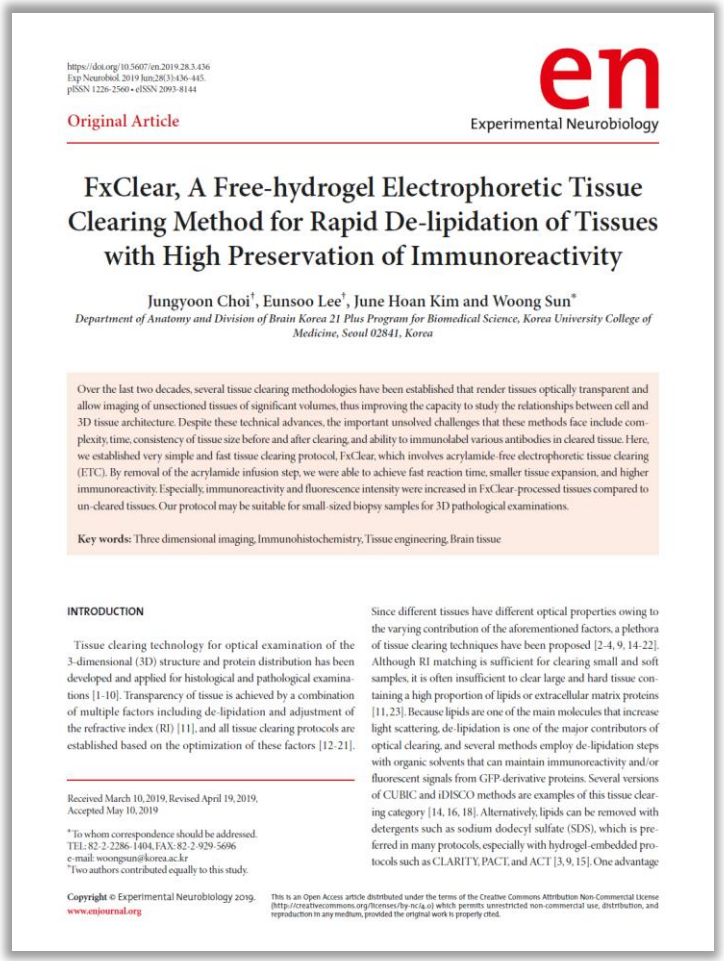
Sample Tissues:
Mouse heart

Solution Properties of Histodenz[2]

Percent (w/v)	Refractive Index (20 °C)
0	1.3330
10	1.3494
20	1.3659
30	1.3824
40	1.3988
50	1.4153
60	1.4318
70	1.4482
80	1.4647

Light sheet Cleared Tissue Imaging

FxClear (Jungyoon Choi, Eunsoo Lee, June Hoan Kim, Woong Sun (2019))



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Exp Neurobiol. 2019 Jun;28(3):436-445.
pISSN 1226-2560 • eISSN 2093-8144

Original Article

en
Experimental Neurobiology

FxClear, A Free-hydrogel Electrophoretic Tissue Clearing Method for Rapid De-lipidation of Tissues with High Preservation of Immunoreactivity

Jungyoon Choi†, Eunsoo Lee†, June Hoan Kim and Woong Sun*

Department of Anatomy and Division of Brain Korea 21 Plus Program for Biomedical Science, Korea University College of Medicine, Seoul 02841, Korea

Over the last two decades, several tissue clearing methodologies have been established that render tissues optically transparent and allow imaging of unsectioned tissues of significant volumes, thus improving the capacity to study the relationships between cell and 3D tissue architecture. Despite these technical advances, the important unsolved challenges that these methods face include complexity, time, consistency of tissue size before and after clearing, and ability to immunolabel various antibodies in cleared tissue. Here, we established very simple and fast tissue clearing protocol, FxClear, which involves acrylamide-free electrophoretic tissue clearing (ETC). By removal of the acrylamide infusion step, we were able to achieve fast reaction time, smaller tissue expansion, and higher immunoreactivity. Especially, immunoreactivity and fluorescence intensity were increased in FxClear-processed tissues compared to un-cleared tissues. Our protocol may be suitable for small-sized biopsy samples for 3D pathological examinations.

Key words: Three dimensional imaging, Immunohistochemistry, Tissue engineering, Brain tissue

INTRODUCTION

Tissue clearing technology for optical examination of the 3-dimensional (3D) structure and protein distribution has been developed and applied for histological and pathological examinations [1-10]. Transparency of tissue is achieved by a combination of multiple factors including de-lipidation and adjustment of the refractive index (RI) [11], and all tissue clearing protocols are established based on the optimization of these factors [12-21].

Since different tissues have different optical properties owing to the varying contribution of the aforementioned factors, a plethora of tissue clearing techniques have been proposed [2-4, 9, 14-22]. Although RI matching is sufficient for clearing small and soft samples, it is often insufficient to clear large and hard tissue containing a high proportion of lipids or extracellular matrix proteins [11, 23]. Because lipids are one of the main molecules that increase light scattering, de-lipidation is one of the major contributors of optical clearing, and several methods employ de-lipidation steps with organic solvents that can maintain immunoreactivity and/or fluorescent signals from GFP-derivative proteins. Several versions of CUBIC and iDISCO methods are examples of this tissue clearing category [14, 16, 18]. Alternatively, lipids can be removed with detergents such as sodium dodecyl sulfate (SDS), which is preferred in many protocols, especially with hydrogel-embedded protocols such as CLARITY, PACT, and ACT [3, 9, 15]. One advantage

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†Two authors contributed equally to this study.

Claim:
FxClear is a very simple and fast tissue clearing protocol, which involves acrylamide-free electrophoretic tissue clearing (ETC).

Method:
Samples are processed for electrophoretic tissue clearing (ETC) in 2% SDS and 200 mM boric acid. After tissue clearing, all samples are washed in PBS to remove SDS.

Imaging Solution:
Cubic mouse solution

Refractive Index: 1,38 – 1,48

Sample Tissues:
Mouse brain slices, whole brain, embryos, kidney, liver

Light sheet Cleared Tissue Imaging

Emerging media for plant tissue clearing

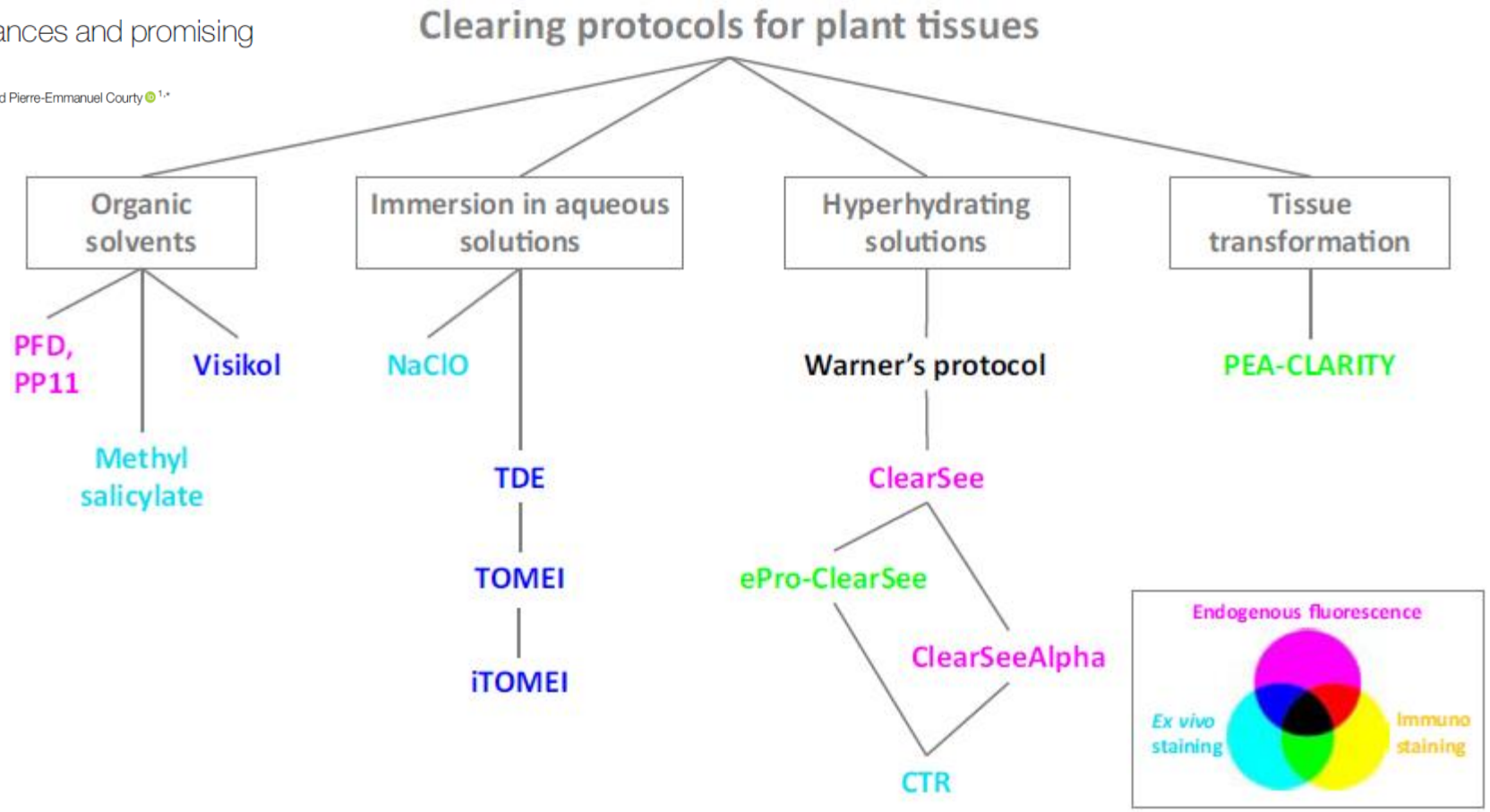
Trends in Plant Science

CellPress

Review

Imaging plant tissues: advances and promising clearing practices

Mathilde Hériché,[1] Christine Arnould,[1] Daniel Wipf,[1] and Pierre-Emmanuel Courty[1,*]



Light sheet Cleared Tissue Imaging

Enable transparency for plant tissue

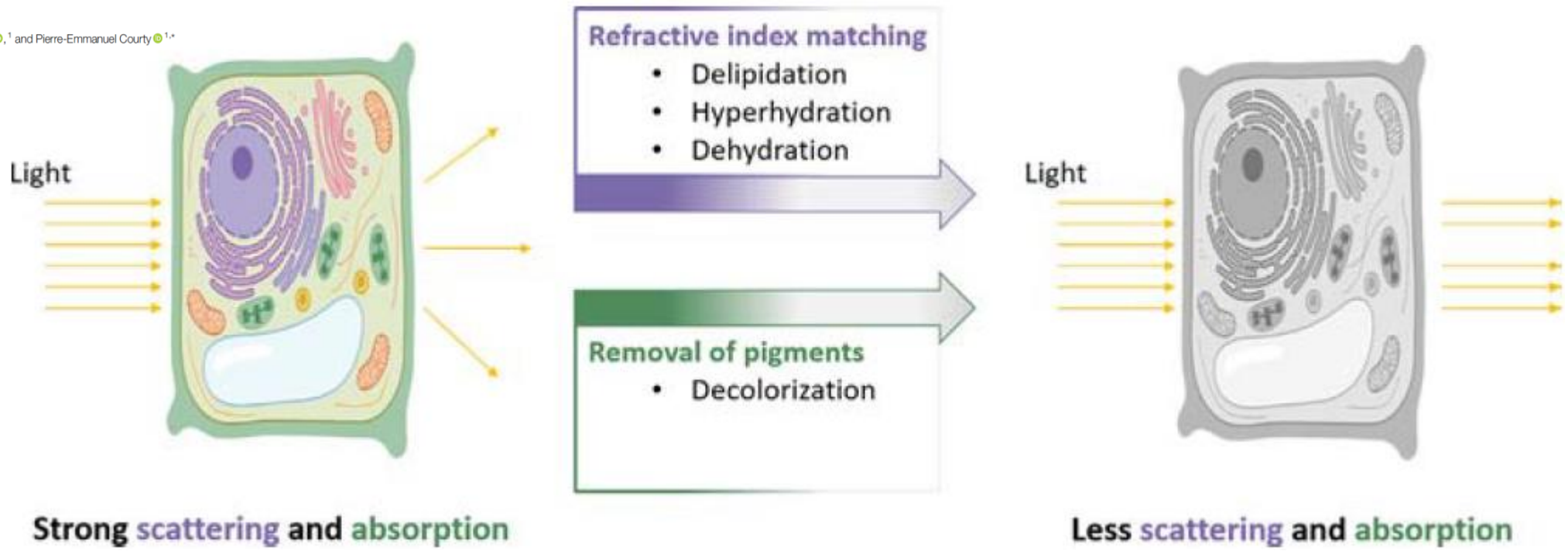
Trends in
Plant Science

CellPress

Review

Imaging plant tissues: advances and promising clearing practices

Mathilde Hériché,[1] Christine Arnould,[1] Daniel Wipf,[1] and Pierre-Emmanuel Courty[1,*]

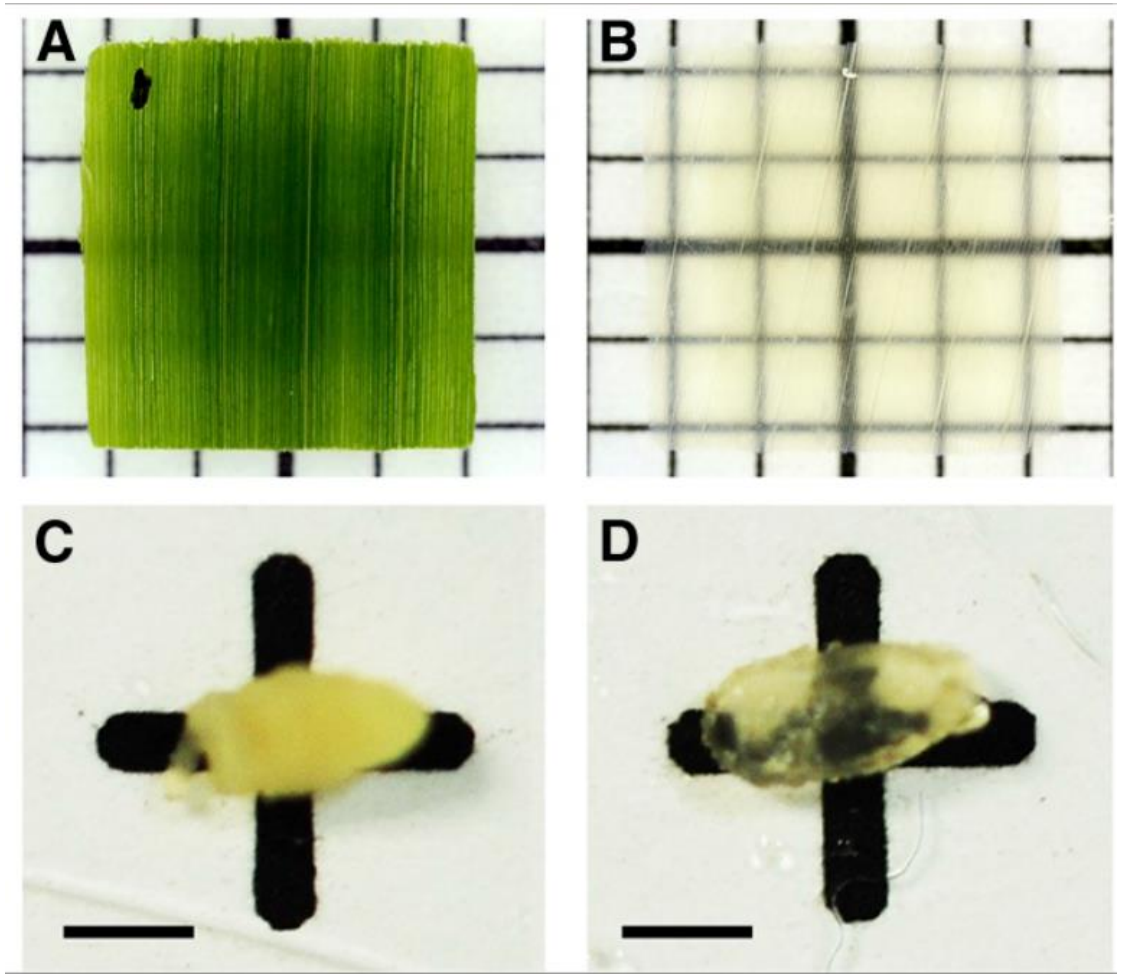


Light sheet Cleared Tissue Imaging

Enable transparency for plant tissue

Maize leaf

Root nodule

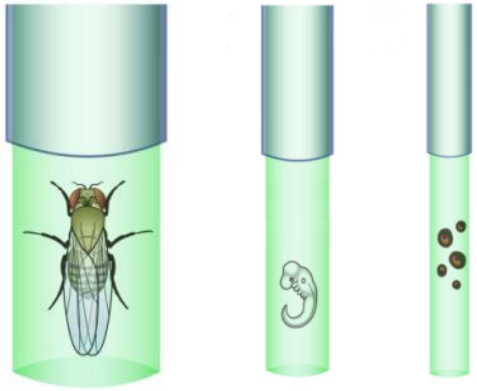


Light sheet sample mounting for horizontal design

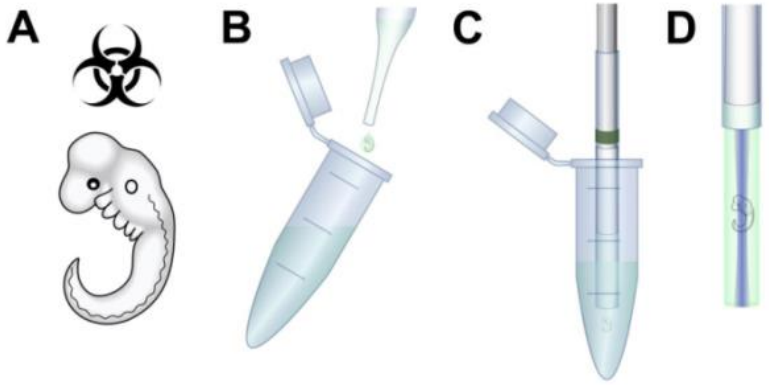
Non-Clearing & Live Animal Samples

Syringe

Glass Capillaries



Typical Procedure:



Start: Put sample into tube. Add liquid agarose.

Bring the capillary close to the sample.

Move the plunger upwards to suck in some agarose, before sucking in the sample.

Proceed gently to not damage the sample at the edge of the capillary while it enters. Hold the capillary horizontally if necessary.

Take the capillary out of the tube when the sample is embedded.
Start rotating the capillary, while holding it horizontally until agarose is solid. Cut off extra agarose .

See full sample preparation movie on You Tube!

Light sheet sample mounting for horizontal design

Non-Clearing & Live Plant Samples (using FEP tubes and capillaries)

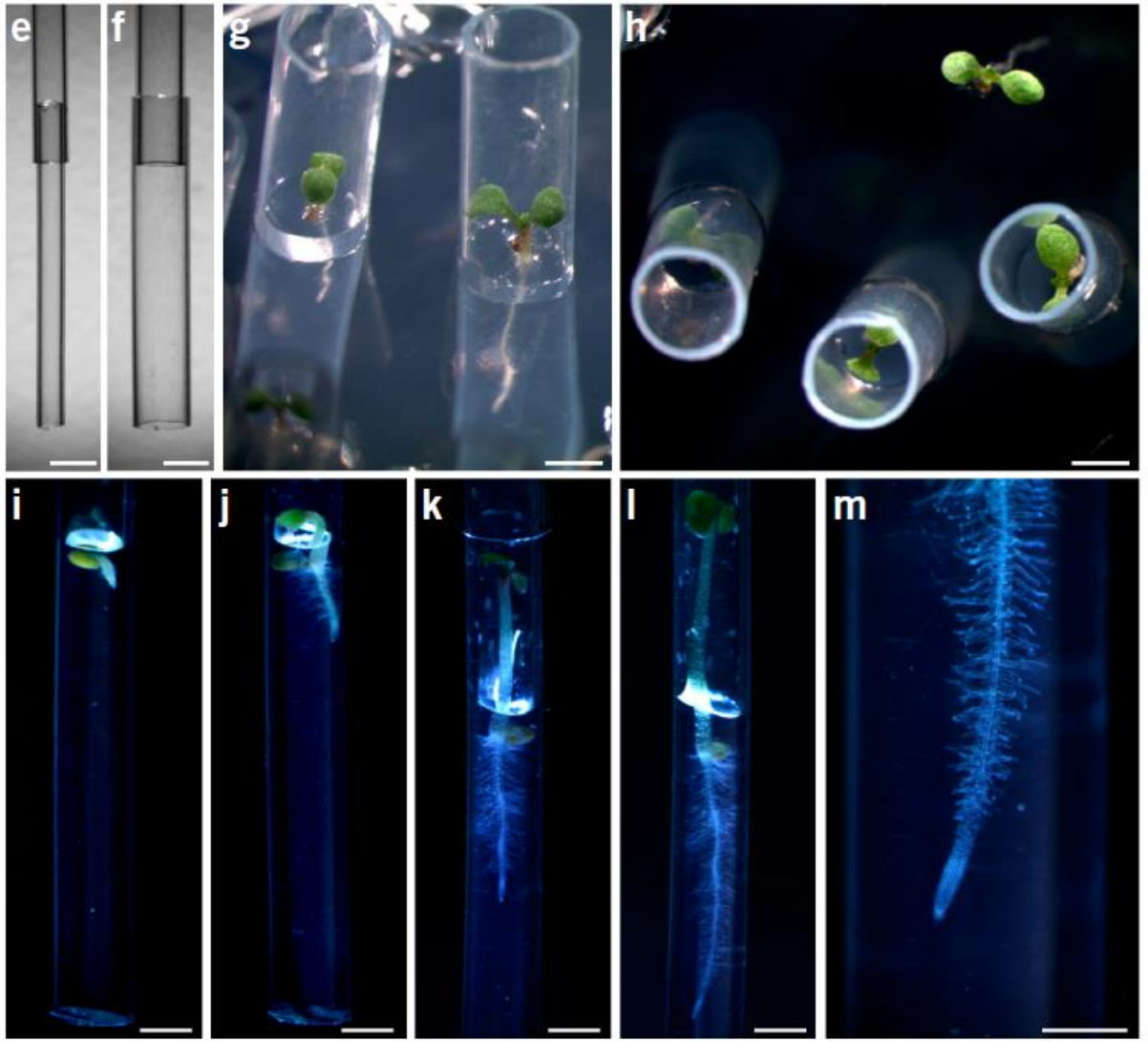
PROTOCOL

Preparation of plants for developmental and cellular imaging by light-sheet microscopy

Miroslav Ovečka[1], Lenka Vaškebová[1], George Komis[1], Ivan Luptovčiak[1], Andrei Smertenko[2] & Jozef Šamaj[1]

[1]Department of Cell Biology, Centre of the Region Haná for Biotechnological and Agricultural Research, Faculty of Science, Palacký University, Olomouc, Czech Republic. [2]Institute of Biological Chemistry, Washington State University, Pullman, Washington, USA. Correspondence should be addressed to J.Š. (jozef.samaj@upol.cz).

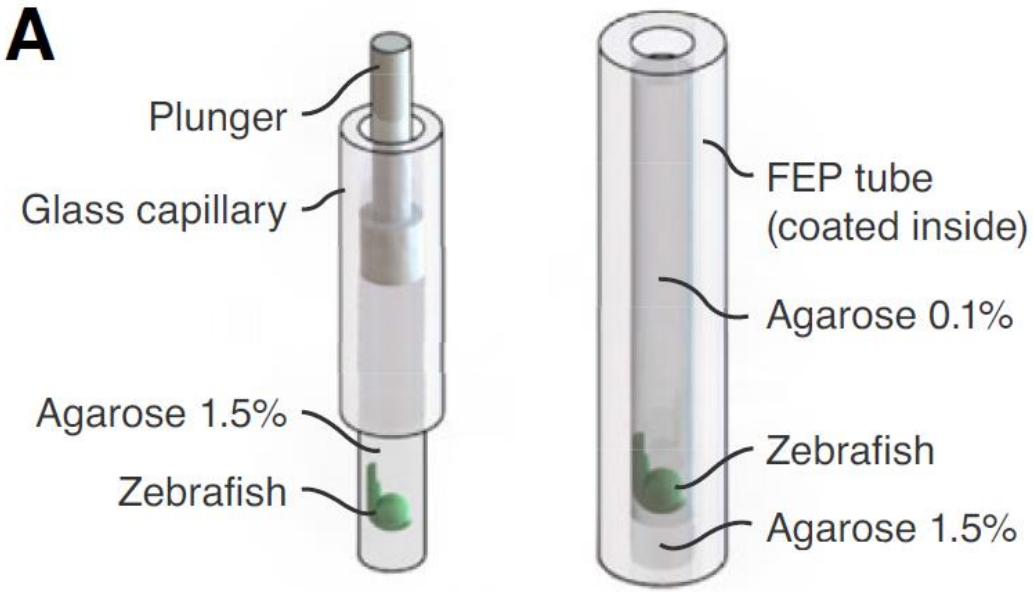
Published online 23 July 2015; doi:10.1038/nprot.2015.081

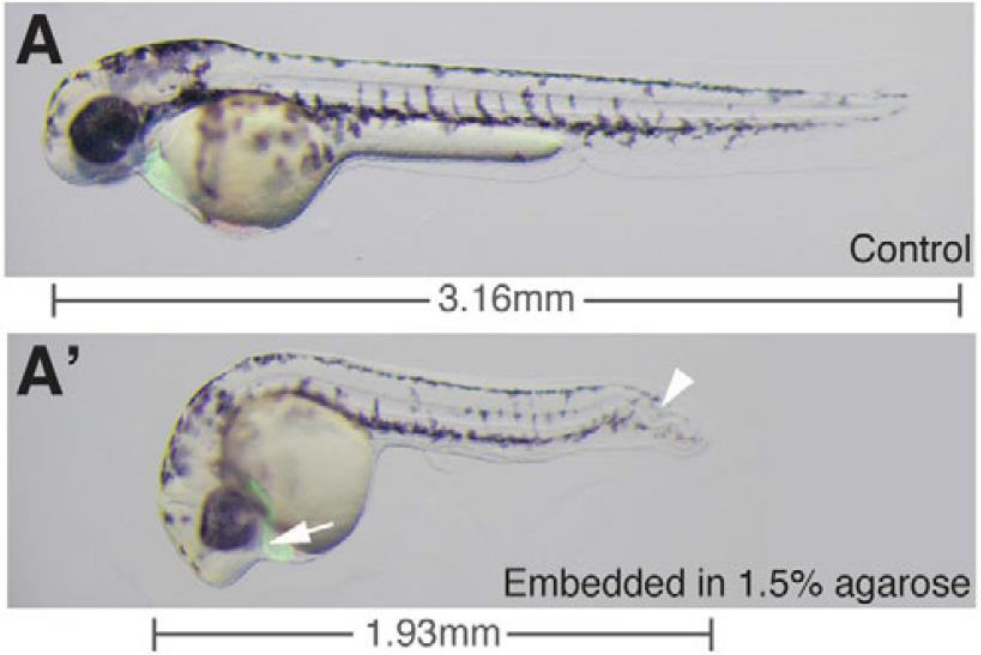


Light sheet sample mounting for horizontal design

FEP tubes for customized sample mounting options

Multilayer mounting enables long-term imaging of zebrafish development in a light sheet microscope
Anna Kaufmann, Michaela Mickoleit , Michael Weber and Jan Huisken
Development 139, 2012

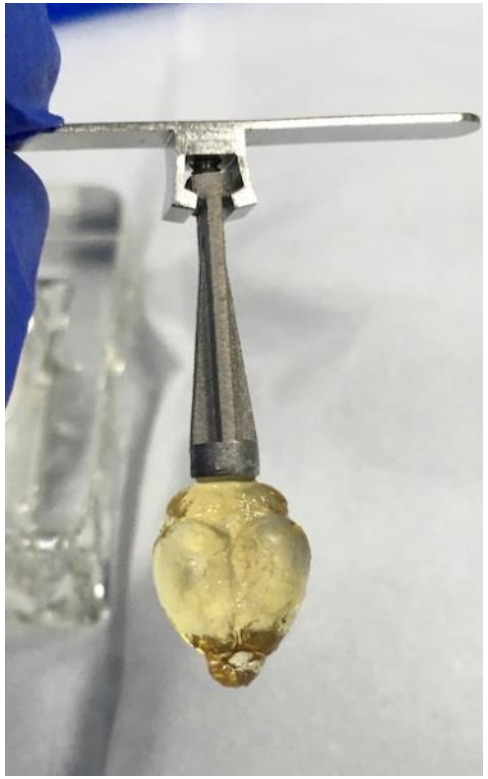




Only with very low agarose concentration does the embryo develop normally.

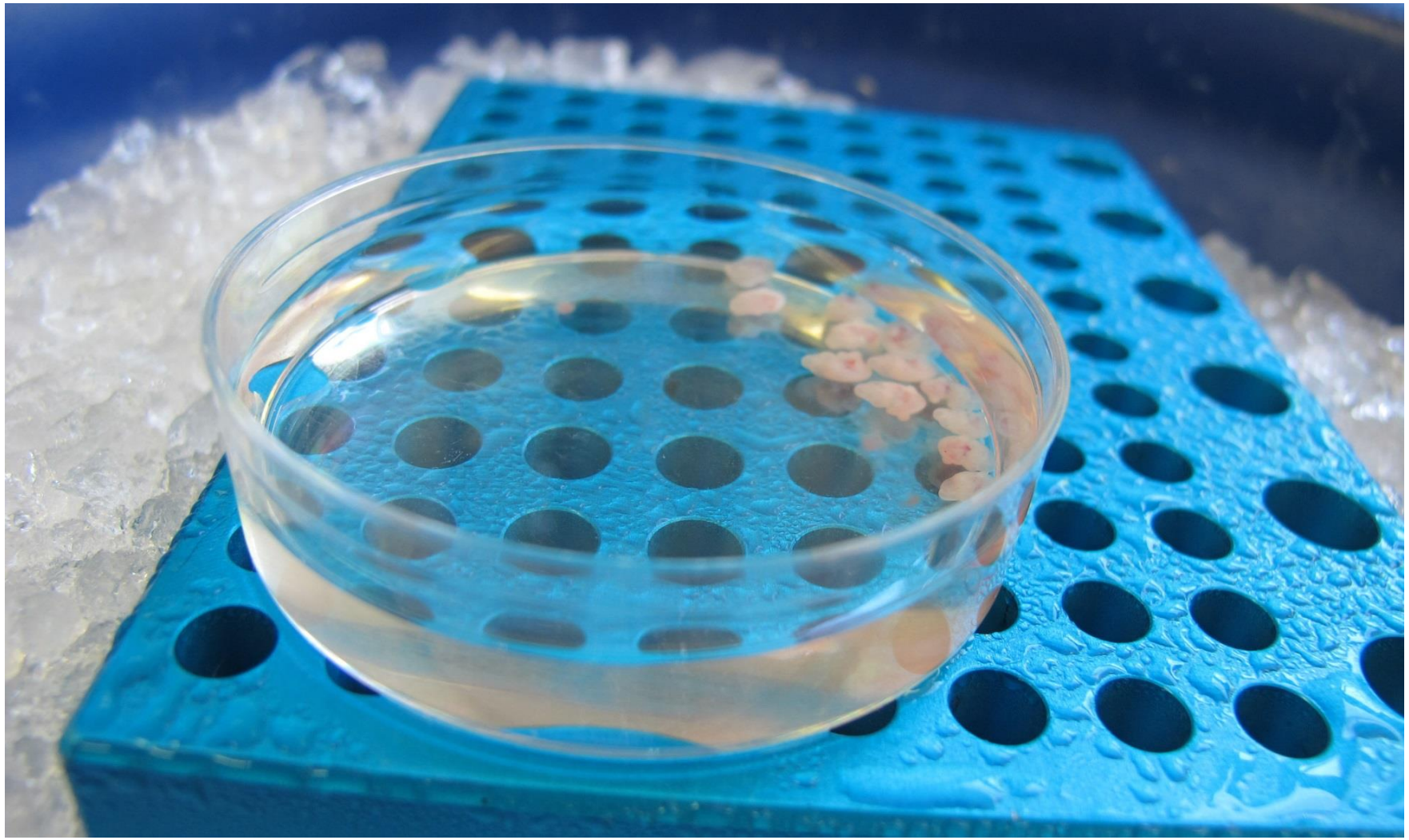
Light sheet sample mounting for horizontal design

Gluing for cleared samples



Light sheet sample mounting for horizontal design

Mounting unknown samples are always a challenge …



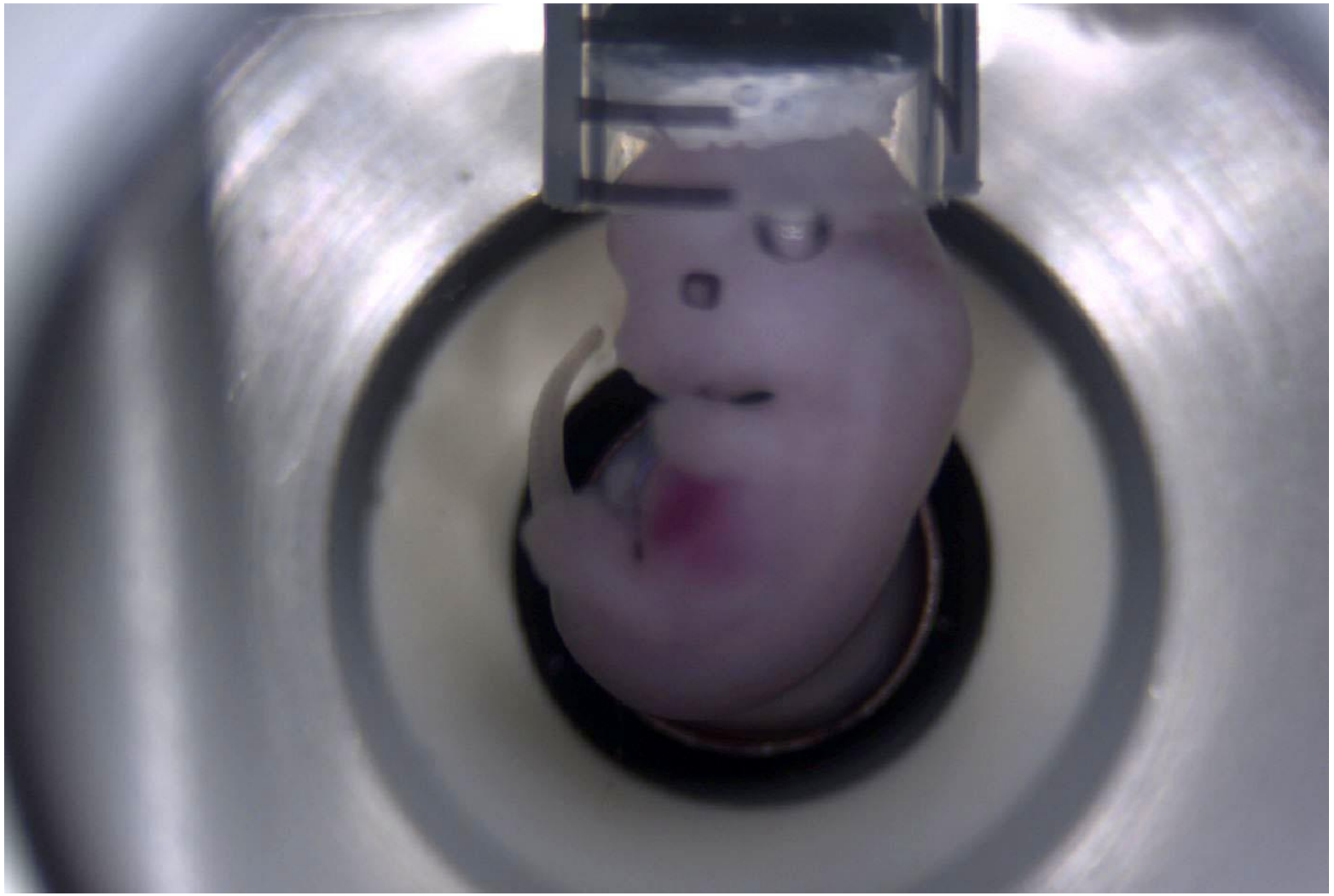
Light sheet sample mounting for horizontal design

... but you will always* find a solution!

* ok, almost always

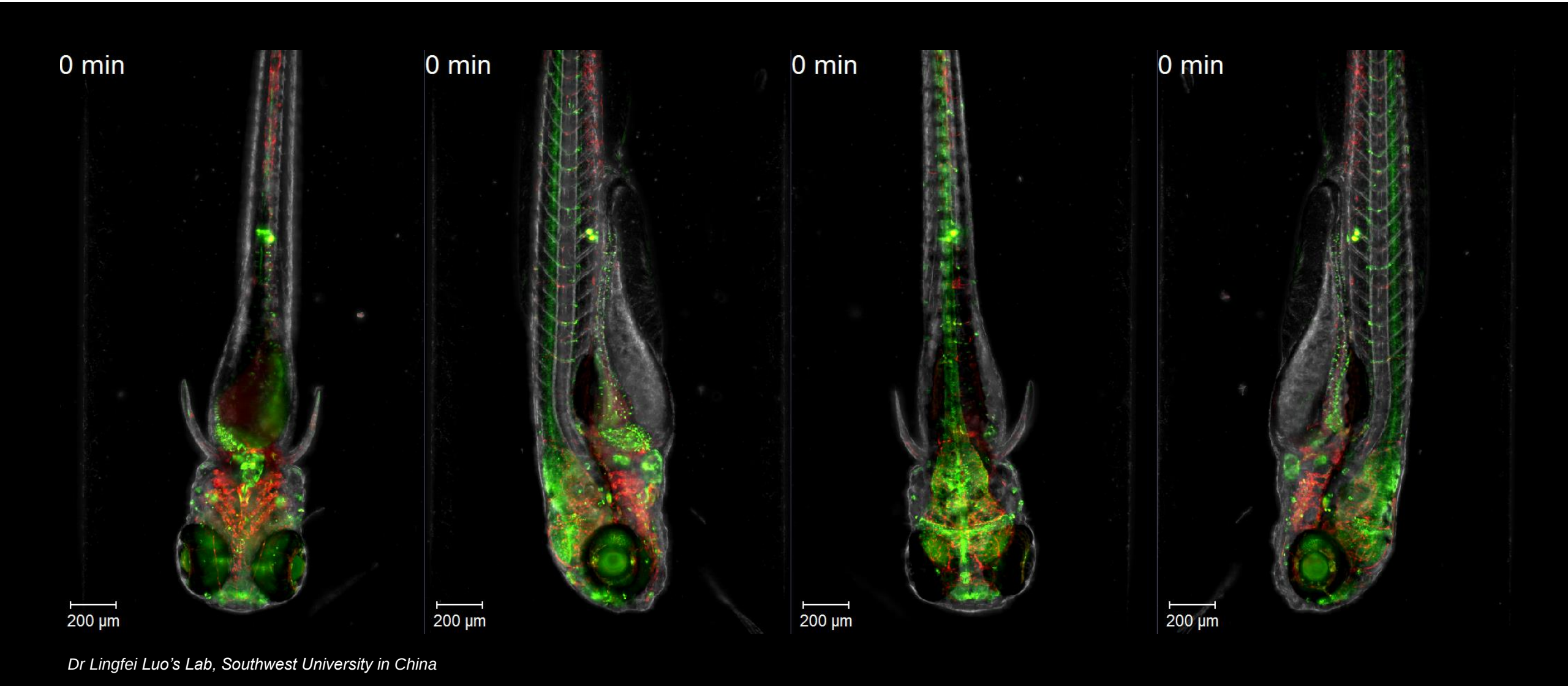
Light sheet sample mounting for horizontal design

It can be surprisingly simple!



Zebrafish

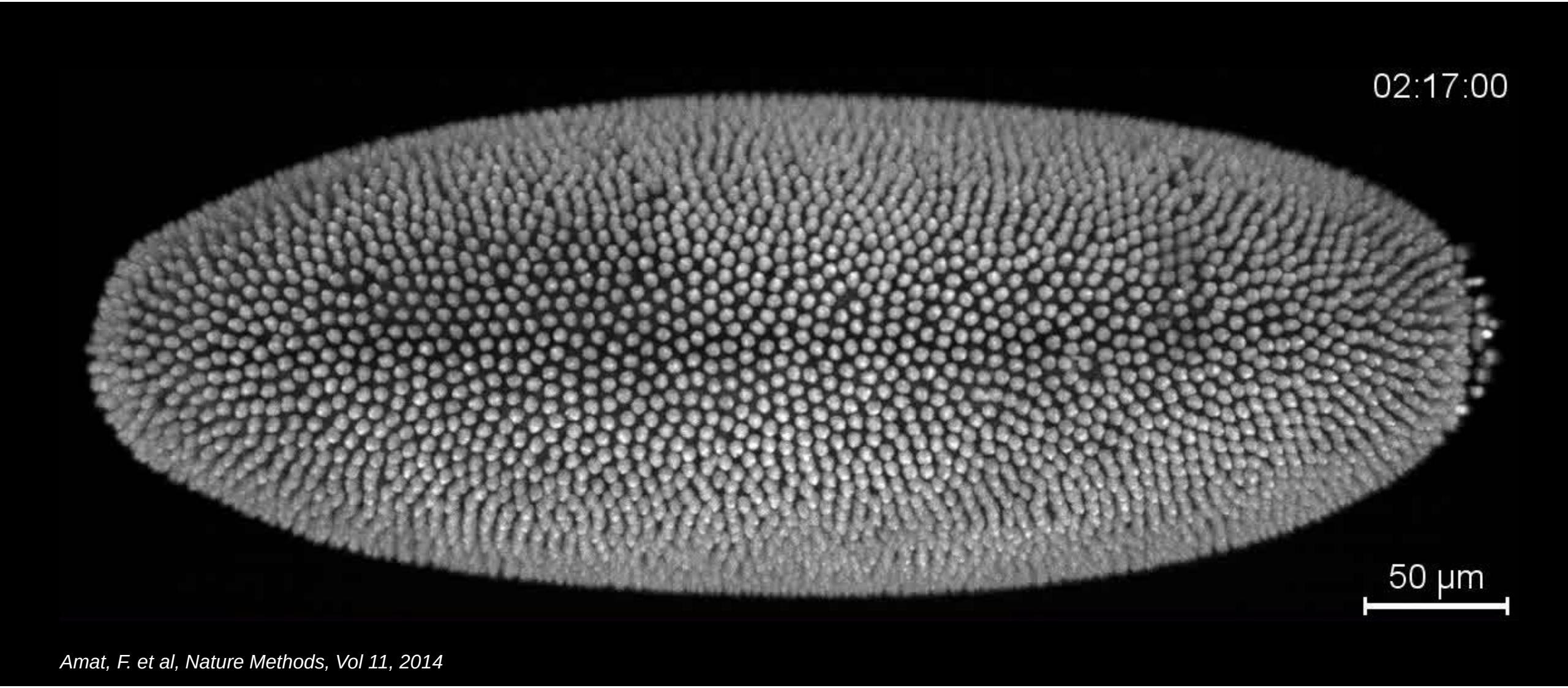
Gentle 3D Imaging of Large Living Specimen



Dr Lingfei Luo's Lab, Southwest University in China

Drosophila Embryo

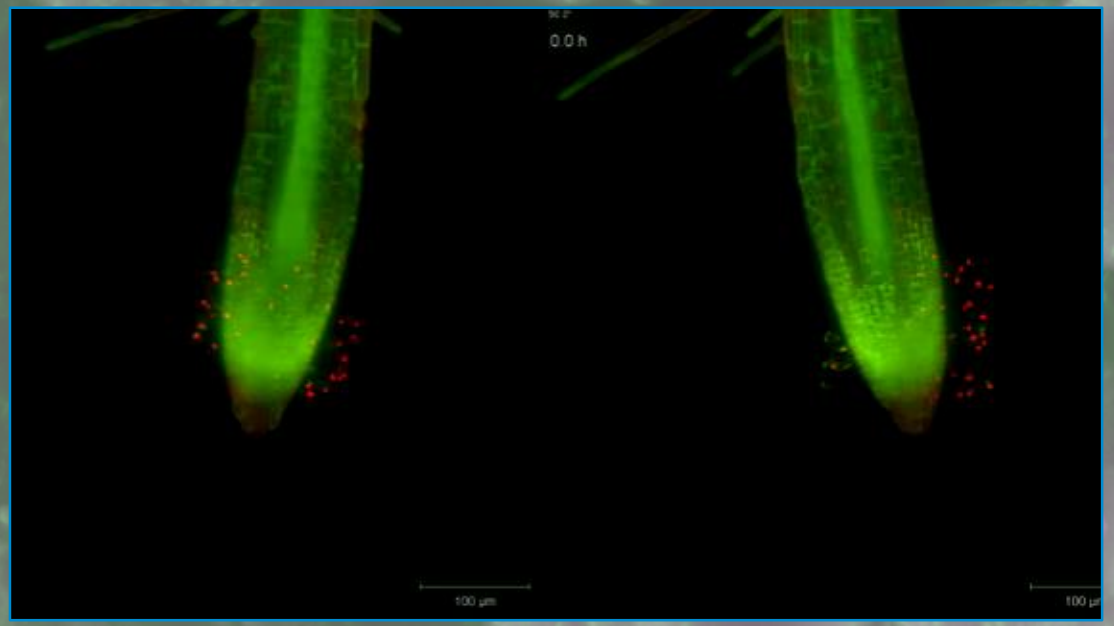
Gentle 3D Imaging of Large Living Specimen

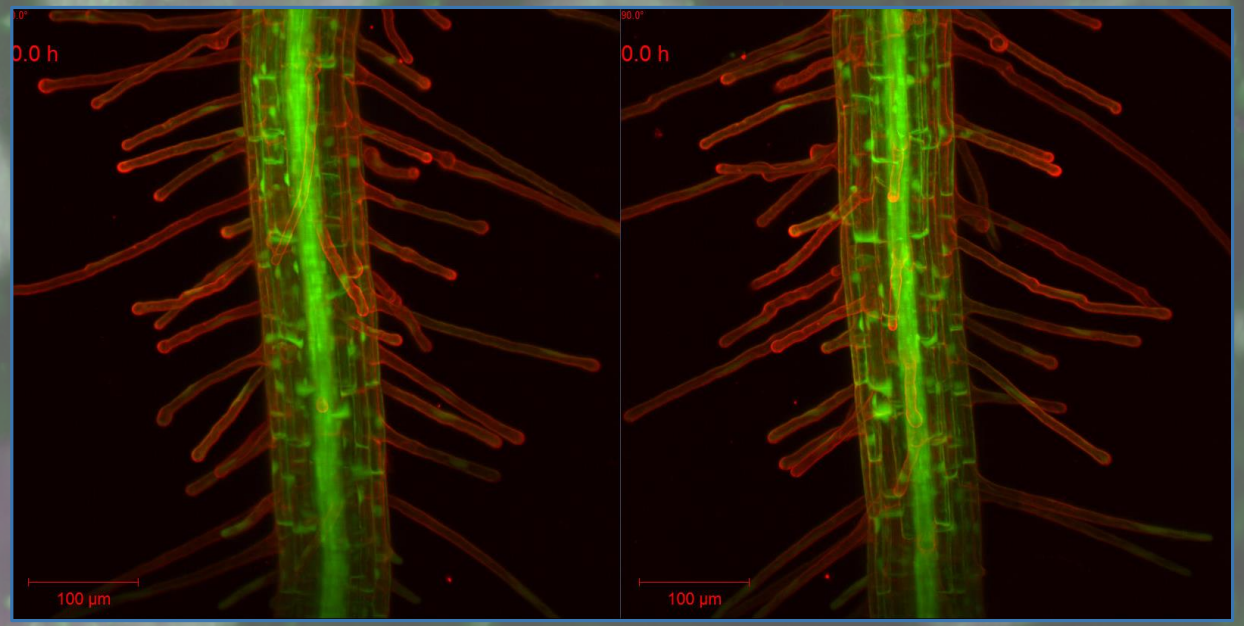


Amat, F. et al, Nature Methods, Vol 11, 2014

Plant Imaging

Natural Growth in a Horizontal Microscope

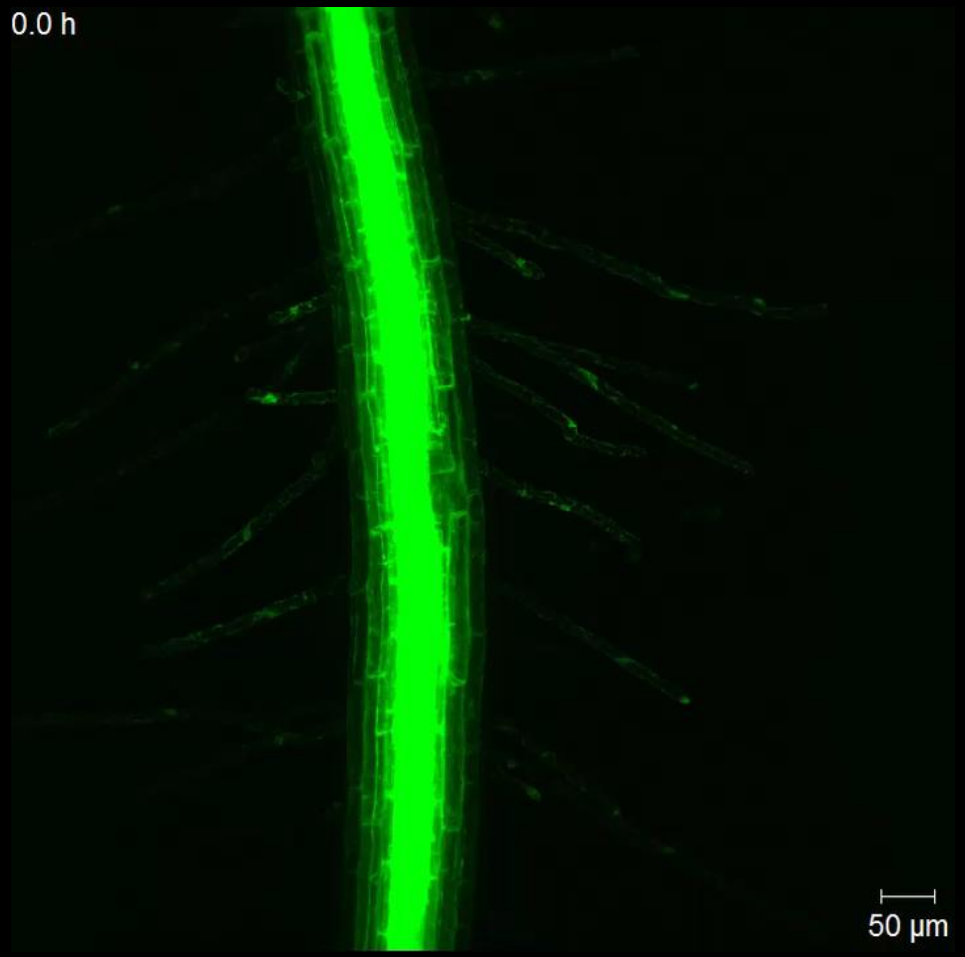




Axel Mithöfer (Max-Planck-Institute for Chemical Ecology, Jena)

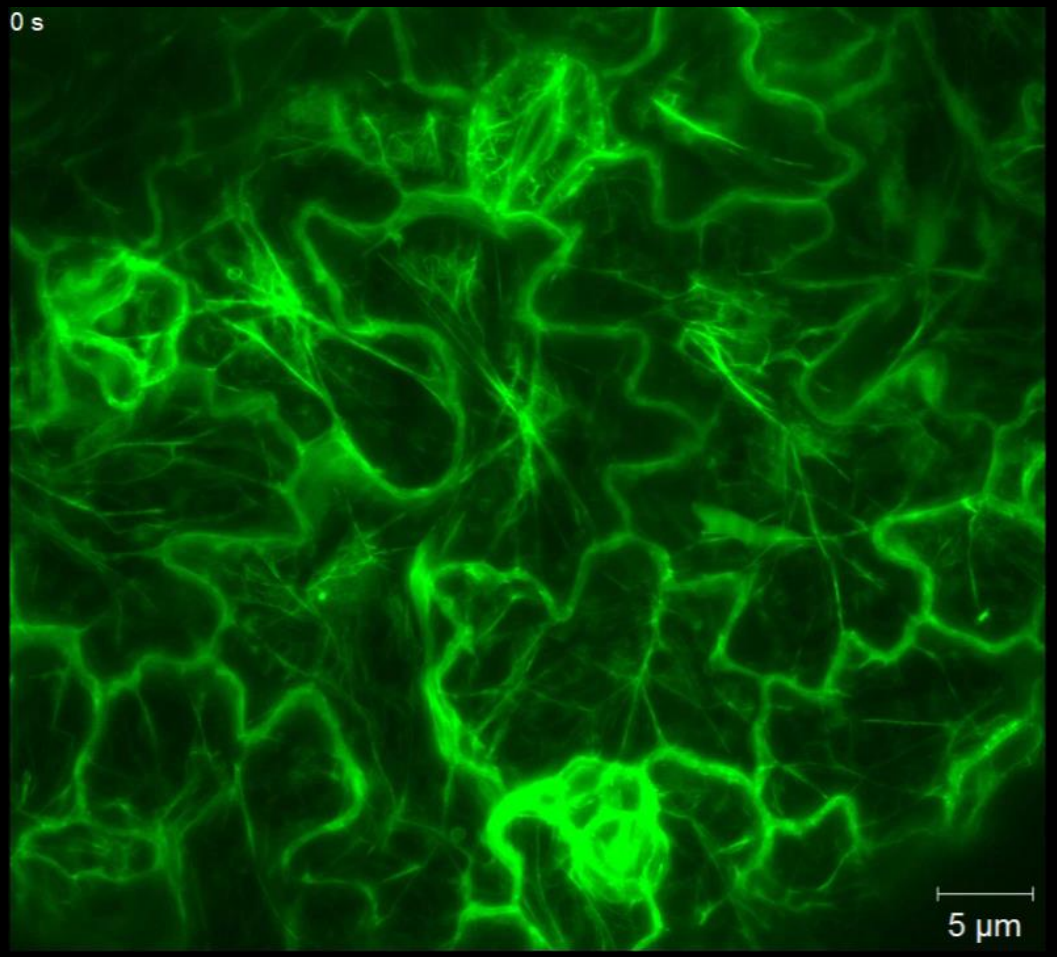
A. Thaliana Model System

Root Formation and Actin Cytoskeleton Dynamics



Lateral root formation in *A. thaliana* line carrying fluorescent microtubule marker GFP-TUA5

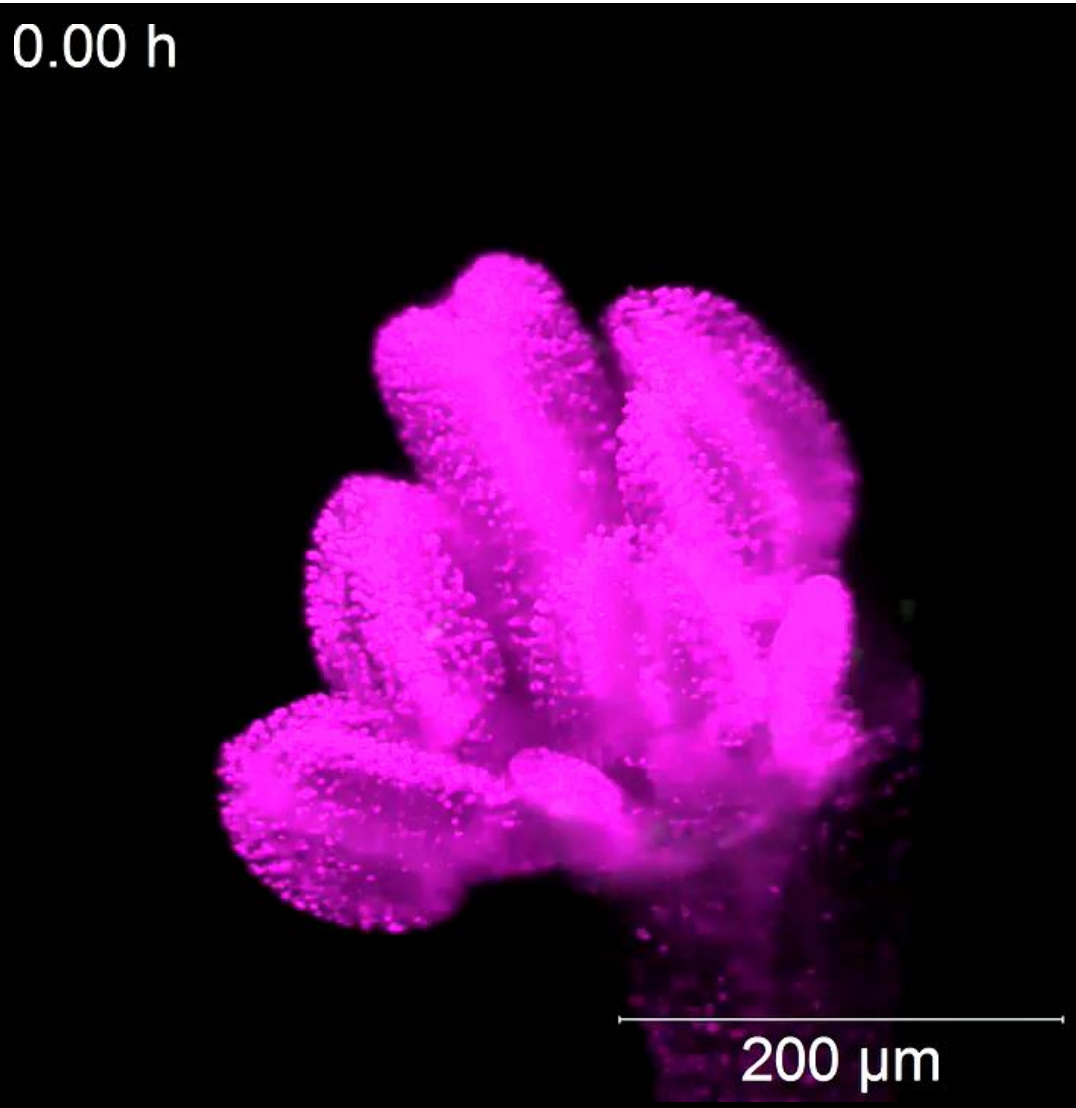
Ovečka, M., Vaškebová, L., Komis, G. et al. Nat Protoc 10, 1234–1247 (2015)



Actin cytoskeleton in cotyledon epidermal cells of light-grown *A. thaliana* seedling carrying fluorescent F-actin marker FABD2-GFP

More Sensitivity and Speed in Live Imaging

Long term Observation of Plant Development



Arabidopsis Flower

Light Sheet Microscopy allows to visualize and study the structural development of entire *Arabidopsis* flowers at subcellular resolution over 5 days.

This is an excellent example of gentle long term live imaging with Light Sheet Fluorescence Microscopy (LSFM).

Labels: H2B:mRuby2 for visualization of somatic nuclei. ASY1:eYFP expressed specifically in meiocytes.

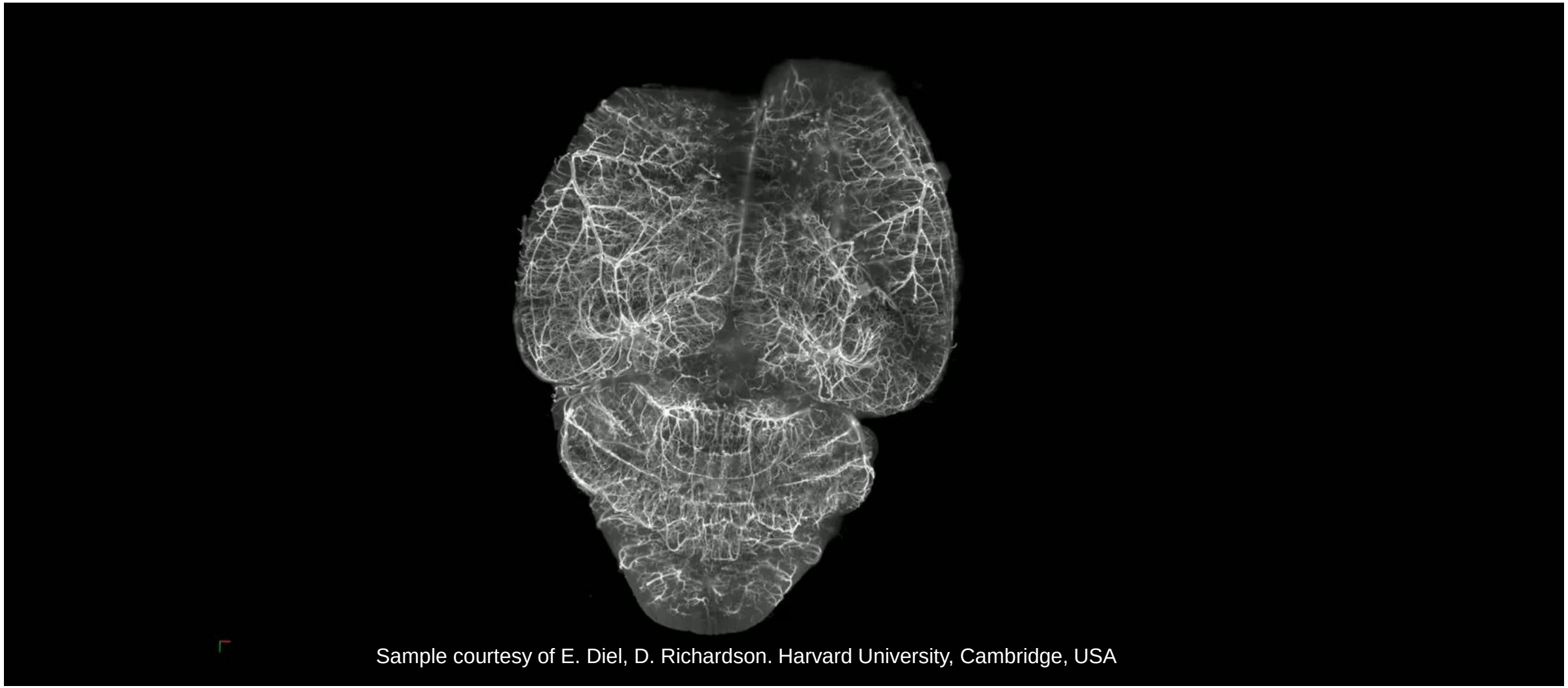
Imaged with W Plan Apo 10x/0.5 and customized sample mounting and incubation chamber.

Illumination: 488 (< 1 %), 561 nm (7%)
pixel dimensions: 1920 x 1920 x 287
scaling: 0.31 x 0.31 x 1.68 micron
Image volume: 587 x 587 x 480 µm

Imaging plant germline differentiation within Arabidopsis flowers by light sheet microscopy.
Valuchova et al., eLife. 2020; 9: e52546., doi: 10.7554/eLife.52546

3D-Reconstruction in 2021

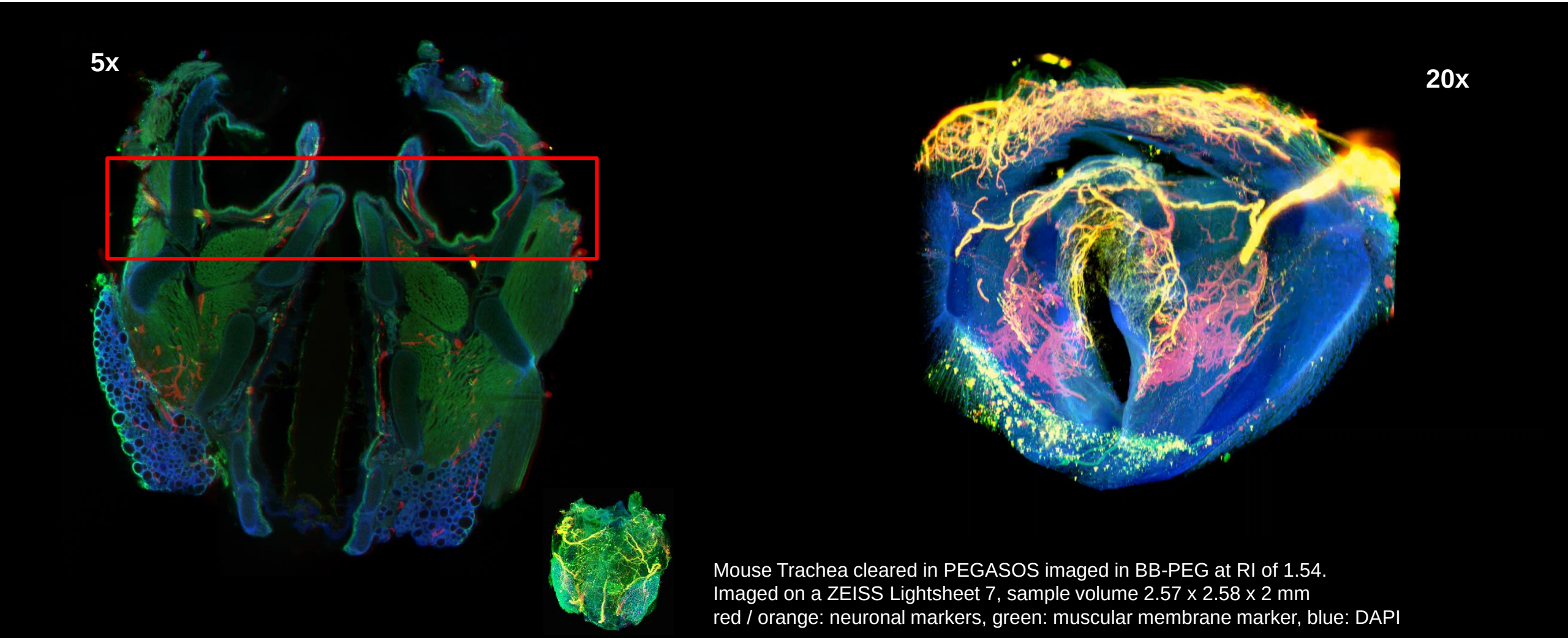
Mouse brain stained with CellTracker™ CM-DiI Dye, cleared with iDISCO+, and imaged in ethyl cinnamate



Sample courtesy of E. Diel, D. Richardson. Harvard University, Cambridge, USA

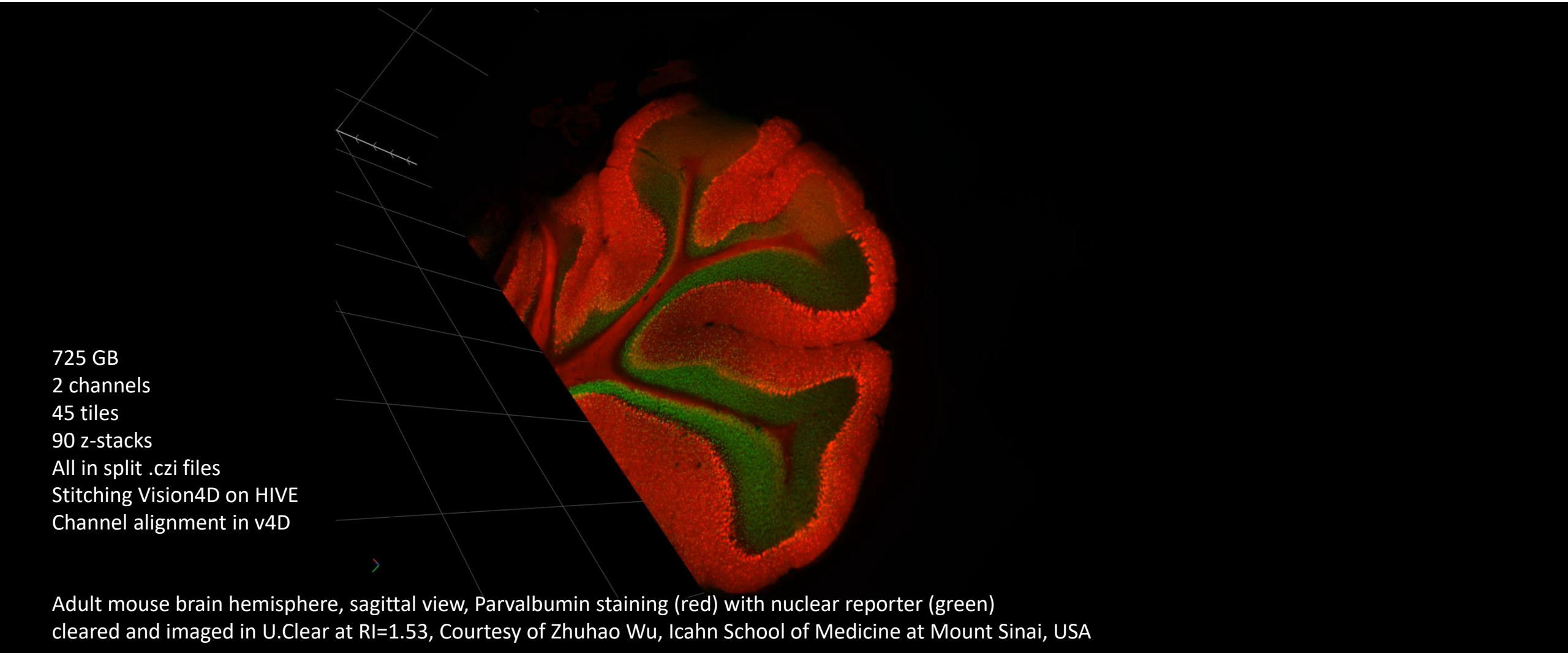
Flexibility in Resolution

Multi-scale Imaging: Overview and detail in one system



Mouse Trachea cleared in PEGASOS imaged in BB-PEG at RI of 1.54.
Imaged on a ZEISS Lightsheet 7, sample volume 2.57 x 2.58 x 2 mm
red / orange: neuronal markers, green: muscular membrane marker, blue: DAPI

Sample courtesy of Prof. C. Birchmeier and Dr. P.-L. Ruffault, Laboratory of Developmental Biology / Signal Transduction, Max Delbrück Center for Molecular Medicine, Berlin, Germany

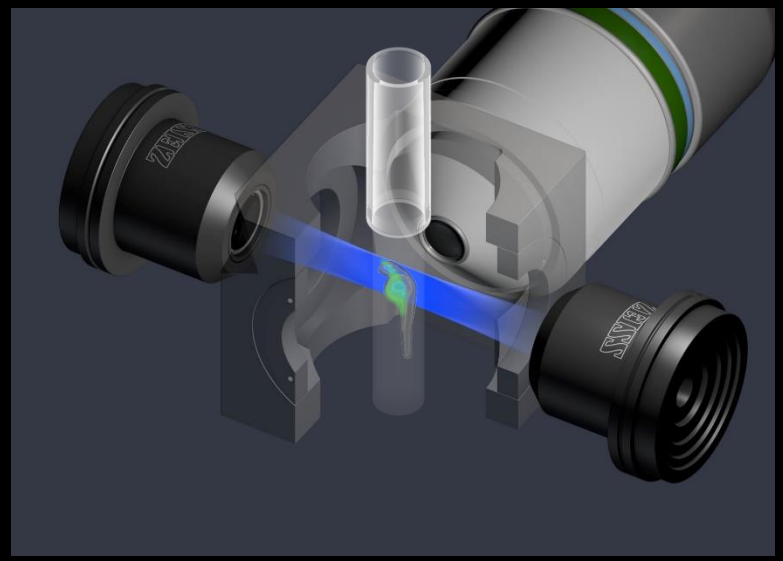


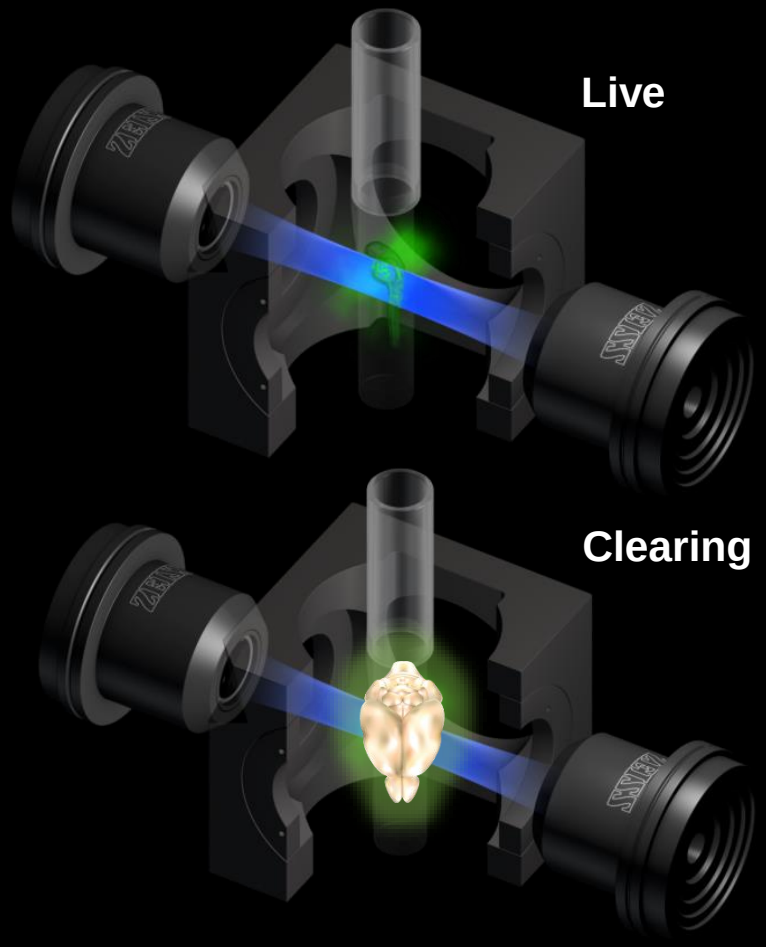
725 GB
2 channels
45 tiles
90 z-stacks
All in split .czi files
Stitching Vision4D on HIVE
Channel alignment in v4D

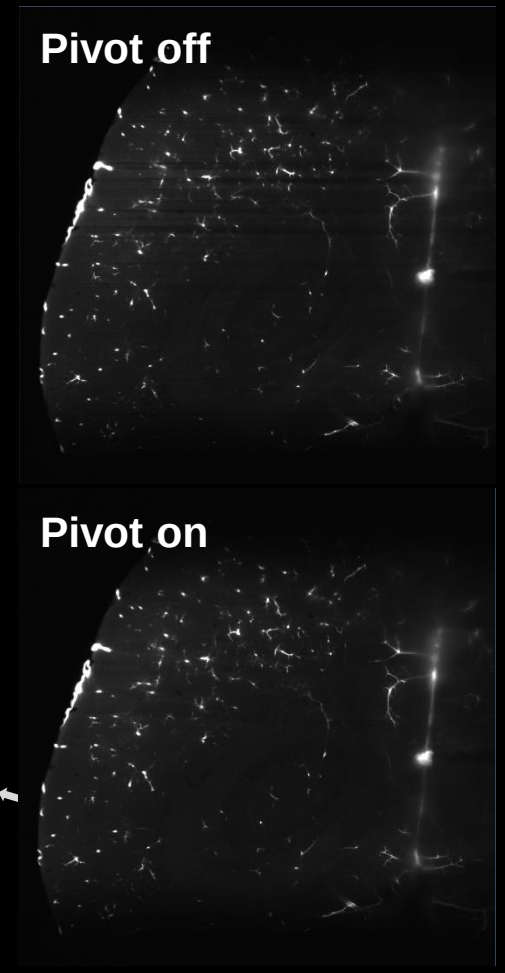
Adult mouse brain hemisphere, sagittal view, Parvalbumin staining (red) with nuclear reporter (green)
cleared and imaged in U.Clear at RI=1.53, Courtesy of Zhuhao Wu, Icahn School of Medicine at Mount Sinai, USA

Lightsheet 7 with Clearing

We kept the horizontal design,
for preserving all our previous strengths







and introduce: New! objectives,
w/ focus rings or correction collars to adapt to RI

Adjustable focus rings or correction collars

illumination

LSFM 5x/0.1 foc

LSFM 10x/0.2 foc

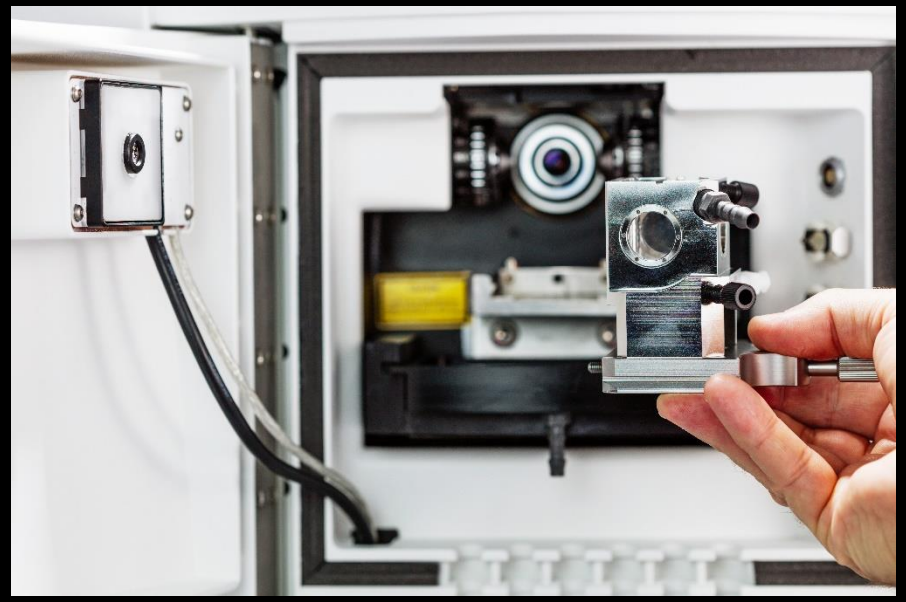
detection

EC Plan-Neofluar
5x/0.16 foc
(WD=10.5 mm)

detection

Clr Plan-Neofluar
20x/1.0 Corr, n=1.53
(WD=6.4 mm)

New! chambers,
w/ spacious interior to mount larger samples



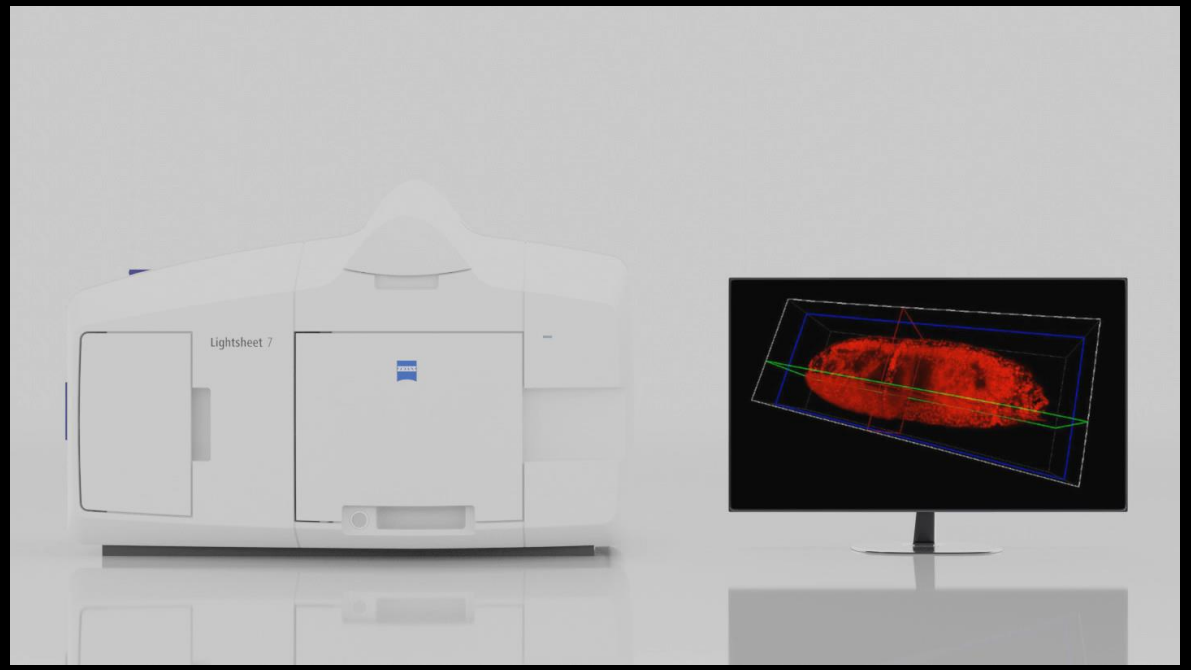
Dedicated to live or clearing applications

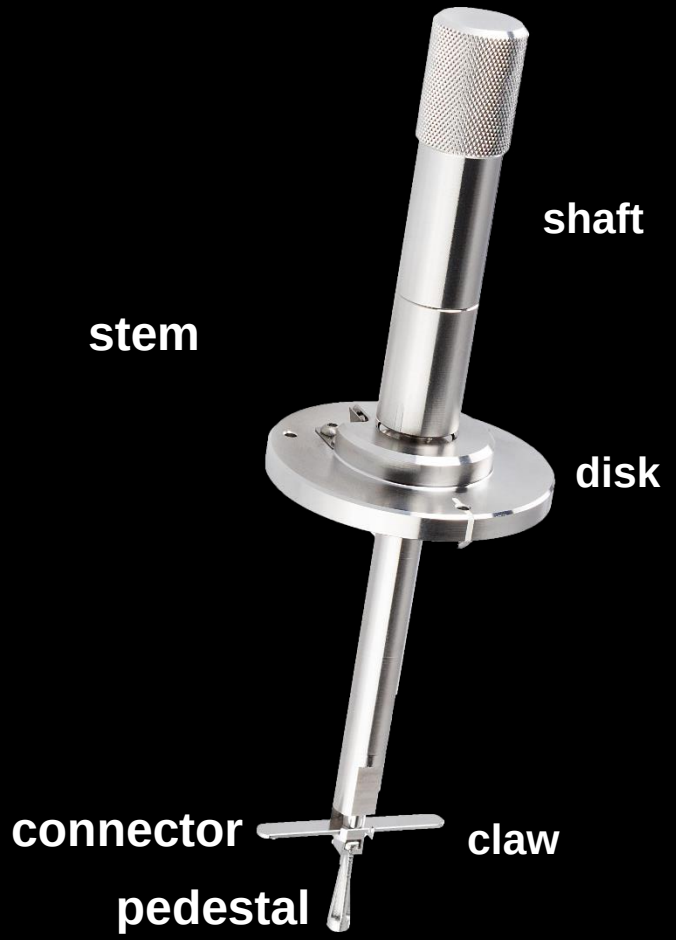
Chamber – Water
n=1.33

Chamber – Clr 20x
n=1.35-1.586

Chamber – Clr Large
n=1.33-1.58
Sample size:
10 x 10 x 20 mm^3

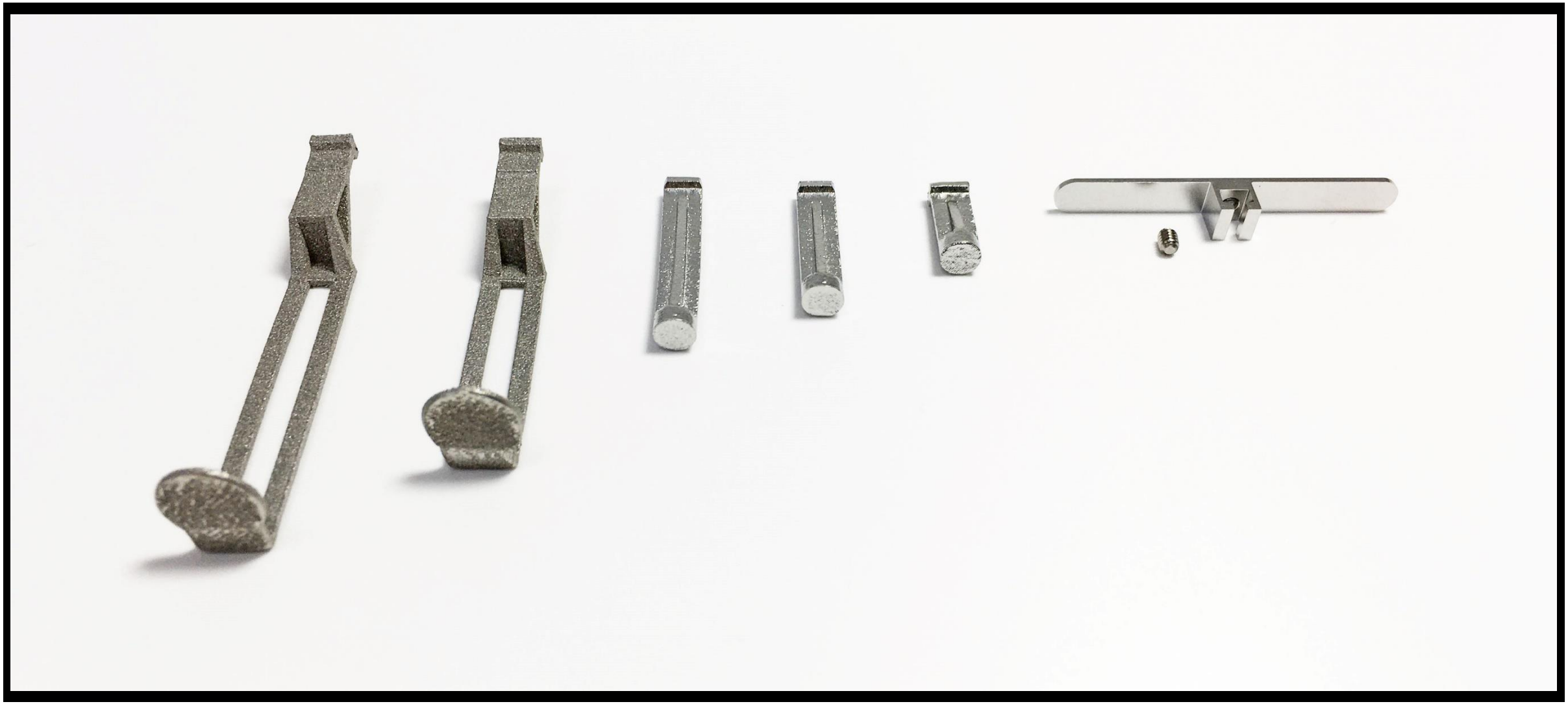
New! smart sample holder,
w/ claw mechanism to load samples outside the system





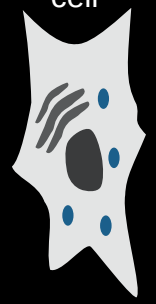
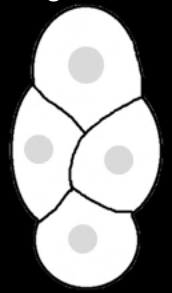
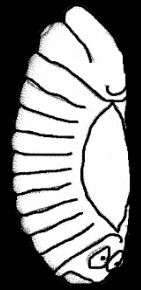
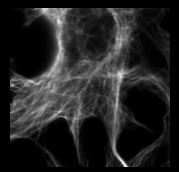
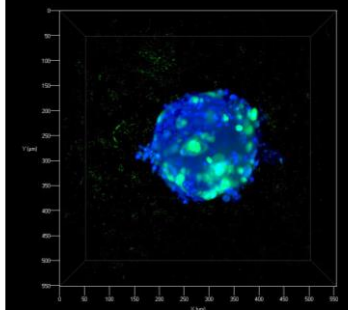
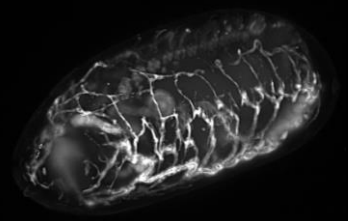
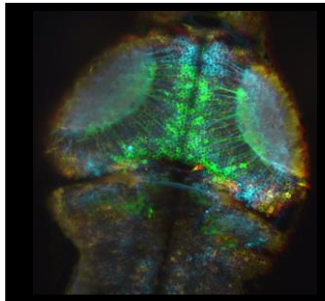
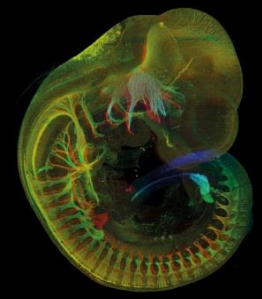
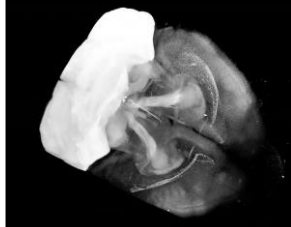
Various 3D-printable sample holder

Tools for „very large“ or very specific samples



From Micro to Meso Scale Imaging

w/ dedicated optics and chambers

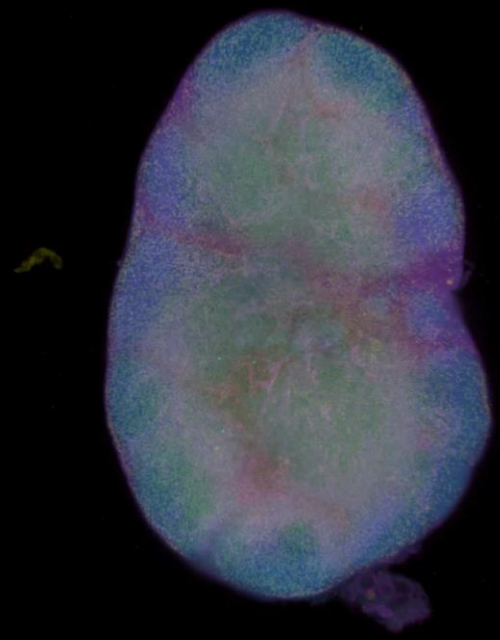
cell	organoid	insect embryo	zebrafish embryo	mouse embryo	adult mouse brain
					
10 µm	50 µm	500 µm	2 mm	4 mm	10 mm
					
U2OS cells microtubule stain Peter Hemmerich FMI, Jena, D	3D cell spheroid Vardan Andriasyan Artur Yakimovich U. of Zuerich, CH	Drosophila melanogaster Trachea development Nipam Patel U. of California, Berhkeley USA	Zebrafish brain Ca^{2+} spikes Christoph Gebhardt Institute Curie, Paris, F	Mouse embryo 10.5 dpf Neuronal precursor cells Jim Swoger EMBL / CRGe, Barcona, E	Mouse brain dtTomato Parvalbumin Erin Diel Harvard U, Cambridge USA

From single color to simultaneous dual color & multicolor

w/ dual camera setup

0ms

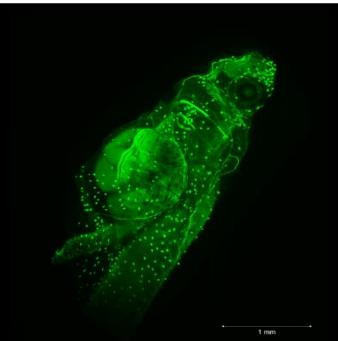
Zebrafish embryo 2d
Endocradium / Blood vessel (red)
Myocardium (green)
Michaela Mickoleit, & Michael Weber
MPI-CBG, Dresden, D



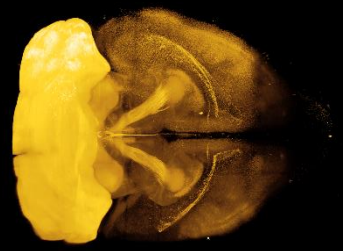
Lymph node
CD8+ cells expressing GFP (yellow)
B cell follicles stinedwith B220 (cyan)
Vasculature network stained via CD31 (magenta)
J.Groom & B.Duckworth,
The Walter and Eliza Hall Institute, Parkville, AUS

From water based imaging media to diverse clearing solutions

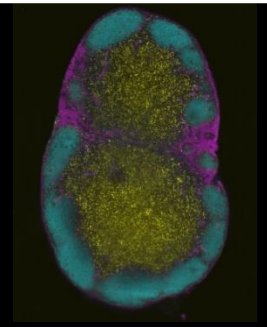
w/ one chamber to rule them all



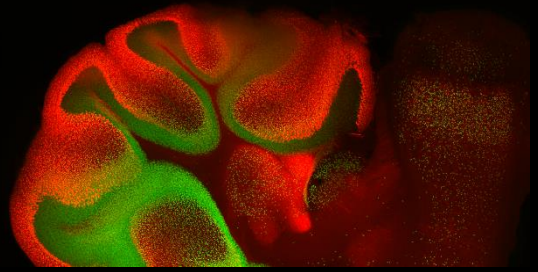
Xenopus laevis, cleared in SeeDB and imaged in PBS at RI=1.33
Jennifer Schmidt & Lennart Olssen, FSU, Jena, D



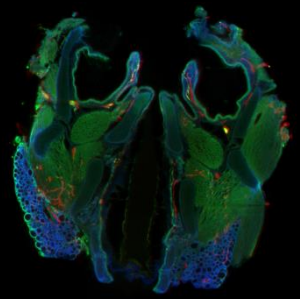
CLARITY cleared mouse brain imaged in Easyindex at RI = 1.46
Erin Diel, Harvard University, Cambridge, USA



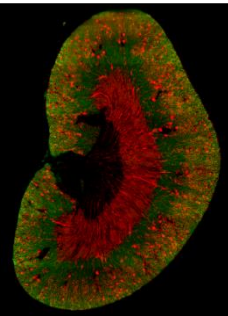
Inguinal mouse Lymph nodes cleared an imaged in Ce3D at RI=1.49
Groom & Duckworth, WEHI, Parkville, Australia



Mouse brain hemisphere, cleared and imaged in U.Clear at RI=1.53
CouZhuhao Wu, Mount Sinai, New York, USAy



Mouse Trachea, cleared with PEGASOS Ppotocol and imaged in BB-PEG at RI=1.54
Courtesy of Birchmeier lab, MDC, Berlin, Germany

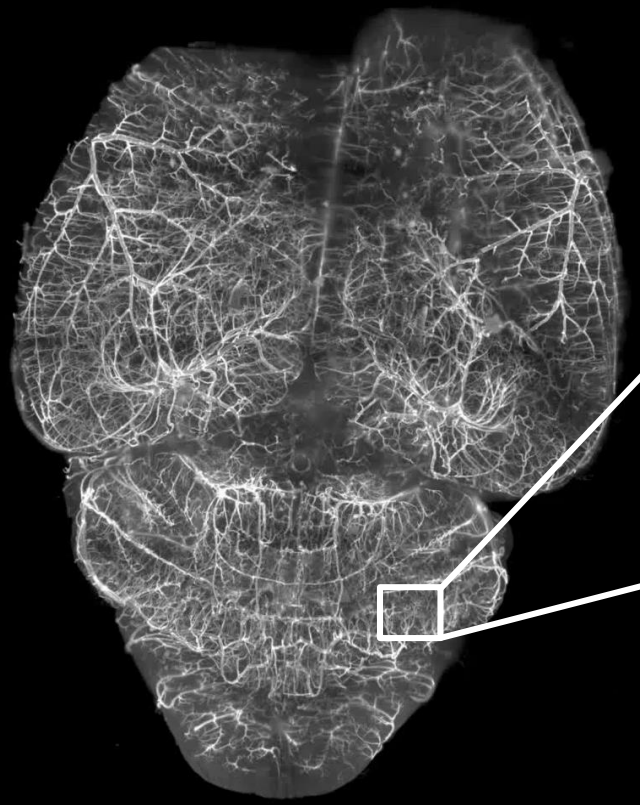


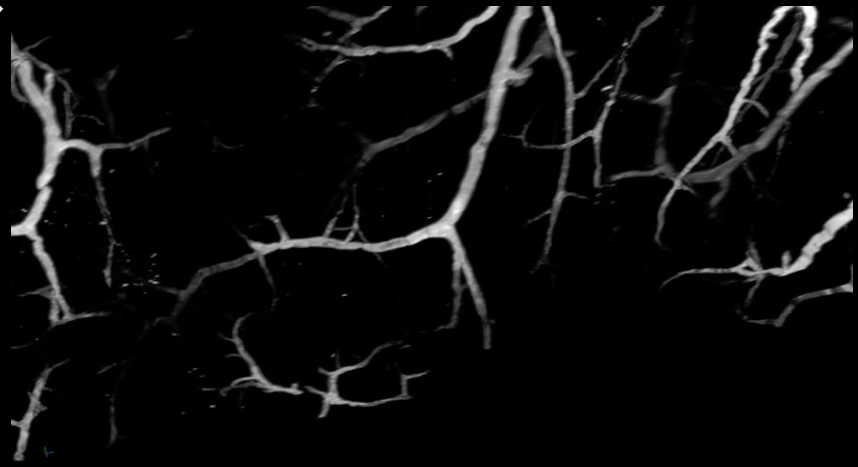
Mouse Kidney, cleared in iDISCO and imaged in Ethyl Cinnamate at RI=1.56
U. Roostalu, Gubra, Denmark

From low resolution to high resolution imaging

w/ detection optics ranging from 2.5x to 20x

2.5x





20x

C57 BL6J mouse perfused with PBS and 4% PFA. Brain

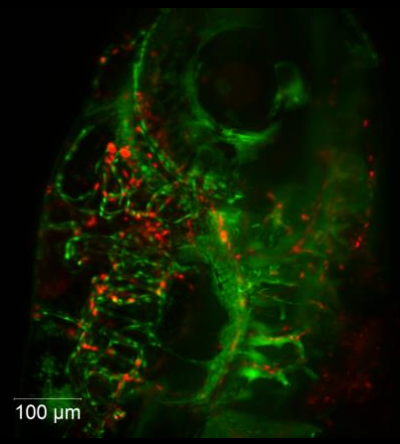
- **cleared using iDISCO+ protocol**
- **stained with CellTracker™ CM-DiI Dye – a lipid dye to stain vasculature membranes**
- **imaged in Ethyl Cinnamate at RI of 1.565.**
- **imaged volume: 13.1 x 13.1 x 6 mm**

Sample courtesy of Erin Diel & Doug Richardson, Harvard Center for Biological Imaging, Harvard University, Cambridge, USA

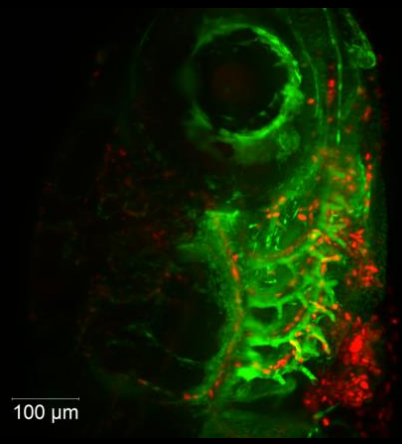
Illumination options

Single side versus dual side illumination

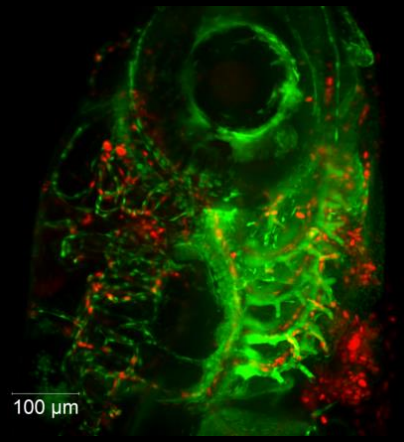
Left Side



Right Side



Dual Side Fusion



Dr. Cathleen Teh.
IMCB, Singapore

Fusion Algorithms:

- Mean Fusion
- FFT Fusion
- Max Fusion
- Subset X Fusion

Fusion Options:

- Post-processing
- Online Processing

Multiview imaging

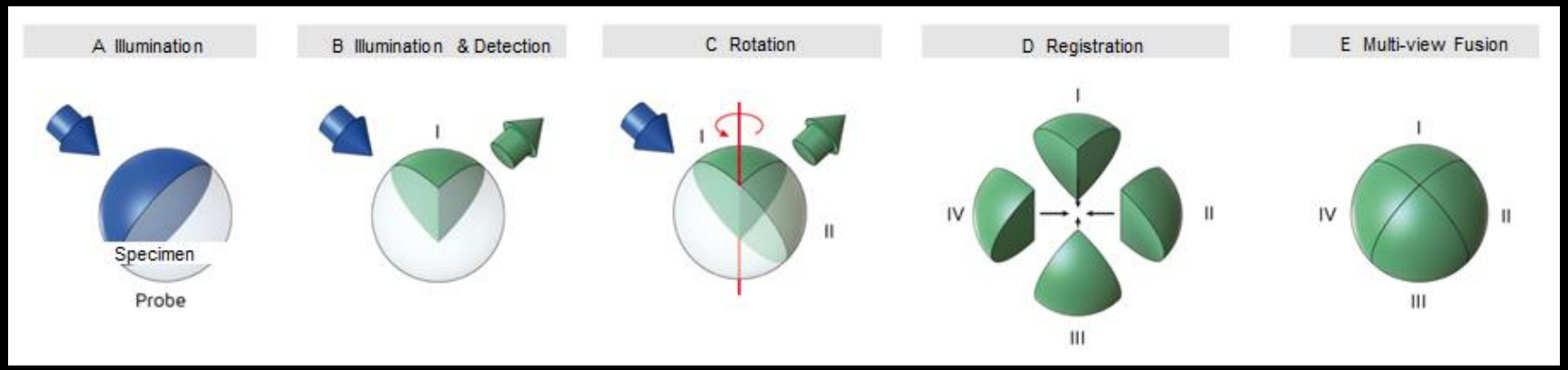
Complimentary information from different viewing angles

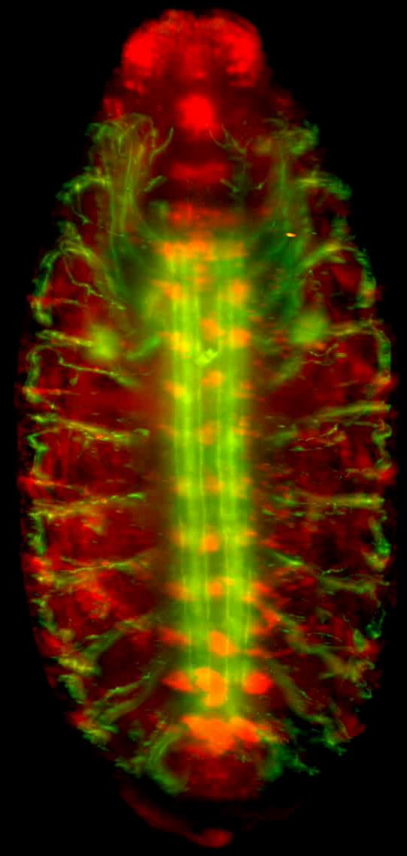
- Free 360° Rotation along gravitational Axis
- Flexibility in Sample Positioning

Multiview imaging

Complimentary information from different viewing angles

Sequential acquisition of multiple z-stacks from different directions via sample rotation.





MultiView Processing Features:

Sample and Data by Dr. Cecilia Lu, OIST, Okinawa, Japan

Registration (Alignment):

- Landmark based (Beads)
- Intensity based (Sample)
- Interactive (Manual)

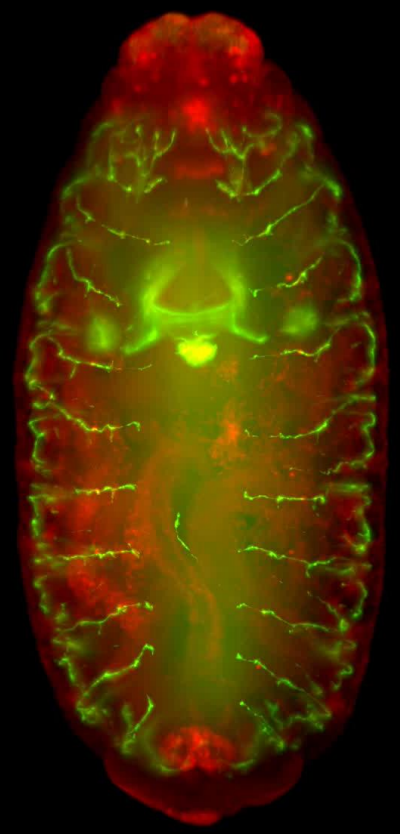
Fusion:

- Full Mean Fusion
- Subset Z Mean Fusion

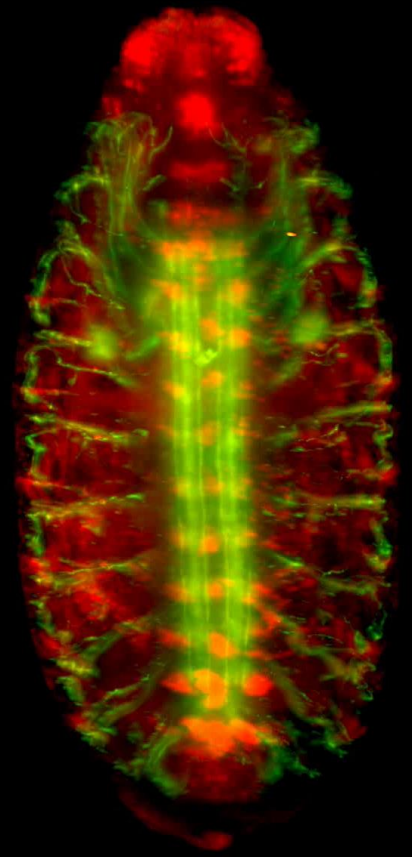
An Additional Degree of Freedom

Multiview: Registration & Fusion

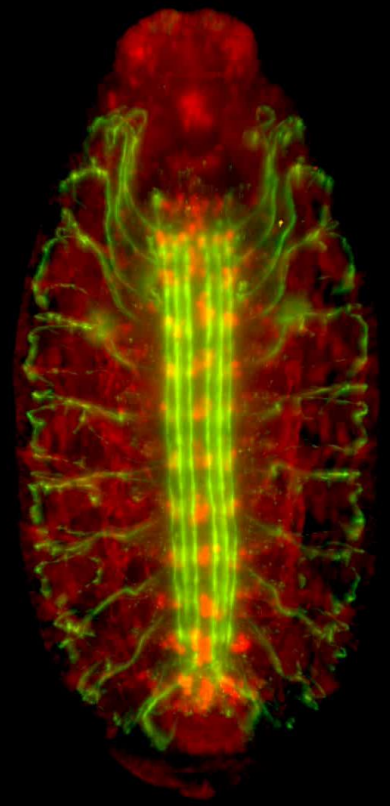
Z-Stack View 1



Registration & Fusion



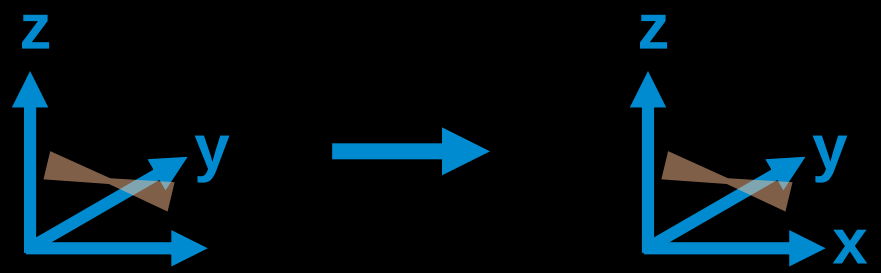
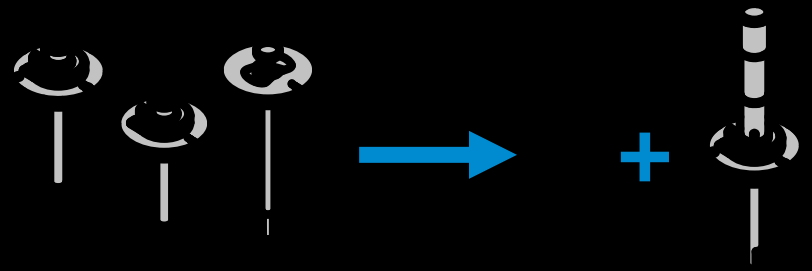
Z-Stack View 3



Sample and Data by Dr. Cecilia Lu, OIST, Okinawa, Japan

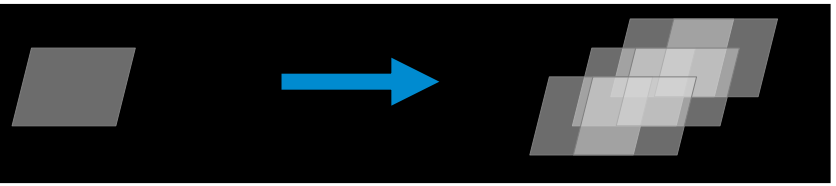
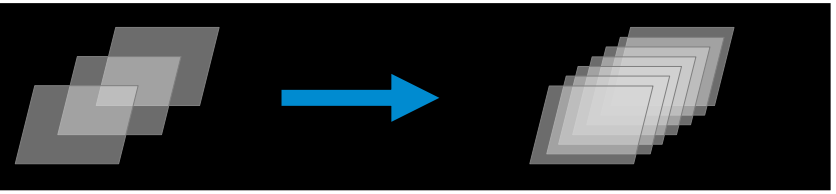
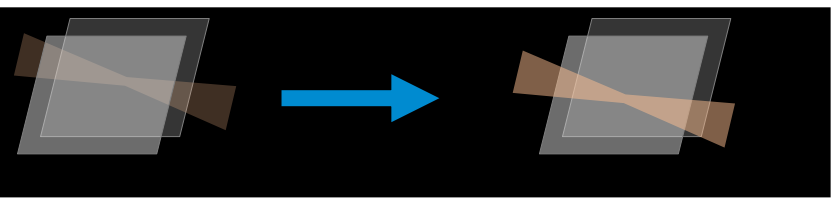
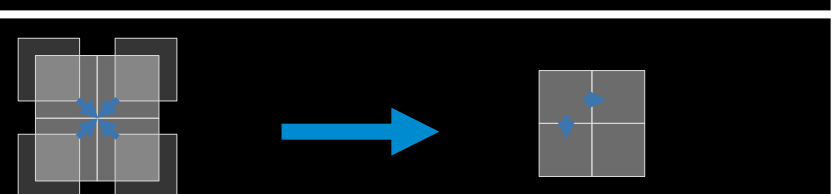
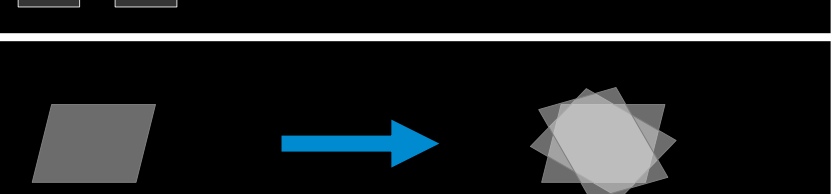
New! guided workflows

w/ semi-automatic user interaction

	**Light sheet adjustment** for optimal position of beam waist	ZEN
	**Sample loading** for contamination free positioning	ZEN

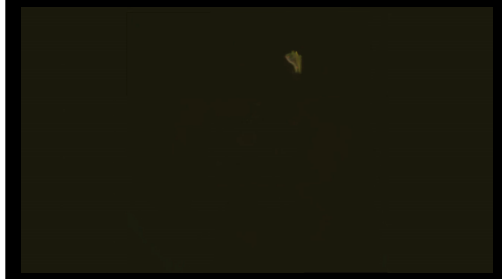
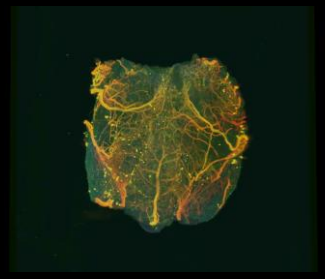
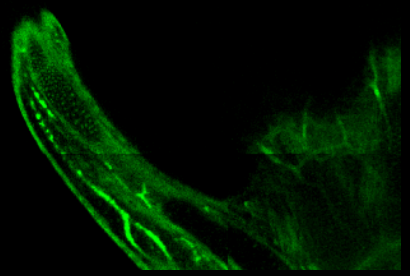
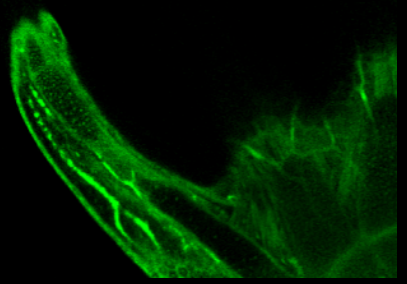
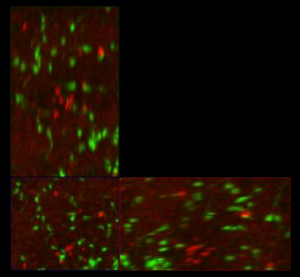
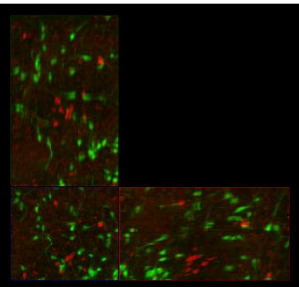
New! data acquisition tools

w/ new possibilities in ZEN Black

	Multidimensional image acquisition - multiview, time series, z-stack, tiling, position - all combinable (w/ exc. multiview & tiling)	ZEN
	3x Z-oversampling - for better deconvolution - for better multiview imaging	ZEN
	Z-offset compensation - for accurate z-stacks - for better depth penetration	ZEN
	Multiview to bona fide Tiling - centered, bounding grid, convex hall - for easy setup for large sample imaging	ZEN
	Stepwise rotation - for defined rotation w/ one click - for easy Multiview setup	ZEN

New! ZEN Blue migration

w/ data analysis and processing

 	**3DxL viewer** - for large data sets	ZEN
 	**Stitching** - for tiled data sets	ZEN
 	**GPU based DCV** - for stacked data sets	ZEN

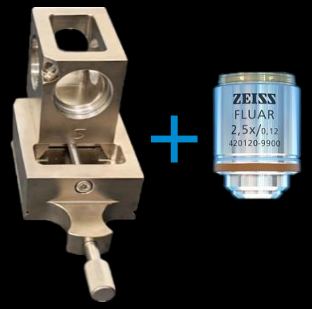
New! Partnering w/ 3rd party,
w/ extension by OEM equipment

ZEISS

ZEN → Vision4D

Arivis Vision4D
- for advanced processing pipelines

arivis



Translucence Mesoscale Imaging System™
- for fast imaging of large samples

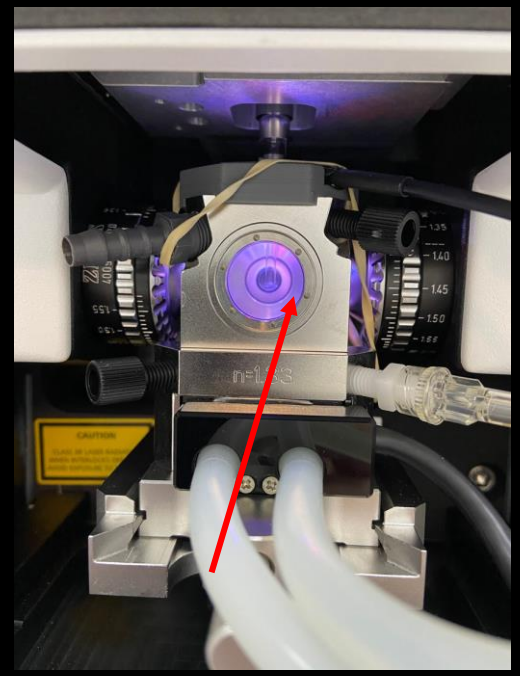
TRANSLUCENCE BIOSYSTEMS

Acquifer HIVE
- for Big Data and upscaling

ACQUIFER

New! addition,

Ring LED for plant growth



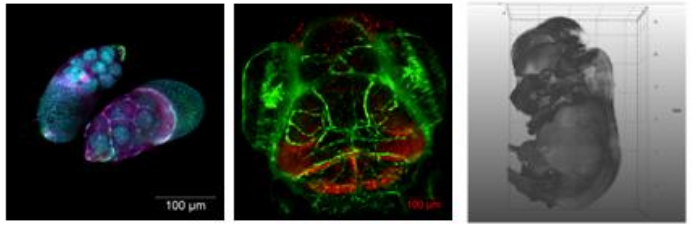
ZEISS Lightsheet 7

Versatility for Multiview Imaging of Living and Cleared Specimens

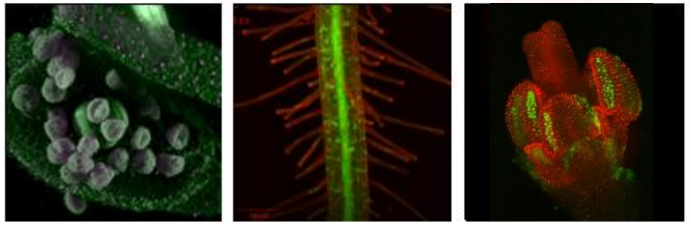
- **Live** organism and **cleared** tissue imaging
- Excellent **image quality**
- **ZEISS Optics with adjustable RI from 1.33 to 1.58**
- Imaging platform from **micro** to **meso** scale
- **Sensitive** and **fast** in image acquisition with fast high QE cameras
- **Robust & stable** design for imaging from minutes to weeks
- Flexible in sample **mounting**
- Efficient in sample **navigation**
- Capable of **acquiring**, **processing** & **analyzing** data
- **Easy-to-use** ZEN Software and integration of 3rd party for **highest productivity** with efficient workflows

Largest Range of Applications

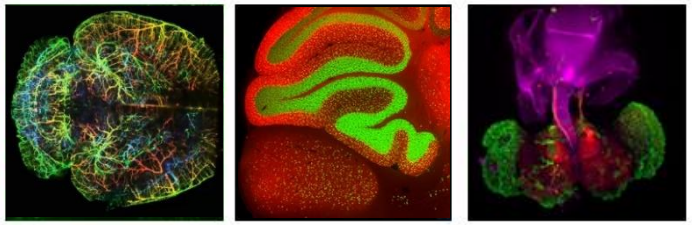
Developmental Biology



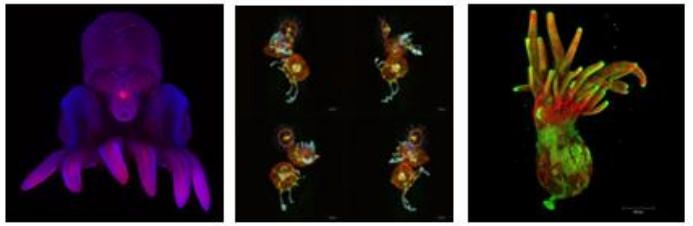
Plant Research



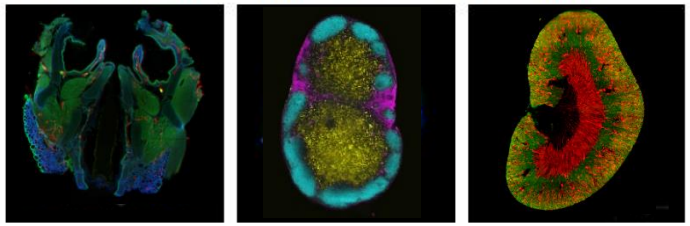
Neuroscience & Brain Research



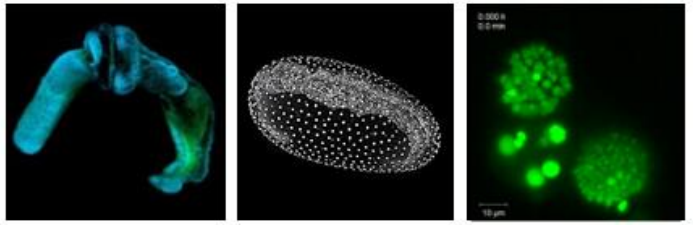
Marine Biology



3D Cell Biology / Cancer & Tissue Research



Worms / Parasites / Other

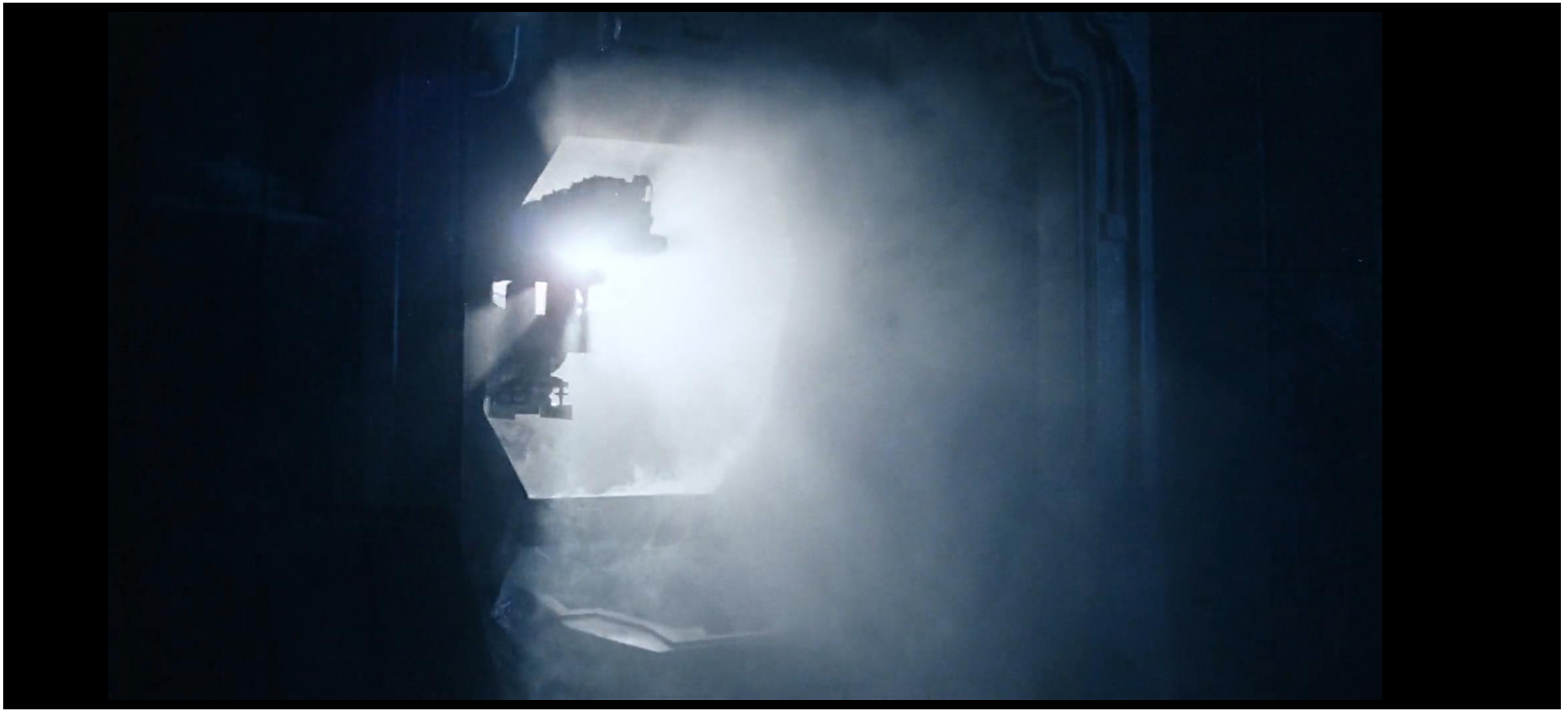


ZEISS Lightsheet 7

Take home message

1. From Live to Cleared Tissue
2. From Micro to Meso Scale
3. From Low to High Resolution

Thank You for you attention!

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