WILEY
Volume 19
March
2017
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Imaging & Microscopy

RESEARCH • DEVELOPMENT • PRODUCTION

Reprint

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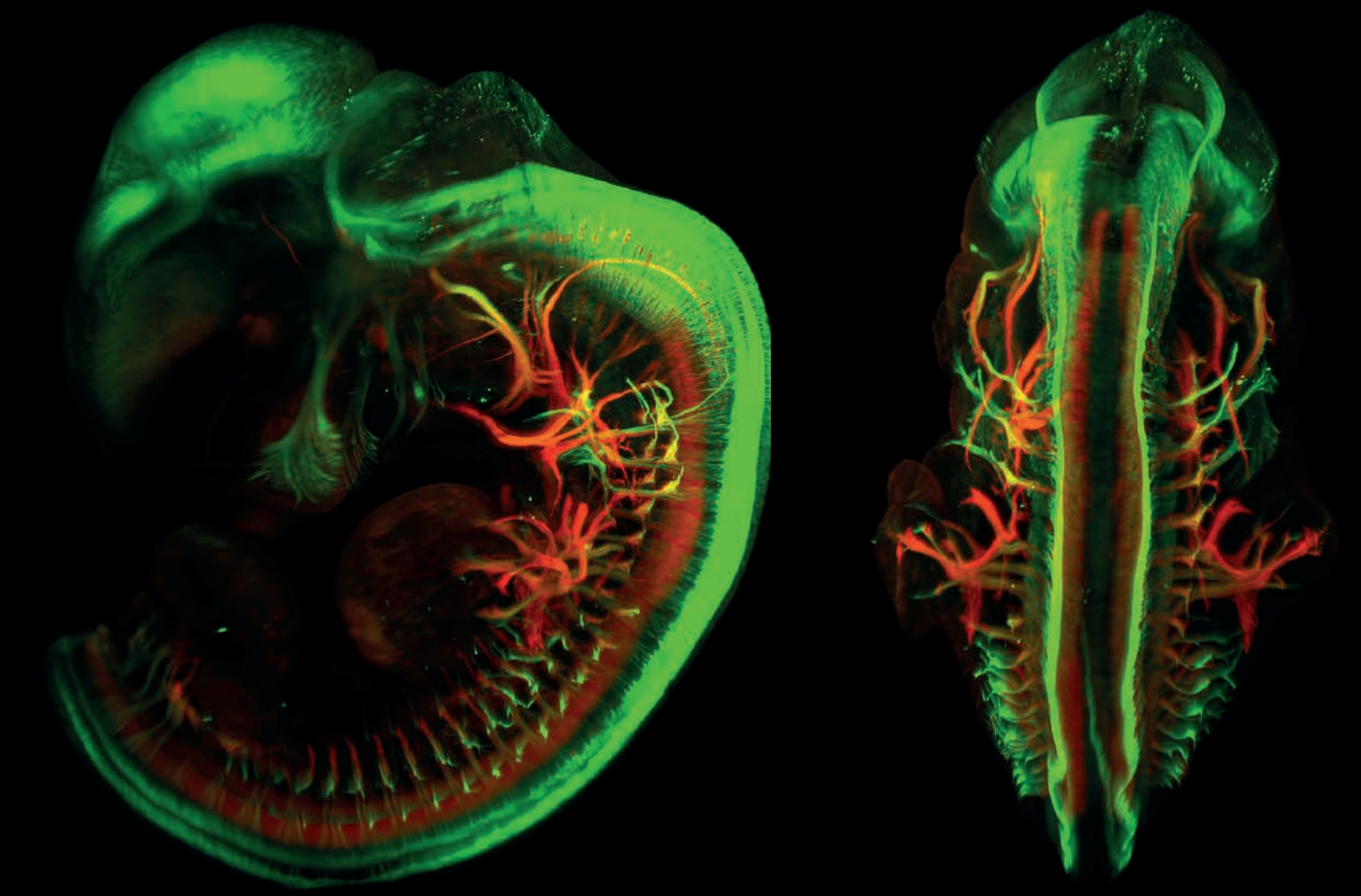

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Insights Tissue Clearing

An Epitome on Current Clearing Protocols

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Today, imaging entire cleared samples is a vital necessity to understand biological systems and processes. Several protocols have been published describing different clearing protocols. The article will address some of the frequently used techniques.

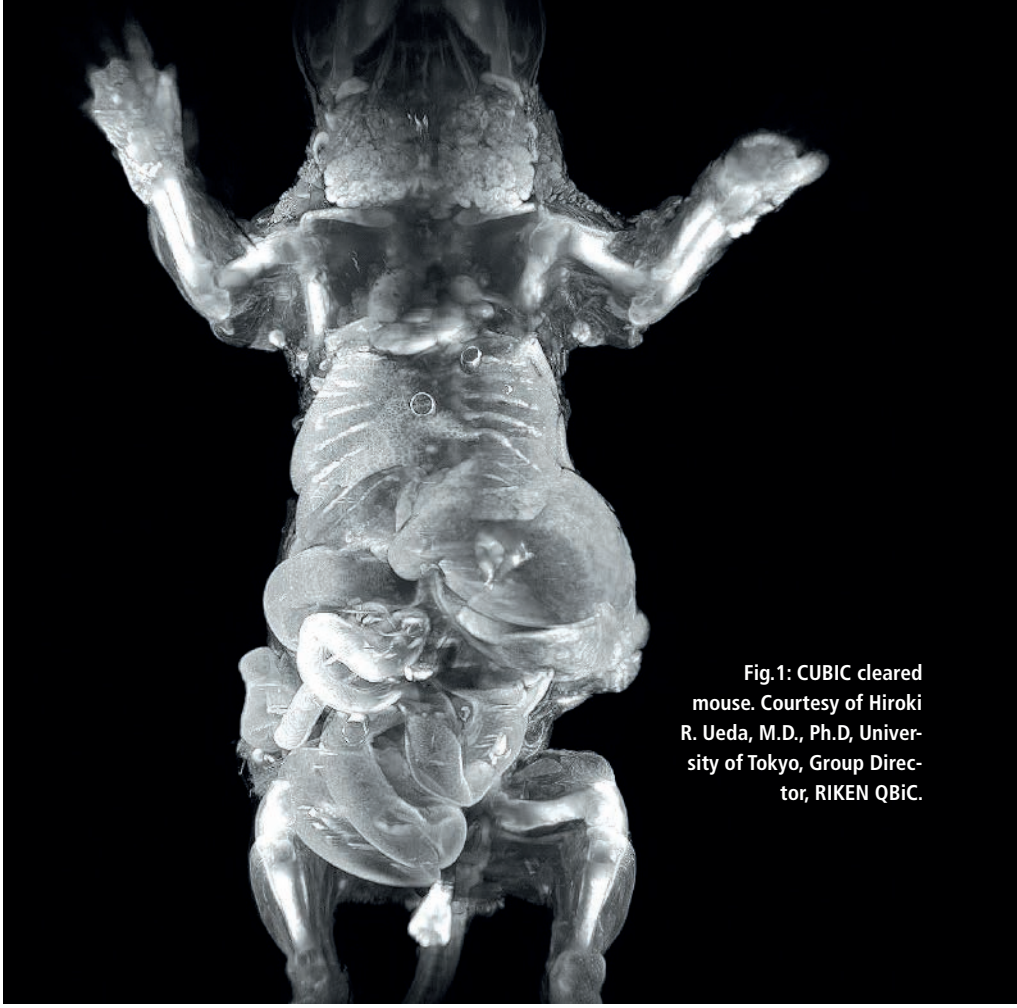

Fig.1: CUBIC cleared mouse. Courtesy of Hiroki R. Ueda, M.D., Ph.D, University of Tokyo, Group Director, RIKEN QBiC.

Why to Clear Samples

Imaging large samples normally starts with destroying them by sectioning into thin slices for further microscopy analysis. This procedure should be a kind of remedy considering the amount of tissue which is irretrievable gone due to the sectioning process which itself introduces irregular contortions. But how to get realistic three dimensional information if scattering, absorption and reflection avert whole mount imaging? Clearing the sample is the attempt to overcome these hurdles. The idea of clearing has been described in detail already in 1911 by the anatomist Werner Spalteholz. He developed one of the first clearing protocols based on a mixture of Methylsalicylate/Benzylbenzoate (5:3). Before researchers applied clove oil or Xylol in balsam of fir for clearing. Today we are dealing with about 30 different clearing procedures divided into two large groups of aqueous buffer-based protocols and organic solvent procedures.

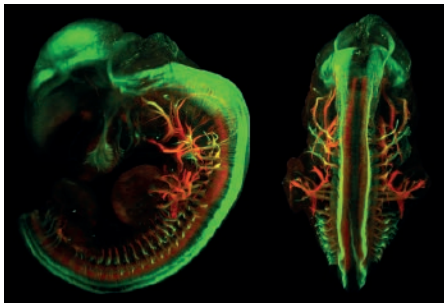

Fig. 2: iDISCO cleared mouse embryo E12 immunostained sensory and motor neurons. Courtesy of Chloé Dominici and Alain Chédotal, Institut de la Vision, Paris, France.

Aqueous Buffer-Based Protocols

The group of aqueous buffer clearing protocols is heterogeneous and can be classified based on the prevalent clearing principle as there are the hydrogel subgroup, the hyperhydration and the immersion subgroup. CLARITY [1], PACT & PARS [2], SE-CLARITY [3], SWITCH [4], EDC-CLARITY [5] and ACT-PRESTO [6] can be assigned to the hydrogel group. Hydrogel is used to embed the sample before it is transferred into the SDS buffer for an electrophoresis step to remove lipids as described within the CLARITY protocol. Endogenous fluorescent proteins are preserved while the sample expansion is insignificant. Several different protocol-parameters have to be considered and optimized individually for each sample. For imaging tissue can be immersed into FocusClear, RapiClear, 80% glycerol, 63% TDE, sRIMS or RIMS. Costs for these imaging solutions vary from US-$ 8 to US-$ 6,000 for 500 ml. The US-$ 8 sRIMS solution or the 63% TDE are frequently used. Depending on the sample size, an equilibration step overnight is necessary to avoid different refractive indices impairing the imaging.

Within the hyperhydration group the CUBIC [7] protocol includes urea and amino alcohols for clearing samples like a mouse brain or even an entire mouse. Incubation in reagent-1 is followed by incubation in reagent-2 and will take about 12 days depending on the sample size. CB-perfusion [8] with diluted reagent-1 improves the result by de-colorizing hem-rich tissue. For subsequent imaging tissue can be immersed in CUBIC reagent-2. On long term, samples can be stored and imaged in a mixture of 50% mineral oil and 50% silicone oil. Other hyperhydration methods are Scale [9], ScaleA2 [9] and ScaleS [10] Depending on the protocol, incubation may take several weeks while the sample may expand significantly.

By using high concentrations of fructose, tissue can be cleared based on immersion as it is described for SeeDB [11] or FRUIT [12] protocol. In case of adult

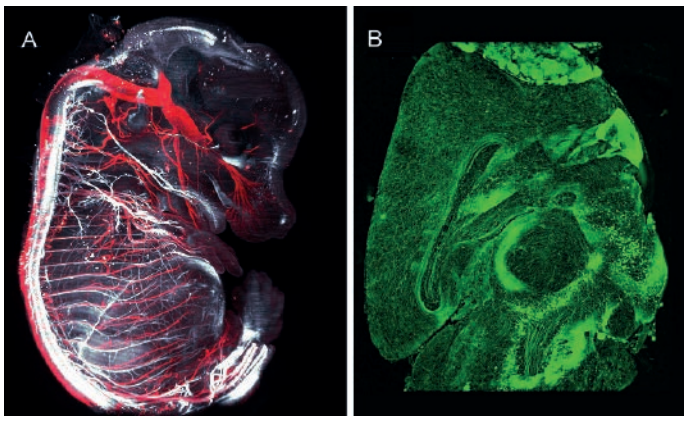

Fig.3: (A) Embryonic day 14 mouse embryo, from the Hb9-GFP transgenic line, cleared with iDISCO+ and immunostained with anti-TrkA (red) and anti-GFP (white), revealing the nociceptive and motor projections respectively. Courtesy of Nicolas Renier, The Rockefeller University, now at the Brain and Spine Institute in Paris. (B) Adult mouse brain, cleared with iDISCO+ and immunostained for Tyrosine Hydroxylase to reveal the adrenergic and dopaminergic systems. Courtesy of Nicolas Renier, The Rockefeller University, now at the Brain and Spine Institute in Paris.

or dense tissue the background may increase significantly and samples will show reduced translucency. The viscose immersion buffer may impair the imaging due to trapped air bubbles. SeeDB2 [13] is based on iohexol solution supplemented with saponin. Clear^{T} [14] is a simple protocol to reduce opacity of embryonic tissue.

Organic Solvent-Based Clearing

Certain aqueous buffer-based protocols may be cost and time intensive. Depolymerizing agents could cause an extreme expansion of the sample. In some cases sample´s opacity or mushiness impede further processing. Facing these issues organic solvent-based protocols might be the right alternative for improvements. Depending on the sample size first clearing results can be achieved within hours. Dense and large samples like tumors or organs from adult animals can be cleared within two days. Costs for most of the organic solvent clearing protocols are within range of a few Dollars. The majority of organic solvent clearing procedures has a simple two step mechanism in common. The first step is dehydration of the sample removing water and therefore the refractive index of 1.33. Within a second step refractive indices are matched. The very first clearing protocol by Spalteholz has been subsequently modified and optimized to meet today's standards in science. While Methylsalicylate/Benzylbenzoate as well as BABB clearing erase the endogenous fluorescence, 3DISCO [15] clearing, as it was developed by Dodt, can preserve endogenous fluorescence for certain time. It was the initial spark of an entire series of new clearing protocols. Dodt himself has been working on stabilized DISCO (sDISCO, 2015 SfN abstract) procedure preserving the endogenous fluorescence even better than the original 3DISCO. In 2013, Alain Chédotal modified the 3DISCO protocol. Bringing this back to mind he said: "Our contribution was to show that the 3DISCO method that we learnt from Frank Bradke was compatible with immunostaining and found a trick that allows performing whole-mount staining of large samples." In 2014 the first results of this procedure were published by Jean-François Brunet in *Science*. Nicolas Renier, a former member

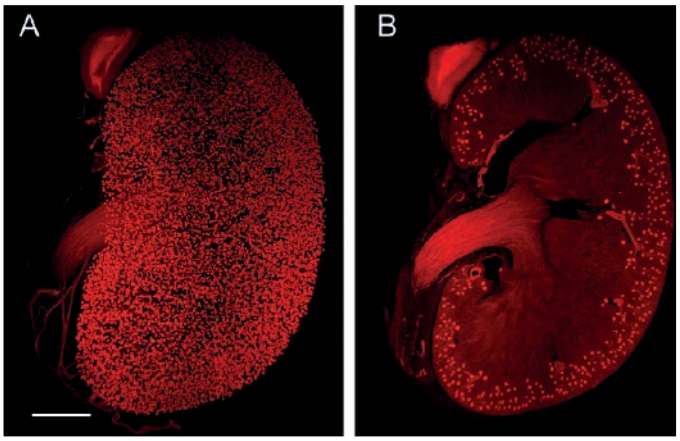

Fig.4: ECi cleared mouse kidney immunostained with anti-CD31 (Alexa Fluor 647). (A) 3D rendering showing 15.618 glomeruli. (B) Centered cross section of the same kidney. Scale bar: 1500 µm [19].

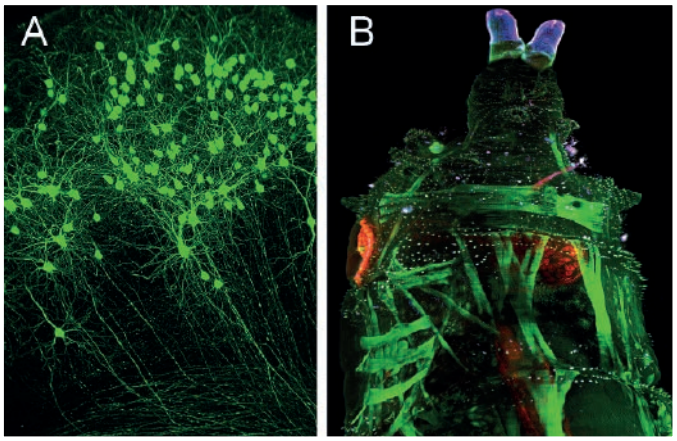

Fig.5: (A) iDISCO cleared mouse cortex, Anti-GFP-Alexa647. (B) 3DISCO cleared *Drosophila melanogaster* larvae, GFP & autofluorescence. (Schröer, Lavision Biotec GmbH)

of the Chédotal Lab, optimized the protocol for adult tissue in the Lab of Mark Tessier-Lavigne and named it iDISCO [16]. Further, he developed iDISCO+ protocol. Nicolas Renier explains the advantages of this clearing protocol: "There is in most clearing protocols a compromise to find between optical transparency, which requires harsh treatments for lipid removal, and preservation of the endogenous fluorescence of proteins, which requires milder tissue treatments. With iDISCO+, we decided to build upon the great transparency offered by the original 3DISCO and forgot the protein fluorescence completely to build a protocol centered around immunostainings, which allow us to get both strong signal from the conjugated stable fluorophores and strong lipid extraction from the organic solvents." Using iDISCO protocols, weak endogenous fluorescence can be strongly intensified by immune labelling. Other organic solvent methods like FluoClearBABB [17], uDISCO [18], and recently ECi [19] preserve the endogenous fluorescence. uDISCO introduces a significant shrinkage allowing an easier imaging of large samples like entire mice. In some cases an extended shrinkage is not favored. With the nontoxic ECi protocol shrinkage is minimized considering that the fixation itself already causes certain shrinkage of the tissue. ECi even clears bones for imaging the marrow inside. Visikol HISTO is a commercial organic solvent clearing kit which is reversible and non-destructive to tissue morphology, such that tissues can be imaged in three-dimensions followed by traditional histological processing.

Conclusion

Clearing will always introduce certain artefacts due to the extensive alteration of the sample characteristics. Protocols have to be individually optimized for each sample. Comparing aqueous buffer and organic solvent-based methods 3DISCO and ECi are faster and cheaper. Endogenous fluorescence preserving protocols like CLARITY or CUBIC deliver excellently cleared samples but temporal and pecuniary aspects are less attractive compared to fluorescence preserving protocols like sDISCO, uDISCO, or ECi. For imaging deep into large samples iDISCO protocol shows remarkable results using a far-red labelling like Cy7 or Alexa790. DAPI should be replaced by TO-PRO-3. Standard dehydration with ethanol followed by an incubation step in methanol reduces shrinkage and background compared to other dehydration methods.

Acknowledgements
The author thanks the researchers for contributing their UltraMicroscope data.

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