SnapShot: Tissue Clearing

Douglas S. Richardson^{1,2} and Jeff W. Lichtman^{1,2,3}



³Center for Brain Science, Harvard University, Cambridge, MA 02138, USA



Pretreatment Decolorization/bleaching Autofluorescence quenching Tissue protection via hydrogel Permeabilization/delipidation -Detergents: Triton X-100, SDS, Saponin Solvents: THF, DCM, Tert-butanol, DMSO

Immunolabeling -Simple diffusion Pressure enhanced Electric field enhanced RI matching (clearing) Sugars: Glucose, Sucrose, Fructose, Sorbitol Solvents: TDE, DBE, BABB, DPE, BB

Non-ionic contrast agents: lohexol, Diatrizoic acid, Idodixanol Others: Glycerol, Nicotinamide +Antipyrine

	Method	Pretreatment	Permeabilization/ lipid removal	Immunolabeling	RI matching	Fluorescent protein emission	Size change	Ref
Solvent-based	iDISCO plus	Methanol dehydration, H ₂ O ₂ bleaching	Methanol, dichloromethane	Simple diffusion	Dibenzyl ether (RI = 1.56)	No	Minimal reduction	Renier et al., 2016
	uDISCO	Tert-butanol dehydration	Tert-butanol, dicholoromethane	Simple diffusion	Diphenyl ether, ethanol, benzyl benzoate (R = 1.57)	Yes	Up to 65% reduction	Pan et al., 2016
Simple immersion	SeeDB2G	-	Saponin	Simple diffusion	Iohexol~ (RI = 1.46)	Yes	Minimal	Ke et al., 2016
	SeeDB2S	-	Saponin	Simple diffusion	Iohexol~ (RI = 1.52)	Yes	Minimal	Ke et al., 2016
	CUBIC-Cancer	Decoloring with N-butyldiethaloamine*	Triton X-100*	Simple diffusion	Nicotinamide, antipyrine (RI = 1.52)	Yes	Minimal	Kubota et al., 2017
	FAST-Clear	-	SDS	Simple diffusion	47% TDE v/v (RI = 1.42) 70% w/v sorbitol (RI = 1.46) DBE (RI = 1.56)	Yes	Minimal	Liu et al., 2017
	C _e 3D	-	0.1-0.3% Triton X-100 present throughout protocol	Simple diffusion	40% (v/v) N-methylacetamide 86% (w/v) Iohexol (RI=1.49)	Yes	Minimal	Li et al., 2017
Hyperhydration	Scale/S	Cholesterol removed with cyclodextrin, collagen relaxed with N-acetyl-L-hydroxyproline	Sorbitol, urea, Triton X-100, DMSO*	Simple diffusion	Sorbitol, urea, Triton X-100, DMSO* (RI = 1.45)	Yes	Minimal	Hama et al., 2015
Hydrogel embedding	Bone CLARITY	Demineralization with EDTA, Sample embedded in acrylamide hydrogel, decolorized with amnioal- cohols**	SDS	Simple diffusion	lohexol~ (RI = 1.47)	Yes	Minimal	Greenbaum et al., 2017
	ACT-PRESTO	Sample embedded in acrylamide hydrogel	SDS	Pressure enhanced	Iohexol~ (RI = 1.47)	Yes	Expansion	Lee et al., 2016
	SWITCH	Sample acidified and fixed in glutaraldehyde	SDS	Simple diffusion with delayed activation of binding	Diatrizoic acid, iodixanol^ (RI = 1.47)	No	Minimal	Murray et al., 2015
	SCM	Sample embedded in acrylamide hydrogel	SDS	Simple diffusion	Iohexol~ (RI = 1.47)	Yes	Expansion	Sung et al., 2016
	Stochastic electrotransport	Sample embedded in acrylamide hydrogel	SDS + rotating electric field	Electric field enhanced	Diatrizotic acid, sorbitol (RI = 1.47)	Yes	Transient expansion	Kim et al., 2015
Hydrogel embedding and hyperhydration	iExM	Sample embedded in acrylamide/acrylate hydrogel, protein denatured and digested	Triton-X100	Simple diffusion	H ₂ O (expands and clears tissue) (RI = 1.33)	No	Up to 20x expansion	Chang et al., 2017
	МАР	Sample embedded in acrylamide/acrylate hydrogel, enzyme-mediated protein digestion	SDS	Simple diffusion	H ₂ O (expands and clears tissue) (RI = 1.33)	Yes	4-5x expansion	Ku et al., 2016

SnapShot: Tissue Clearing

Douglas S. Richardson^{1,2} and Jeff W. Lichtman^{1,2,3}

¹Harvard Center for Biological Imaging, ²Department of Molecular and Cellular Biology,

³Center for Brain Science, Harvard University, Cambridge, MA 02138, USA



Equalization of Refractive Index to Minimize Light Bending in Random Directions

Light waves are bent (direction of travel altered) whenever they contact matter. These newly scattered waves are then able to interact constructively or deconstructivity with one another. When similar molecules are tightly packed (for example: pure water) light scattered by any molecule in any direction other than the initial direction of propagation will be out of phase with light scattered from another molecule half a wavelength in distance away and will interfere deconstructivity. Only in the direction of propagation of the original wave is the interference constructive. When substances with dissimilar scattering properties (i.e. different refractive indices) are mixed (for example: air bubbles or lipid droplets in water), the deconstructive interference of the scattered light is reduced and light entering the substance is reemitted in all directions (Richardson and Lichtman, 2015). This is easily visualized by shining a laser pointer through two cubes, one filled with pure water, the other containing water and a few microliters of milk. Although both liquids appear clear to the eye, the laser beam will be visible in the second cube where the milk's proteins and lipids reduce the deconstructive interference of bent laser light in all directions.

Common Steps in Tissue Clearing

Although a vast number of tissue clearing techniques now exist, they primarily consist of 2-4 of the following steps: sample pretreatment, permeabilization and/or delipidation, immunolabelling and the final refractive index matching (clearing) step. Samples are often pretreated to remove pigments that will absorb light or autofluoresce, which masks the signal of interest. Additionally, a number of techniques require the tissue to be protected in a hydrogel prior to delipidation.

Permeabilization aids in the penetration of the final clearing solution throughout a sample and is often performed using non-ionic detergents or DMSO. As lipids represent the greatest refractive index inhomogeneity in biological tissue, their removal is essential for clearing large samples. This can be accomplished with a detergent such as SDS or solvents like THF, DCM, tert-butanol, and DMSO.

The permeabilized and/or delipidated sample is now more compatible to immunostaining as the tissue has become more porous allowing for better antibody penetration. Immunolabelling is most often accomplished by passive diffusion. More recently, rotating electrical fields have been shown to enhance the speed of antibody penetration and improve the distribution of antibodies throughout a sample (Kim et al., 2015). Centrifugal force has also been used to enhance antibody penetration by applying pressure to the sample (Lee et al., 2016). Murray et al., have demonstrated a method for improving antibody distribution in large tissues by inhibiting their binding during diffusion. Once the antibodies are evenly distributed throughout the tissue they are "SWITCHED ON" to bind their epitopes (Murray et al., 2015).

Finally, the refractive index of the remaining material is equilibrated producing a transparent sample using a variety of solutions that contain high refractive index components.

Next-Generation Clearing Techniques

Since our previous publication (Richardson and Lichtman, 2015) a number of next generation clearing techniques have been published (displayed here). This new cohort of protocols is more likely to maintain fluorescent protein emission and offer better control over the expansion or contraction of tissue during the clearing process. In addition, a number attempt to simplify the process by reducing redundant treatments or chemicals. It is reasonable to assume that progress will continue to be made and further advances will be forthcoming.

Abbreviations

BABB, benzyl alcohol, benzyl benzoate; DBE, dibenzyl ether; DPE, diphenyl ether; DMSO, dimethyl sulfoxide; DCM, dichloromethane; EDTA, ethylene diamine tetra-acetic acid; FP, fluorescent protein; Ref, reference; RI, refractive index; RIMs, refractive index matching solution; SDS, sodium dodecyl sulfate; THF, tetrahydrofuran; TDE, thiodiethanol; w/v, weight per volume

Footnotes

- *These steps are performed in unison.
- **This step is performed after delipidation.
- ~lohexol, Histodenz, and Omnipaque refer to the same compound.
- ^lodixanol, Optiprep, and Visipaque refer to the same compound.

REFERENCES

Chang, J.B., Chen, F., Yoon, Y.G., Jung, E.E., Babcock, H., Kang, J.S., Asano, S., Suk, H.J., Pak, N., Tillberg, P.W., et al. (2017). Nat. Methods 14, 593-599.

Greenbaum, A., Chan, K.Y., Dobreva, T., Brown, D., Balani, D.H., Boyce, R., Kronenberg, H.M., McBride, H.J., and Gradinaru, V. (2017). Sci. Transl. Med. 9, eaah6518.

Hama, H., Hioki, H., Namiki, K., Hoshida, T., Kurokawa, H., Ishidate, F., Kaneko, T., Akagi, T., Saito, T., Saido, T., and Miyawaki, A. (2015). Nat. Neurosci. 18, 1518–1529.

Ke, M.T., Nakai, Y., Fujimoto, S., Takayama, R., Yoshida, S., Kitajima, T.S., Sato, M., and Imai, T. (2016). Cell Rep. 14, 2718–2732.

Kim, S.Y., Cho, J.H., Murray, E., Bakh, N., Choi, H., Ohn, K., Ruelas, L., Hubbert, A., McCue, M., Vassallo, S.L., et al. (2015). Proc. Natl. Acad. Sci. USA 112, E6274–E6283.

Ku, T., Swaney, J., Park, J.Y., Albanese, A., Murray, E., Cho, J.H., Park, Y.G., Mangena, V., Chen, J., and Chung, K. (2016). Nat. Biotechnol. 34, 973–981.

Kubota, S.I., Takahashi, K., Nishida, J., Morishita, Y., Ehata, S., Tainaka, K., Miyazono, K., and Ueda, H.R. (2017). Cell Rep. 20, 236-250.

Lee, E., Choi, J., Jo, Y., Kim, J.Y., Jang, Y.J., Lee, H.M., Kim, S.Y., Lee, H.J., Cho, K., Jung, N., et al. (2016). Sci. Rep. 6, 18631.

Li, W., Germain, R.N., and Gerner, M.Y. (2017). PNAS 114, E7321-E7330.

Liu, A.K.L., Lai, H.M., Chang, R.C., and Gentleman, S.M. (2017). Neuropathol. Appl. Neurobiol. 43, 346-351.

Murray, E., Cho, J.H., Goodwin, D., Ku, T., Swaney, J., Kim, S.Y., Choi, H., Park, Y.G., Park, J.Y., Hubbert, A., et al. (2015). Cell 163, 1500–1514.

Pan, C., Cai, R., Quacquarelli, F.P., Ghasemigharagoz, A., Lourbopoulos, A., Matryba, P., Plesnila, N., Dichgans, M., Hellal, F., and Ertürk, A. (2016). Nat. Methods 13, 859-867.

Renier, N., Adams, E.L., Kirst, C., Wu, Z., Azevedo, R., Kohl, J., Autry, A.E., Kadiri, L., Umadevi Venkataraju, K., Zhou, Y., et al. (2016). Cell 165, 1789-1802.

Richardson, D.S., and Lichtman, J.W. (2015). Cell 162, 246-257.

Sung, K., Ding, Y., Ma, J., Chen, H., Huang, V., Cheng, M., Yang, C.F., Kim, J.T., Eguchi, D., Di Carlo, D., et al. (2016). Sci. Rep. 6, 30736.