Improve image quality in 2D and 3D biological samples

CytoVista Clearing System and ProLong Glass Antifade Mountant.

The development of reliable 2D and 3D cell and tissue culture techniques and reagents has produced a plethora of options for studying the brain and other neural tissues. However, as the tissues and cultures become more complex, the experimental samples become more challenging to investigate. New approaches are needed for imaging spheroids, organoids, and thicker tissue sections.

One key challenge with imaging neural tissue is uneven light scattering caused by cell organelles and components such as nuclei, mitochondria, and membranes [1], as well as by lipids, which are heterogeneously distributed. It is this heterogeneity of the biological sample that contributes to the uneven scattering of light, making the sample appear milky white or opaque under the microscope [2]. Opaqueness is the result of an inherent mismatch between the refractive indices of the objective, medium, and cells or tissue. In fluorescence microscopy, opaqueness limits axial (z-dimension) resolution and focal depth during imaging. Therefore, it becomes imperative to match the refractive index of each component in order to capture high-quality images.

Optical clearing for sharp images

Several clearing techniques have been developed to match the refractive index of the sample to that of coverslips, immersion oil, and objectives. An effective clearing treatment for cells, spheroids, organoids, and tissue must meet specific criteria. First, it cannot change the overall morphology of the sample. Second, it must be compatible with immunofluorescence (IF), immunocytochemistry (ICC), and immunohistochemistry (IHC) techniques, including incubations with fixatives, permeabilization reagents, and antibodies. Third, the resulting refractive index from the clearing treatment needs to be closely matched to common microscope objectives, and the instrumentation needed to process and image the sample must be easily available in research laboratories. With the increased use of fluorescent proteins in neuronal studies, it is also important that clearing treatments do not diminish the fluorescence of these proteins [1].

Table 1. Selection guide for clearing reagents.

	ProLong Glass Antifade Mountant	CytoVista 3D Cell Culture Clearing/ Staining Kit	CytoVista Tissue Clearing Reagent and Enhancer
Form	Hard-setting, ready to use	Soft-setting, ready to use	Soft-setting, ready to use
Media type	Aqueous	Solvent	Solvent
Refractive index	~1.52 after curing	1.48	Reagent alone: 1.50 With enhancer: 1.53
Sample archiving time frame	Months to years	Weeks to months	Weeks to months
Imaging depth	Up to 150 µm	Up to 1 mm	Up to 10 mm
3D cell culture	Yes	Yes	No
Tissue sections up to 150 µm	Yes	No	Yes
Tissue sections up to 10 mm	No	No	Yes
Signal-to-noise ratio	Best	Good	Good
Photobleaching protection	Best	None	None
Mounted microscope slides	Yes	Yes	Yes
Microplate imaging	No	Yes	No
Sample preparation time	Overnight to 4 days	30 min to a few hours	1–3 days
Reversibility/tissue recovery	Yes	Yes	Yes
Cat. No.	P36980	V11325	V11324

CytoVista Clearing Reagents

Thermo Fisher Scientific recently released the Invitrogen™ CytoVista™ Clearing System (Table 1), a family of products developed to minimize the impact of refractive index

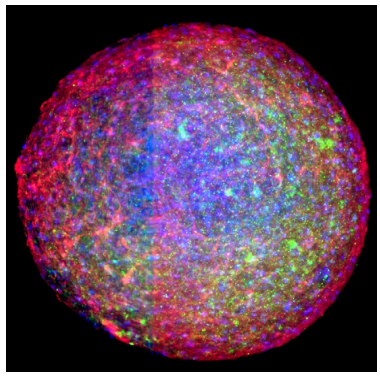

Figure 1. Clearing and fluorescent staining of brain spheroids. StemoniX™ 3D microBrain™ cortical neuronal spheroids (~500 µm diameter) were generated from induced pluripotent stem cell (iPSC)-derived neural progenitor cells. Neuronal bodies were stained with Invitrogen™ NeuroTrace™ Green Fluorescent Nissl Stain (green, Cat. No. N21480). Glial fibrillary acidic protein (GFAP) was detected with an anti-GFAP primary antibody and labeled with Invitrogen™ Alexa Fluor™ Plus 647 goat anti–rabbit IgG secondary antibody (red, Cat. No. A32733). Nuclei were stained with DAPI (blue, Cat. No. D21490). The tissue was cleared and mounted with the Invitrogen™ CytoVista™ 3D Cell Culture Clearing/Staining Kit (Cat. No. V11325). This image is a composite of z-stack imaging using the Thermo Scientific™ CellInsight™ CX7 LZR HCA Platform and HCS Studio™ Software. Videos of the z-stack images can be found at **thermofisher.com/cytovista**.

mismatch when imaging cells, spheroids, organoids, or tissue. The CytoVista clearing workflow is compatible with most fluorophores, including fluorescent proteins, that are detected with common fluorescence imaging instruments such as widefield, confocal, and light sheet microscopes, and high-content analyzers. Features of the CytoVista family of tissue and 3D cell culture clearing reagents include rapid clearing of fluorescently labeled cells, spheroids, organoids, and tissue for 2D and 3D imaging, as well as minimal shrinkage, expansion, or other morphological changes to cells and tissue. In addition, cell and tissue clearing with CytoVista reagents does not require any special equipment and is compatible with IF, ICC, and IHC protocols. After the fluorescence analysis is complete, if needed, the cells or tissue clearing can be reversed and samples can be further analyzed for other histological studies.

The CytoVista 3D Cell Culture Clearing/Staining Kit clears fluorescently labeled 3D cultured cells such as organoids and spheroids, enabling the acquisition of sharp, bright images on samples up to a depth of 1,000 µm using fluorescence instrumentation (Figure 1). This kit includes CytoVista 3D Cell Culture Clearing Reagent, penetration buffer, wash buffer, blocking buffer, and antibody dilution buffer, and can be used to clear and image samples on microscope slides or in microplates or chambers. With this system, most samples can be cleared in as little as 30 minutes, depending on the thickness of the sample.

For thicker samples, the CytoVista Tissue Clearing/Staining Kit can be used to clear fluorescently labeled tissue up to 10 mm thick prior to fluorescence imaging. This kit contains both CytoVista Tissue Clearing Reagent and CytoVista Tissue Clearing Enhancer, which together can be used to clear most fluorescently labeled tissue types, as well as the buffers needed for IHC protocols. The clearing process is relatively fast, again depending on the thickness of the sample. For example, a whole mouse brain, which is approximately 8 mm thick, can be cleared in 24 hours, while a 1 mm section can be cleared in 2 hours.

ProLong Glass Antifade Mountant

Invitrogen™ ProLong™ Glass Antifade Mountant (Table 1) is a glycerol-based, hard-setting, ready-to-use mountant that can be applied directly to fluorescently labeled cells or tissue samples on microscope slides or coverslips. Prolong Glass mountant has a refractive index of 1.52 after curing, similar to that of glass coverslips, compatible immersion oil, and oil immersion objectives. Due to the close match in the refractive indices of the objective, medium, and cells or tissue, ProLong Glass mountant enables superior axial and lateral resolution in fluorescence imaging applications. ProLong Glass mountant also provides exceptional photobleaching protection across the visible to near-infrared spectra, and it is compatible with most organic dyes and fluorescent proteins. ProLong Glass mountant is ideal for producing bright, high-resolution z-stack, 3D, and 2D images of any cell or tissue sample up to 150 µm in thickness without adding extra steps to the fluorescence imaging workflow. Figure 2 shows the use of ProLong Glass mountant for imaging along the axial dimension of a rat brain section (100 µm thick) without loss of target resolution throughout the entire sample.

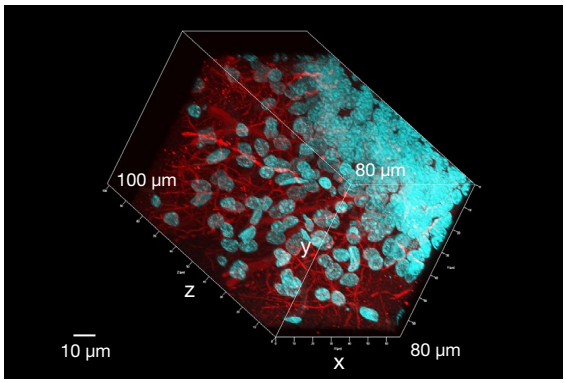

Figure 2. 3D imaging using ProLong Glass Antifade Mountant. Formalin-fixed, paraffin-embedded (FFPE) 100 µm rat brain sections were stained with Invitrogen™ β-3 tubulin primary antibody (Cat. No. MA1-118) and Invitrogen™ Alexa Fluor™ 594 goat anti–mouse IgG secondary antibody (red, Cat. No. A11032), with overnight incubations for each antibody. Nuclei were stained with DAPI (blue, Cat. No. D1306). The stained tissue sections were placed on a coverslip, and Invitrogen™ ProLong™ Glass Antifade Mountant (Cat. No. P36980) was added. The samples were allowed to air-dry uncovered overnight on a flat, dry, dark surface. The following day, ~30 µL of 100% glycerol was applied on the surface of the hardened mountant, microscope slides were placed on top, and the samples were left to cure for 1 hr. Sections were imaged on a Zeiss™ LSM 710 confocal microscope using a Plan-Apochromat 63x/1.4 NA oil immersion objective. Images were processed using Ziess™ ZEN software.

Choosing the right clearing solutions

The CytoVista Clearing System and ProLong Glass Antifade Mountant offer unique solutions for improving 2D and 3D imaging quality and resolution. Table 1 provides a selection guide to help you choose the right product for your experiment. For more information on clearing reagents and mountants, go to **thermofisher.com/cytovista**. ■

References

1. Richardson DS, Lichtman JW (2015) *Cell* 162:246–257.
2. Zhu X, Xia Y et al. (2017) *Neurosci Bull* 33:95–102.

Hitting the mark with histone antibodies

Specificity analysis of antibodies that recognize histone posttranslational modifications.

The nucleosome is the fundamental repeating unit of the eukaryotic chromosome. It functions to package DNA into units of ~150 base pairs wrapped around two copies each of histones H2A, H2B, H3, and H4, while also significantly contributing to the regulation of gene expression. Histones have a remarkable assortment of posttranslational modifications (PTMs)—including methylation, acetylation, phosphorylation, ubiquitination, SUMOylation, and ribosylation [1]. Due to the large number of modified histone residues, and the additional complexity resulting from the different methylation states of lysine or arginine residues, studying the epigenome requires a set of highly specific and validated tools [1].

Antibodies specific for histone PTMs are essential reagents in a variety of experimental techniques, including chromatin immunoprecipitation (ChIP), western blotting, immunofluorescence, and immunohistochemistry. ChIP is extensively used to assess protein–DNA interactions and to analyze the occupancy of chromatin modifications on a genome-wide scale. Because certain histone modifications may display similar DNA-binding patterns, the accuracy of a ChIP experiment depends upon the specificity of the antibody and its ability to distinguish between subtly different PTMs, such as dimethylation versus trimethylation. Recent studies aimed at testing the quality of commercially available histone PTM antibodies have raised concerns regarding their specificity, which is of paramount importance when analyzing the association of histone modifications and disease [2-5]. Thus, rigorous specificity analysis and functional validation of histone PTM antibodies are needed.

Here we describe our analysis of Invitrogen™ histone PTM antibodies. We are conducting extensive specificity testing of our antibodies using peptide arrays and functional ChIP validation assays, and comparing their performance to that of other widely cited, commercially available histone PTM antibodies. Table 1 lists a set of recently tested Invitrogen histone PTM antibodies that performed as well as or better than corresponding histone PTM antibodies from other manufacturers in histone peptide arrays; a detailed explanation of how this comparison was performed is discussed in Figure 1.

Table 1. Invitrogen histone PTM antibodies validated on peptide arrays.

Antibody target	Abbreviated target name	Host and class	Cat. No.*
Methylation antibodies			
Methyl-Histone H3 (Lys4)	H3K4me1	Rabbit monoclonal	701763
		Rabbit oligoclonal	710795
Methyl-Histone H3 (Lys9)	H3K9me1	Rabbit oligoclonal	710814
Methyl-Histone H3 (Lys27)	H3K27me1	Rabbit polyclonal	491012
Di-Methyl-Histone H3 (Lys4)	H3K4me2	Rabbit monoclonal	701764
		Rabbit oligoclonal	710796
Di-Methyl-Histone H3 (Lys9)	H3K9me2	Rabbit polyclonal	491007
Di-Methyl-Histone H3 (Lys36)	H3K36me2	Rabbit monoclonal	701767
Tri-Methyl-Histone H3 (Lys9)	H3K9me3	Rabbit polyclonal	491008
Tri-Methyl-Histone H3 (Lys27)	H3K27me3	Rabbit monoclonal	MA511198
Acetylation antibodies			
Acetyl-Histone H3 (Lys9)	H3K9ac	Rabbit monoclonal	701269
		Rabbit oligoclonal	710293
Acetyl-Histone H3 (Lys14)	H3K14ac	Rabbit polyclonal	720094
Acetyl-Histone H3 (Lys18)	H3K18ac	Rabbit polyclonal	720095
Acetyl-Histone H4 (Lys8)	H4K8ac	Rabbit polyclonal	720105
		Rabbit oligoclonal	710828
Phosphorylation antibodies			
Phospho-Histone H3 (Ser10)	H3pS10	Rabbit monoclonal	701258
Phospho-Histone H4 (Ser1)	H4pS1	Rabbit polyclonal	720100

*All antibodies listed here perform as well as or better than corresponding antibodies from other suppliers.

Methods for comparing histone PTM antibodies

To compare the specificity of antibodies for a particular histone PTM, we tested antibodies from various manufacturers using histone peptide arrays, which contain 384 peptides from the N-terminal tails of histones featuring 59 posttranslational modifications. This peptide array assay was followed by an analysis of the functional performance of the histone PTM antibody in ChIP. Commercially available antibodies were chosen based on citations and their applicability in peptide arrays, peptide dot blots, ChIP-qPCR, and ChIP-Seq, as well as in additional applications such as western blotting and immunocytochemistry.

Specificity analysis using peptide arrays

Figure 1A shows representative peptide arrays that have been incubated with either the Invitrogen™ Di-Methyl-Histone H3 (Lys4) Antibody (anti-H3K4me2 rabbit oligoclonal antibody) or a commercially available antibody from another supplier purported to have the same specificity.