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New Non-curing Mounting Media Couples Improved Axial Resolution with Cross-Spectrum Photobleach Protection Enabling High Quality Deep Tissue Imaging

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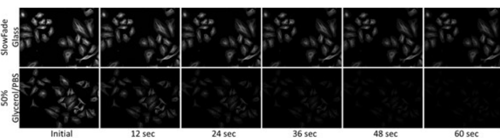
ABSTRACT

Fluorescence microscopy offers unparalleled insight into the inner workings of specimen tissue by illuminating sub-cellular structures. The ability to visualize and interrogate specimen tissue is complicated by factors including photostability and tissue thickness. These factors are more pronounced when imaging thicker tissue, >30 microns, which is often of greater interest than thinner tissue samples. The longer signal acquisition times required for thicker tissue place increased demand on the signal-generating fluorophores leading to a greater potential for photobleaching. Fluorophore photobleaching occurs after repeated excitation cycles and results in the loss of signal. To mitigate photobleaching, reagents such as free-radical scavengers and antioxidants are included in the mounting media to minimize fluorophore degradation. Image distortion is caused by poor optical matching between the lens objective, glass cover slip, and the sample itself. The refractive index (RI) is a characterization of how materials in the signal path interacts with light, and any mismatch in this pathway limits the axial resolution and focal depth of the imaging process. Cover glass and immersion oil objectives (RI=1.52) minimize refraction of light traveling to and from the sample. Most commonly used mounting media have a RI near 1.45 resulting in a mismatch. In thicker biological samples it is vital to match the refractive indexes of your materials to acquire a high-quality image. Images acquired with mismatched materials are blurry, distorted, and lack the desired focal depth. Presented here is a new mounting media formulation with an RI=1.52 developed to provide both protection against photo-degradation and RI-matching in a non-curing format. To demonstrate resolution improvement with RI-matching, point spread functions of sub-resolution microspheres were measured to quantitatively compare samples mounted with the new mounting media and current alternatives. Images of 100 micron thick tissues were mounted with the new formulation and compared to non-curing mounting media with RIs=1.45. The ability to look deeper into samples and cross-spectrum photobleach protection enables acquisition of high-resolution images of thick tissue specimens. These experiments seek to educate and empower microscopy users with additional considerations when choosing antifade mounting media.

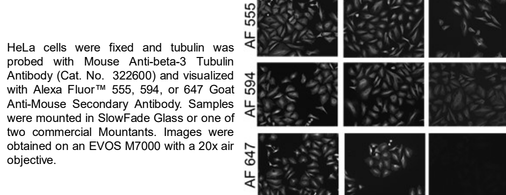
RESULTS

Maintaining photobleach protection during acquisitions



A sixty second time-lapse showing the enhanced resistance to photobleaching. HeLa cells were fixed and tubulin was probed with Mouse Anti-beta-3 Tubulin Antibody (Cat. No. 322600) and visualized with Alexa Fluor™ Plus 647 Goat Anti-Mouse Secondary Antibody (Cat. No. A32728). Samples were mounted in SlowFade™ Glass PBS/Glycerol. Images were acquired at 12-second intervals using a 20x objective with continuous illumination from a standard 100-watt Hg-arc lamp.

Improved Red Channel Imaging

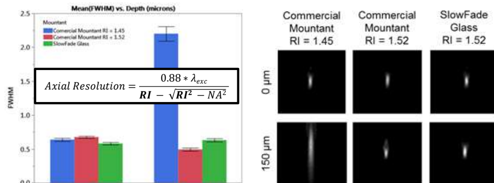


HeLa cells were fixed and tubulin was probed with Mouse Anti-beta-3 Tubulin Antibody (Cat. No. 322600) and visualized with Alexa Fluor™ 555, 594, or 647 Goat Anti-Mouse Secondary Antibody. Samples were mounted in SlowFade Glass or one of two commercial Mountants. Images were obtained on an EVOS M7000 with a 20x air objective.

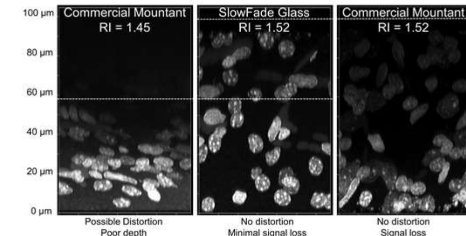
Optical tissue clearing of 1-mm thick mouse brain section

1-mm-thick mouse brain section was mounted in SlowFade Glass non-curing antifade mountant (Cat. No. S36917). Images were taken at 0, 16 and 48 hours, and as demonstrated enough optical tissue clearing is achieved with only 16 hours of incubation, making it easy to do deep tissue imaging.

Refractive index matching improves focal depth and resolution

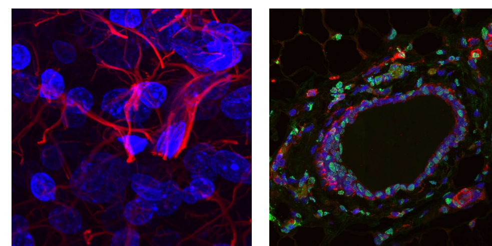


To quantify axial resolution, sub-resolution fluorescent yellow (Ex/Em: 505 nm/515 nm) 170-nm microspheres were absorbed onto the surface of a glass coverslip and a microscope slide. Microspheres were mounted in SlowFade Glass (RI ~1.52) or one of two commercial Mountants and coverslips were adhered to the microscope slides with paraffin. Z-stacks of individual microspheres (five at each focal depth) were collected on a Zeiss LSM 710 confocal microscope using a 63x Oil objective. SlowFade Glass maintains a higher axial resolution similar to that of the 1.52 RI commercial mountant, both of which are far superior to the lower RI commercial mountant (RI 1.45). The maximum theoretical axial resolution of the microscope is 500 nm.



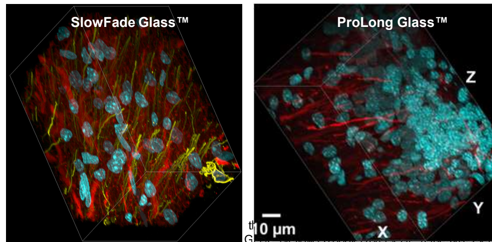
Cryo-preserved 100-µm-thick rat brain sections were stained with DAPI nuclear stain (Cat. No. D1306). Stained samples were mounted with non-curing antifade mounting media. Tissue sections were imaged on a Zeiss LSM 710 confocal microscope using a Plan-Apochromat 63 × /1.4 NA Oil objective sampling at a rate of 71 nm in the X and Y dimensions and 100 nm in the Z dimension, with a pixel size of 0.07 µm.

Image high resolution tissue sections with confidence



100 micron thick-tissue samples were stained and mounted with SlowFade Glass and Images were acquired a Zeiss LSM 710 confocal microscope. Left) Cryo preserved rat brain tissue section immunostained for GFAP with rabbit anti-GFAP and detected using Alexa Fluor 594 Plus goat anti-rabbit. Nuclei were counter-stained with DAPI. A 63X oil immersion objective was used and the resulting image represented as a maximum intensity projection. Right) Mouse mammary tissue section probed with mouse anti-histone H3 and rabbit anti-pan actin. Targets were detected with Alexa Fluor 488 Goat Anti-Mouse (Cat. No. A11029) and Alexa Fluor 594 Goat Anti-Rabbit (Cat. No.A11012) secondary antibody conjugates. The tissue was counter-stained with SYTOX Deep Red (Cat. No. S11381). A 40X oil immersion objective was used and the resulting image represented as a maximum intensity projection.

Deep tissue imaging with SlowFade Glass™ and Prolong Glass™



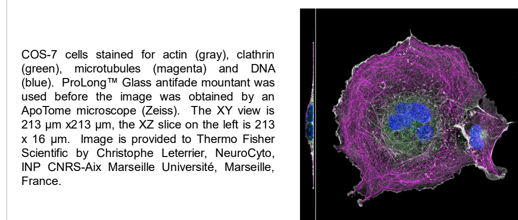
06100). Targets were detected with Alexa Fluor Plus 594 Goat Anti-Mouse (Cat. No. A-11032) and Alexa Fluor Plus 647 Goat Anti-Rabbit (Cat. No. A-32733). Nuclei (cyan) were stained with DAPI (Left, Cat. No. D1306) or SYTOX Deep Red (Right, Cat. No. S11381). Slides were mounted and imaged with a Zeiss LSM 710 confocal microscope using 63 × oil Immersion.

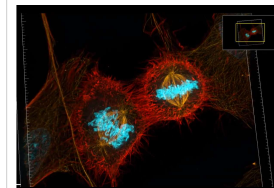
CONCLUSIONS

- As a non-curing mounting media, SlowFade Glass™ can be used right after mounting samples to obtain high quality images with photobleach protection, enabling long acquisition times.
- The matched refractive index of SlowFade Glass™ enables higher quality deep tissue imaging while preserving photobleach protection.
- ProLong Glass™ is a curing mounting media that offers refractive index matching with archivability, and idea for tissue and high-resolution techniques.

ProLong Glass™ images from customers

COS-7 cells stained for actin (gray), clathrin (green), microtubules (magenta) and DNA (blue). ProLong™ Glass antifade mountant was used before the image was obtained by an ApoTome microscope (Zeiss). The XY view is 213 µm x213 µm, the XZ slice on the left is 213 x 16 µm. Image is provided to Thermo Fisher Scientific by Christophe Leterrier, NeuroCyto, INP CNRS-Aix Marseille Université, Marseille, France.





Immunofluorescence of mitotic HeLa cells stained with primary mouse anti beta-Tubulin HeLa, with capital L
antibody and secondary goat anti mouse F(ab)2 Alexa Fluor 568 (A11019). F-actin is stained with ATTO643-Phalloidin (ATTO-TEC #AD 643-81) and chromosomes with Hoechst 33342 (H1399). Mounted with ProLong glass antifade mountant (P36980). 3D-Animation of 100nm slices taken by Structured Illumination Microscopy with Zeiss Elyra S.1. Total range of the stack z=5.6µm. In courtesy of Lisa Behringer-Ließ and Dr. Markus Sauer, Dept. of Biotechnology and Biophysics, University of Wuerzburg.

Fixed HeLa cells labeled with SYTOX Deep Red nuclear dye (S11380) and anti-tubulin (A11126) detected using Goat anti-Mouse Alexa Fluor 488 ReadyProbes Secondary Antibody (R37120), were imaged by super resolution structured illumination microscopy (SR-SIM). Samples were prepared using Image-iT Fixation/Permeabilization Kit (R37602) and mounted using ProLong Glass Antifade Mountant (P36980). Image shown is a 3D projection from a z-stack (magenta – SYTOX Deep Red; green – alpha tubulin). Image courtesy of Jeffrey Caplan and Kun Huang, University of Delaware

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QUESTIONS AND COMMENTS

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