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Mountants and Antifades

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1 Introduction

This document is a summary of information from the Confocal listserver archives, Cytometry Archives and the Histonet archives. A more detailed review of of antifade reagents has been published by Ono *et al.* (1) and a paper describing the effect of refractive index mismatch has been published by Diaspro *et al.* (2).

The blue hyperlinks in this document should take you to the original articles.

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1.1 Refractive Index Mismatch

One of the major causes of image degradation in microscopy is due to improper matching of immersion medium and mountant.

Mismatching the refractive index of mountant and objective immersion medium results in spherical aberration and signal loss.

	Refractive Index
Cells	
Water	1.333
glycerol	1.466
Glass	~1.52
Zeiss Oil	1.515
Diamond	2.42

From Zeiss webpage on objectives:

“Oil objectives are designed and corrected on the assumption that the refractive indices of the immersion and embedding media are equal (n = 1.52). For water objectives, this index is assumed to be n = 1.33 for both media.”

The mountant and the immersion medium should be matched within 0.01-0.05 [LINK] ideally to three decimal places [LINK].

2 Mountants

Mountants comprise two or three main ingredients: a base, an antifade reagent and sometimes a plasticizer to set. Commercial products often include sodium azide as a preservative.

2.1 Base constituents

The base component of a mountant is either aqueous (RI ~1.34); glycerol (RI~1.47); natural oil (RI~1.53); or plastic (RI~1.51). The base-ingredient is the major determinate of the refractive index of the medium. [LINK].

There is some suggestion that phycoethyrin fluorescence is quenched by glycerol. Glycerol may be incompatible with specimens containing lipophilic plasma membrane stains like DiI which may leach [LINK][LINK].

2.2 Antifade constituents

The bleaching process is thought to involve reaction of the excited-dye molecule with singlet oxygen. Most antifade reagents are reactive oxygen species scavengers. However, sometimes the antifade itself will quench the fluorescence of the dye [GRAPH][LINK].

Agents such as NPG (N-propyl gallate), DABCO (1,4-diazabicyclo[2.2.2]octane), 4-POBN (-(4-Pirydyl-1-oxide)-N-tert-butyl nitrone) and PPD (P-phenylanediamine) reduce bleaching and are often added as 'antifade' reagents to mounting media. However, they also reduce the intensity of the initial signal (White et al., 1987; Longin et al., 1993; Song et al., 1996). They may also be of little use with DNA-binding dyes, as they and their solvents, which are both nonpolar, may interfere with dye binding. For example, Vectashield (which contains PPD in buffered glycol; Longin et al., 1993) reduces the signal of PI bound to DNA, whereas NPG in 80% glycerol eliminates the red luminescence from RNA in cells stained with acridine orange (not shown).

2.2.1 p-Phenylenediamine (PPD)

This is the most effective antifade compound. However, it can react with cyanine dyes (especially Cy2) and cleave away half of the cyanine molecule [LINK]. Use of phenylenediamine as an anti-fading reagent in mounting media may result in weak and diffused fluorescence after storage of the stained slides.

2.2.2 n-Propyl gallate (NPG)

Not very soluble, needs heating and/or overnight to dissolve. Non-toxic and can be used with live cells. However, it is protective against apoptosis and so may interfere with the biological process under study [LINK].

2.2.3 DABCO

1,4-Diazabicyclo-octane, also known as triethylenediamine. Not as effective as PPD but less toxic. Suggested to be used in live cell work but would be predicted to have the same anti-apoptotic properties of NPG.

2.3 To-Harden or Not-to-harden?

Some mountants harden as they dry. This facilitates handling and storage but does result in significant changes in the RI over time as the mountant hardens – e.g. see figure 2 in the *Prolong* product information PDF [GRAPH]. During the hardening process, there can also be shrinkage and tissue damage. However, this shrinkage may be insignificant compared to shrinkage due to dehydration during the fixation process. [LINK].

If you opt to use a mountant that does not harden, you will need to seal the coverslip to prevent drying and leakage of the mountant, and my also need to use spacers to prevent the coverslip from squashing the specimen.

2.3.1 Sealants

2.3.1.1 Nail varnish

There are many accounts of nail varnish quenching GFP [LINK][LINK] but others do not see this [LINK][LINK]. The quenching is possibly linked to the water soluble isopropyl alcohol in the varnish [LINK].

Allow at least 30 minutes for nail polish to dry. Sealants that have not been thoroughly dried will damage the lenses. If you do get varnish on the objective lens it may be chipped off with a toothpick – the wood is reported to be soft enough not to damage the lens. Try this on a practice area first though ;-) [LINK].

2.3.1.2 Entellan

[Product LINK]

Entellan is sold as a xylene based mounting medium like DPX (see below) but contains poly(methyl methacrylate) rather than polystyrene (80kD) which is the styrene in DPX [LINK].

Although sold as a mountant, Entellan has been recommended as a sealant as it sets quickly [LINK] (20 minutes). Reported to be best sealant for glycerol based mountants [LINK].

2.3.1.3 Dental or modelling wax

This technique is safe and easy and easily reversed. The wax comes as pink coloured sheets. Heat the sheet in a glass beaker and it will become liquid at 40ºC - 60ºC. Use a small paintbrush for sealing the coverslip. When the wax is in touch with the coverslips at room temperature it became solid immediately [LINK].

2.3.1.4 VALAP

To seal living samples, VALAP (equal mixture of Vaseline, lanolin, and paraffin mixed on a heating plate 60°C) is reported to work well. It melts at low temperature, so it will not heat the slide and sample when applied. It forms a watertight seal. You can make the slide permanent later by perfusing in glutaraldehyde by capillary action (poke small hole at either end of seal and use filter paper at one end to draw fluid through the sample) and then sealing cover glass with nail polish over the VALAP. This has worked well in live cell experiments. [LINK].

2.3.2 Spacers

Broken coverslips have been recommended as cheap, permanent spacers (#1 = 0.13-0.17 mm; #1.5 = 0.16-0.19 mm; #2 = 0.17-0.25 mm thick). Cut with a diamond-pen [LINK].

Gaskets are commercially available, or you can make your own from latex gloves [LINK] or Parafilm [LINK]. Molecular probes sell thick (0.5 mm and up) silicone gaskets as spacers. These are not cheap (~US$85 for 40).

Scotch tape has also been reported to work as a 60-100 µm spacer [LINK][LINK] but may be autofluorescent [LINK].

Custom made metal spacers have been found to be useful in live cell work where they can be re-used [LINK][LINK].

2.4 Commercial Products

2.4.1 Aqueous

2.4.1.1 Gel/Mount

[Product LINK]

Gel/Mount is a non-glycerol based mounting medium. The manufacturers say that it is specially formulated without glycerol to avoid the deleterious effects of this compound in some of the phycobiliprotein based detection systems. I am having trouble tracking down information on glycerol affecting phycobiliproteins. There is some suggestion that glycerol can cause the phycobiliprotein to dissociate from its membrane [LINK]. Gel/Mount does not contain phenylenediamine which may interfere with cyanine dyes.

2.4.1.2 Fluoromount- G

[Product LINK]

2.4.1.3 Fluorsave

[Product LINK]

Comes highly recommended. Hardens after about 1 hour [LINK]. As with other hard-setting mountants, shrinkage after 1 week can damage tissue though [LINK]

2.4.2 Glycerol-based

2.4.2.1 Prolong

[Product LINK].

Reported not to not quench fluorescence like some other antifades [LINK].

Said to be good for the Alexa dyes but not so good for fluorescent proteins [LINK].

It is reported that Prolong stores better than Vectashield and probably uses PPD as an antifade [LINK]. Very expensive though.

Reported instance of UV induced autofluorescence with Prolong [LINK].

2.4.2.2 *Vectashield*

Comes as regular [Product LINK] or hard set [Product LINK].

Not good for Cyanine dyes – suggesting that its antifade reagent is PPD? Vectashield suffers from a blue autofluorescence when excited with UV. It is suggested to fade quickly though. [LINK]. Autofluorescence may be exacerbated by storage at -20°C [LINK].

2.4.2.3 *MOWIOL*

[Product LINK]

MOWIOL is a PVA-based medium originally designed for EM. It's not good for 3D imaging. The refractive index is inconsistent between batches [LINK]. Possible linked to the fact that it arrives as a powder and requires reconstitution in glycerol and buffer (see Appendix for recipes).

MOWIOL has been reported to be involved in PFA-fixation induced redistribution of GFP-tagged transmembrane proteins [LINK].

2.4.3 Plastic

2.4.3.1 *DPX*

[Product LINK]

A mixture of **d**istyrene (a polystyrene), a **p**lasticiser (tricresyl phosphate), and **x**ylene, called DPX, was introduced in 1939 and later modified by the substitution of a more satisfactory plasticiser, dibutylphthalate (butyl, phthalate, styrene - BPS) [LINK]. Always perform procedure in a fume hood and use forceps to handle the slides: DPX contains xylene.

DPX requires dehydration of your tissue (which compresses structures in the z-axis) and which will extract any lipid-soluble fluorophores.

For TRITC, Lissamine rhodamine, rhodamine Red-X, Cy2, Cy3, or Cy5, DPX is reported to work fine. However, poor results are reported with FITC or AMCA. [LINK].

2.4.3.2 *Permount*

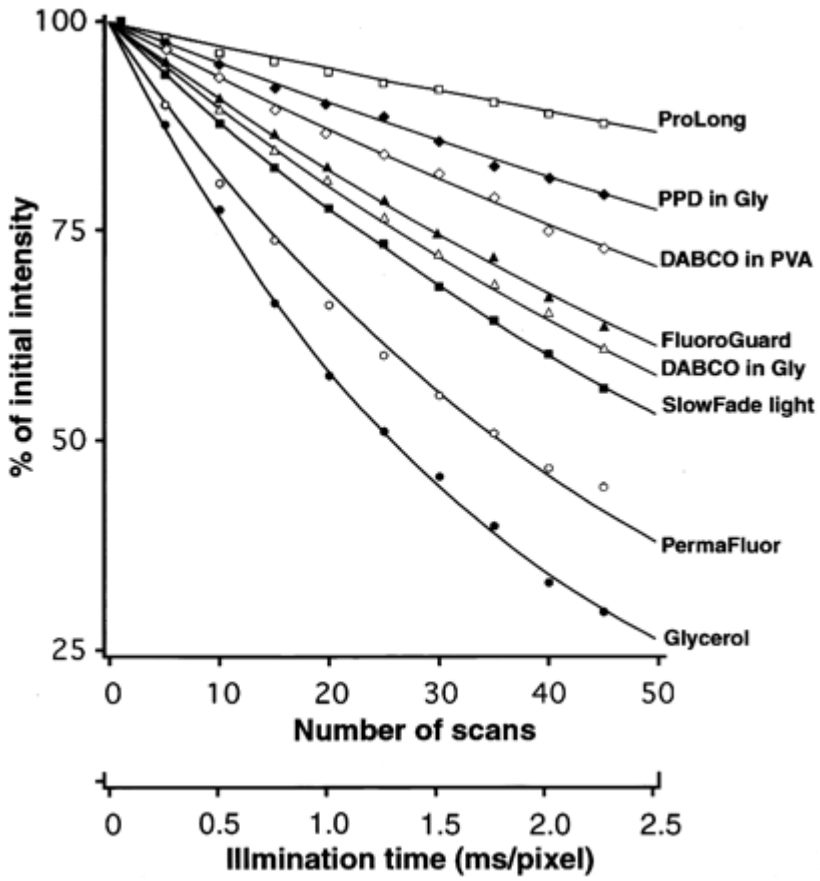
[Product LINK].

Toluene-based synthetic resin mounting medium. [LINK] or based on natural oil? [LINK]. Quick setting and lower viscosity than DPX. Can craze after 20 yrs (!) [LINK]

2.4.4 Summary

Mountant (Click for product link)	Manufacturer/Supplier	Hard-set?	Base	RI[1]	Cost/unit (US$)	Purchased volume (ml)	Cost/ml[2]
Fluorsave	Calbiochem	Y	Aq		$ 72.00	20	$ 3.57
Fluoromount-G	Southern Biotech Assoc	Y	Aq	1.393	$ 48.00	25	$ 1.92
Gel/Mount	Biomeda	N	Aq	1.358	$ 33.00	20	$ 1.65
Vectashield Hard Mount	Vector Labs	Y	Glyc	1.440	$ 125.00	10	$ 12.50
Vectashield	Vector Labs	N	Glyc	1.458	$ 105.00	10	$ 10.50
MOWIOL	Calbiochem	Y	Glyc	1.490[3]	$ 110.00	24[4]	$ 4.58
Prolong	Molecular Probes	Y	Glyc	1.455	$ 98.00	10	$ 9.80
DPX	Electron Microscopy Sciences[5]	Y	Plas	1.5251	$ 48.00	100	$ 0.48
Fluorescence Mounting medium	DAKO	Y	Plas?	1.47-1.50	$ 20.25	15	$ 1.35
Entellan	Merck	Y	Plas	1.500	$ 35.00	100	$ 0.35
Permount	Biomeda	Y	Oil	1.54	$ 25.00	100	$ 0.25

[1]From Confocal list, suppliers websites and Servicio de Microscopía Óptica y Confocal
[2]Prices from March 2004.
[3]Varies with recipe
[4]One unit is 2.4g which is dissolved in 24 ml.
[5] and many other suppliers



from Ono *et al.*(2001)

3 Web resources

EMS information on various products [LINK].

Summary of Confocal Archive info from Wes Wallace [LINK] .

Microscopía Optica y Confocal Refractive Index table for a number of mounting and immersion media [LINK]

4 Appendix 1: Recipes

Most of these are glycerol based. There are reports that some batches/sources of glycerol will autofluoresce so check new bottles before making up mounting media. [LINK]

4.1 PPD-Glycerol

The key to using para-phenylenediamine is pH if the pH is below ~8.0, you will see fading and background. We buffer our medium with bicarbonate to keep the pH up.

0.1 - 0.01% p-phenylenediamine (Sigma #P1519)
50-70% glycerol in PBS
50% glycerol in 20 mM phosphate buffer (pH 8.5)

Aliquot and store frozen -80°C (if it goes dark throw it out). [LINK]

GD Johnson et al., J. Immunol.Meth. 55: 231-242, 1982.

meta-Phenylenediamine has been reported to react in the presence of weak acid to form a yellow-fluorescing basophilic stain. Avoid anything that might be a mixture of the para- and meta- isomers. M. Wessendorf (Conf Arch) [LINK].

4.2 DABCO-PVA

2.5% DABCO, 10% polyvinyl alcohol (PVA) (Sigma; Type II), 5% glycerol, and 25 mM Tris buffer, pH 8.7

Day 1

1. Add 4.8 g of polyvinyl alcohol (PVA) to 12 g of glycerol and mix well.
2. Add 12 ml of distilled water and leave it on a rotator at room temperature over night.

Day 2

3. Add 24 ml of 0.2M Tris-HCl at pH 8-8.5.
4. Heat in a water bath to 50°C while mixing for about 30 minutes.
5. Add 1.25g of DABCO and mix well.
6. Centrifuge at about 2000 rpm for 5 minutes.
7. Aliquot the supernatant (we use 1 ml aliquots which is enough for 15-20 slides) and store at -20°C.

Since it polymerizes upon contact with air you should not refreeze the aliquots. Thaw it just before use and throw away what you do not use. Sigma has both the PVA (#P8136) and DABCO (#D2522), and so has Chemicon (#151938 RT for PVA and #195137 for DABCO). Johan Wassélius (Conf List.)

4.3 NPG-Glycerol

2% n-propyl-gallate (Sigma)
49% PBS
49% glycerol
pH=7.4.

4.4 DABCO-Glycerol

1% DABCO in 90% glycerol, phosphate buffered. Johnson, G.D. et al, J Immunol Meth 55(2):231-42. Samples seem to last for weeks to years if kept in the dark. [LINK]

4.5 MOWIOL-DABCO

Recipe
-Add 2.4g of MOWIOL 4-88 [Calbiochem] to 6 g of glycerol. Stir to mix
-Add 6ml of water, leave stirring @RT for several hours
-Add 12ml of 0.2M Tris [pH8.5} and heat to 50C for 10 min with occasional mixing
-Clarify by centrifugation @ 5,000g for 15 minutes
-OPTIONAL: Add DABCO [1,4,-diazobicycli-[2.2.2]-octane, Aldrich] to 2.5% w/v to reduce fading of fluorophores
-Aliquot into airtight containers and store @-20C

Stable @RT for a few weeks if in airtight tubes. This remains stable at -20šC for 12 months. Once thawed, stable for one moth at RT.

use a pH 8.0 Trizma buffer system and 4% n -propyl gallate as an

5 Reference List

(1) Ono M, Murakami T, Kudo A, Isshiki M, Sawada H, Segawa A. Quantitative Comparison of Anti-Fading Mounting Media for Confocal Laser Scanning Microscopy. J Histochem Cytochem 2001; 49(3):305-312.

(2) Diaspro A, Federici F, Robello M. Influence of refractive-index mismatch in high-resolution three-dimensional confocal microscopy. Appl Opt 2002; 41(4):685-690.