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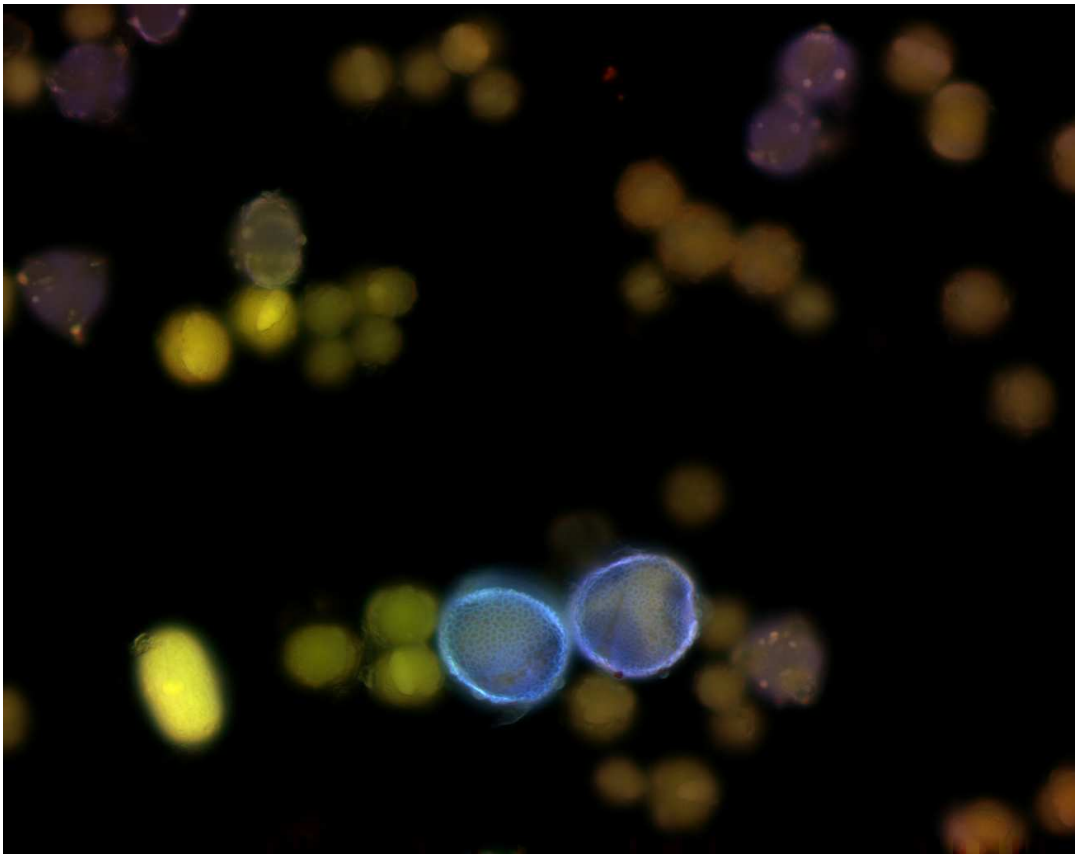
Autofluorescence: Causes and Cures



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1 INTRODUCTION

This document is a summary of information from the Confocal listserver archives, Cytometry Archives and the Histonet archives. Particular mention should go to Dr John A. Kiernan (London, ON, Canada) from the Histonet archives and Dr Martin Wessendorf (Univ. Minnesota, USA) from the Confocal Archives. A more detailed review of this subject has been published by Billington and Knight (Billinton and Knight 2001).

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What we most often mean by "autofluorescence" is "natural fluorescence" or "fixative-induced fluorescence". The emission spectra of natural fluorescence and fixation-induced fluorescence is very broad compared to the spectra of the dyes, probes and proteins we are interested in, making it difficult to separated wanted from unwanted fluorescence by traditional filtering methods (although linear un-mixing may help address this issue).

The best ways to address the issue of autofluorescence in order of preference:

1. Avoid it
   - not always possible.
2. Try to filter it out during image acquisition
   - difficult due to the broad emission spectrum.
3. Chemically remove it
   - can also reduce "real" signal.

2 NATURAL FLUORESCENCE

Natural fluorescence (as seen in living tissue cultures, for example) is due in large part to substances like flavins and porphyrins and (in plants) chlorophyll. These compounds are generally extracted by solvents and aren't much of a problem in fixed, dehydrated sections. They may persist (and be redistributed) in frozen sections that have passed through various aqueous reagents. I don't know of any published study, but there could well be several. There's nearly always some "background" fluorescence (for unknown reasons) in anything, especially with a broad-band blue excitation. Narrow-band filters and lower wattage (50 rather than 200) mercury lamps reduce this. If it isn't too bright, the background can be helpful in seeing where you are in the section. Published fluorescence micrographs are always printed so that the background is black and only the significant fluorescence is visible. Lipofuscin is a native autofluorescent material that persists even in paraffin sections. It can be especially annoying in certain large neurons in the CNS. (*JAK – Histonet Archive*). [LINK].

2.1 Lipofuscins

Brown in colour. Stains red in colour with Sudan Black and is PAS-positive [LINK]. Fluorescence properties: Excitation: UV-blue; Emission: green-yellow-orange.

Prominent in neurons, glial cells cardiac muscle cells but found in a wide range of cell types. Predominately post-mitotic cells. Lipofuschin has an enigmatic chemistry and stains positive for proteins, carbohydrates and lipids (!) (Billinton and Knight 2001).

Lipofuscin is the breakdown product of old red blood cells – an "aging" pigment. It usually occurs as small, punctate intracellular structures that are strongly fluorescent under any excitation ranging from 360nm to 647 nm. The colour should appear orange under UV excitation, green or yellow under blue excitation, or red under green excitation. Lipofuscin, should be less prevalent in younger (e.g., early adult) animals. It may be quenched with either $CuSO_4$ or Sudan Black (Schnell *et al.* 1999). (*MW – Confocal Archive*). [LINK].

2.1.1 Protocols for reducing Lipofuscin fluorescence

2.1.1.1 Picric Acid fix

Fix in a phosphate buffered mix of 2% formaldehyde and 0.2% picric acid.

Clear the fixed tissue with a graded series of ethanol through to DMSO or xylene and then back to buffer, you often seem to extract quite a lot of the background fluorescence without losing any of the specific fluorescence. (*Ian Gibbins – Flinders University, Australia. Conf. Arch.*) [LINK]

2.1.1.2 Sudan Black treatment

1. 0.3% Sudan Black (w/v) in 70% EtOH (v/v) stirred in the dark for 2 hours
2. Apply to slide for 10 minutes after the secondary antibody application.
3. Rinse quickly with PBS 8 times and mount
4. For FITC and Alexa 594 this does not reduce the emission signal noticeably. [LINK]

Also see). (Romijn *et al.* 1999).

2.2 Elastin and Collagen

Excitation: UV-green; Emission: green-yellow.

Typically from blood vessel walls. Elastin contains several fluorophores, one of which is a cross-linking tricarboxylic amino acid with a pyridinium ring (Deyl *et al.* 1980). This is a similar fluorophore to that found in collagen.

Nearly all vessels on the arterial side of the circulation have an internal elastic lamina that lies between the endothelium and the innermost layer of smooth muscle cells. Bigger vessels also have an external elastic lamina lying just outside the outermost smooth muscle cells, although it is usually much thinner than the internal lamina. The largest vessels have a series of layers of elastin between the smooth muscle layers. Even smaller "muscular" arteries often have strands of elastin between the muscle cells. Elastin is intensely fluorescent over a range of excitation wavelengths.

Most of the extracellular material between the smooth muscle and in the adventitia layer is collagen which is also autofluorescent. In smaller vessels, it's usually not a problem but in larger ones, there can be significant fluorescence from bundles of collagen fibres. (*Ian Gibbins, Flinders University, Conf. Arch)* [LINK].

2.2.1 Protocol for reducing Elastin fluorescence

If autofluorescence is coming from the elastin fibres, try incubating your labelling sections in 0.5% Pontamine sky blue 5BX (C.I. 24400, BDH Laboratories, Gurr) in buffer or saline for 10 minutes followed by a 5 minute wash before mounting. It appears that this shifts the usual green autofluorescence emission of elastin into a red emission. This works well in lung tissue using fluorescein labelled probes but not so well for rhodamine or Texas Red labelled preparations. (*Paul Rigby. U of West. Australia. Conf. Arch.*) [LINK].

Cowen *et al.* (Cowen *et al.* 1985) used a dye called Pontamine Sky Blue (also know as C.I. Direct Blue 1 and 6,6'-[(3,3'-dimethoxy[1,1'-biphenyl]-4,4'-diyl)bis(azo)]bis[4-amino-5-hydroxy-1,3-Naphthalenedisulfonic acid], tetrasodium salt) as a selective quencher of autofluorescence in mesenteric vessels and carotid arteries. The dye is available through BDH or was at least when the authors did the work. (*Ian Clements, Molecular Probes. Conf. Arch.)* [LINK]

Pontamine Sky Blue fluoresces red, presumably working by energy transfer. This also limits its usefulness. [LINK].

Another suggestion is the use of 0.1% toluidine blue for 1-5 minutes [LINK] although this may not work for all vessels [LINK].

3 FIXATIVE-INDUCED FLUORESCENCE

Using a long-pass green emission filter GFP, FITC, Alexa488 and even YFP appear *bright green – not yellow*. With a long-pass green filter fixative-induced fluorescence is dull, muddy-yellow. If it's dull, muddy yellow, it's not what you're interested in. You can tell if you have a long pass or band pass emission filter by turning on the halogen brightfield lamp your green filter in place. If the field of view is green then it's a bandpass filter; if it looks a warm- yellowy white, then you have a long-pass filter (blue has been filtered from the light).

Aldehyde fixatives react with amines and proteins to generate fluorescent products. Glutaraldehyde is worse than formaldehyde. The simplest way to stop aldehyde-induced fluorescence is to use a fixative that does not contain an aldehyde. Carnoy, Clarke and methacarn are examples, but are used only for subsequent paraffin sectioning.

Glutaraldehyde exists as low polymers. When it reacts with and cross-links protein molecules, lots of free aldehyde groups remain. These tissue-bound free aldehyde groups will combine covalently with any amino group offered to them, including terminal and side-chain (lysine) amino groups of proteins being used as histochemical reagents - that means all antibodies, all lectins and all enzymes. Your valuable and highly specific monoclonal primary antibody may bind at sites that contain basic proteins but not the antigen you're after.

The answer to the aldehyde problem is aldehyde blocking. This is done by reducing the -CHO groups to -OH with sodium borohydride or by feeding them bland amino groups (e.g. glycine/lysine).

Aldehyde-induced fluorescence: due to reaction of aldehydes with tissue components. Worse with glutaraldehyde than formaldehyde; worse when fixation is longer or warmer. Fairly uniform, non-punctate distribution across tissue, but may be brighter in some cells than others depending upon the presence of biogenic amines. (*JAK – Histonet Archive*). [LINK].

3.1 Protocols for reducing fixative-induced fluorescence

3.1.1 Sodium Borohydride

The use of these reagents is particularly suited to reduce the reversible Schiff's bases that are formed by the aldehyde-NH2 reaction and lead to autofluorescence, especially when using glutaraldehyde. If you can use paraformaldehyde for fixation, the reduction step is often unnecessary and autofluorescence is low. ***This material has a high potential for explosion and is very caustic.***

The protocol was prepared by Jennifer Kramer and a similar procedure is described by Beisker, *et al.* (Beisker *et al.* 1987).

1. Immediately before use, make up a 1 mg/ml solution of sodium borohydride in a physiological buffer such as PBS. The solution will be fizzy like carbonated water. Preparing this solution on ice and performing all subsequent incubations on ice has also been recommended.
2. Apply this solution immediately (while fizzing) to cells or tissue sections.

For glutaraldehyde fixed cell monolayers incubate in the sodium borohydride solution for 4 minutes. Replace with fresh sodium borohydride solution for another 4 minutes.

For paraformaldehyde fixed paraffin embedded 7 µm sections incubate 3 times, 10 minutes each in sodium borohydride solution.

For thicker tissue sections, more changes of sodium borohydride solution and/ or longer periods of incubation might be necessary.

Rinse many times with physiological saline to remove traces of sodium borohydride.

Continue with blocking steps at this point. Discard any leftover sodium borohydride solution as it loses its reactivity with time.

Ian Clements (Molecular Probes) from the Confocal Archive [LINK]

An alternative is cyanoborohydride. Does the same thing but is gentler, but generates cyanide as a by product. *Dr Mario M. Moronne (NanoMed Technologies) from the Confocal Archives.* [LINK] [LINK].

3.1.2 Trypan Blue treatment

Evan's Blue and trypan blue were used in the earlier days of direct immuno-fluorescence and the study of auto-antibodies. Each was used on tissues/cells at 0.001% to 0.1% in water or buffer. They are soluble in aqueous mounting media and diffuse out quickly. It reduces background significantly; both unwanted fluorescence due to the conjugate and native fluorescence due to structural elements in the tissues/cells. The dyes fluoresce red, abolishes the unwanted conjugate staining by energy transfer but has very little effect on desired specific staining. Therefore this technique may not be suitable for multi-label experiments. *Bob Weimer (http://www.antibodies-probes.com)* [LINK]

1. Trypan Blue 250ug/ml, pH 4.4
2. Dye solution is removed after 1 minute
3. Wash in buffer quickly and mount in aqueous/glycerol mounting media. [LINK]

(Mosiman *et al.* 1997;Wan *et al.* 1993)

3.1.3 Avoiding Aldehyde fixatives

One way to reduce aldehyde related fluorescence is to avoid or use low concentrations of aldehyde (Tokumasu and Dvorak 2003).This protocol uses the diimidoester cross-linking agent dimethyl suberimidate (with low PFA) to minimise the aldehyde-amine reaction that underlies fixation-induced autofluorescence.

1. Fix with 50 mM dimethyl suberimidate (DMS) (Sigma) in a buffer containing 100 mM sodium borate buffer (pH 9.5) and 1 mM $MgCl_2$ for 1 h
2. Wash and add 2% paraformaldehyde (PFA) (Electron Microscopy Science, Fort Washington, PA, U.S.A.) in phosphate-buffered saline (PBS), pH 7.4 for 15-30 minutes.
3. Quench fixation with 0.1 M glycine in PBS, pH 7.4, for 1 h at room temperature (RT).

3.1.4 Pre-bleach treatment

A light-box containing four fluorescent-light tubes, each with different emission peaks (UV to 633 nm) was constructed to bleach the aldehyde-induced fluorescence. The pre-bleach treatment lasted for 12-48 hrs prior to labelling with fluorophores. Specimens may require cooling if the antigens are heat labile (Neumann and Gabel 2002).

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