Long Wavelength Fluorophores Enhance Resolution in Autofluorescent Tissues

David M. Pier, Derek S. Dauphin and Brendon S. Noble
School of Science, Technology and Health, University Campus Suffolk, Ipswich, UK

ABSTRACT
Autofluorescence typical of biological molecules limits the detection of common fluorophores such as DAPI, FITC and rhodamine. Using bone and cartilage as sample tissues we demonstrate that autofluorescent emission spectra overlap that of short wavelength fluorophores. At wavelengths above 650nm autofluorescence emission intensity from both tissues is dramatically reduced, becoming undetectable in conditions that saturate the camera below 450nm thus enhancing the resolution of epifluorescent imaging. Whilst long-wavelength fluorophores have been described for specialized imaging techniques, this study highlights the benefits of far-red fluorophores that are commonly available, and are compatible with most modern epifluorescent microscopes and routine staining protocols.

KEYWORDS
Immunohistochemistry, immunocytochemistry, fluorescence, autofluorescence, far-red, cartilage

ACKNOWLEDGEMENTS
This work is supported by Geron Corporation, The East of England Development Agency and Suffolk County Council. The authors would like to thank Prof. Tim Arnett, University College London, for the gift of bone discs.

AUTHOR DETAILS
David M. Pier, School of Science, Technology and Health, University Campus Suffolk, Waterfront Building, 19 Neptune Quay, Ipswich, IP4 1QJ, UK
Tel: +44 (0) 1473 338672
Email: drdavepier@gmail.com
Website: www.ucs.ac.uk/biotech

INTRODUCTION
Fluorescent immunolabelling techniques are important and widely used techniques to localise biological molecules within tissue and cell samples. The familiarity of researchers with early fluorescein and rhodamine dyes emitting in the visible range has led to the use of these wavelengths often with little consideration of the sample’s innate emission properties [1]. Detection of such fluorophores is limited in tissues displaying high natural fluorescence or ‘autofluorescence’ which can limit detection of the signal of interest, reducing contrast and clarity of molecule detection [2]. A variety of methods have been employed to reduce the deleterious effects of autofluorescence including a number of chemical treatments such as borohydride treatment [3]. However, these treatments can reduce the intensity of immunofluorescent labelling leading to compromises between fluorophore detection and background autofluorescence. Furthermore, some chemical treatments such as Sudan Black B must be applied in solvents that can disrupt the fine structure within cells. Photobleaching has also been suggested as a means of reducing autofluorescence [4]. But this technique is not suitable for imaging live cells and may cause problems with unstable materials. Non-fluorescent, enzyme-linked methods of antibody detection benefit from large signal amplification but suffer from relatively diffuse staining, making them inappropriate for detailed intracellular work.

Fortunately, the excitation and emission wavelengths of common autofluorescent biological molecules is largely restricted to the UV and visible regions of the electromagnetic spectrum [5]. There is little detectable endogenous signal from biological tissues at long wavelengths, such as those in the far-red and infrared end of the spectrum. This property has been exploited in deep tissue imaging [6] but has not yet been described as a way of limiting contaminating autofluorescence in routine microscopy. Here we demonstrate increased resolution of epifluorescent imaging when using far-red fluorophores.

MATERIALS AND METHODS
Cell Culture and Immunofluorescent Staining
Undifferentiated hES-MP cells were cultured on bovine bone discs in DMEM, 10% FBS and 1% L-glutamine (Invitrogen). Cells were maintained in a humidified atmosphere with 5% CO₂ at 37°C. After 24 hours the cultures were fixed with 4% formaldehyde. Vinculin was detected with mouse anti-vinculin (Sigma) and visualized indirectly with a secondary anti-mouse antibody conjugated to Alexa Fluor 350, 488, 568 or 647 (Molecular Probes). Cells were mounted in an antifade reagent (Dako).

Fluorescence Microscopy
Images were acquired using a Carl Zeiss Axio Observer Z1 light microscope equipped with an HXP 120C metal halide light source and an AxioCam MRm cooled monochrome camera used with filter sets 10, 45, 49 and 50.

Absorption and Emission Spectroscopy
Sample absorption and emission spectrum were generated from cryosectioned human cartilage tissue or bovine bone discs using a Horiba Scientific SPEX Fluoromax-3 fluorometer equipped with fibre optic sample illumination. Excitation and emission slits set at 5 nm and data acquired using Fmax3 software.

Image Analysis
Nine identical fields of view were imaged with filter sets and exposure times appropriate for the fluorophore of choice. The intensity of each pixel was measured using Image J software [7] and the standard deviation calculated. Significance was analysed using one-way ANOVA with a Turkey post-hoc test.

RESULTS
Collagen molecules exhibit strong autofluorescence [5] and tissues rich in these proteins can exhibit large autofluorescent signals. For this reason we chose tissues containing high levels of collagen (bone and cartilage) as model tissues to examine autofluorescence. Indeed the autofluorescent properties of articular cartilage is so prominent it has been used as a method for examining the structure (and implied health) of the tissue [8].

To determine the excitation wavelengths that would not elicit a significant autofluorescence emission, we measured the emission spectrum of the highly autofluorescent tissue cartilage (Figure 1a). Excitation of untreated cartilage tissue with wavelengths throughout the visible spectrum produced a broad emission profile. Emission intensities were reduced at longer wavelengths, with intensities in the far-red defined to less than 3% of peak signal intensity in the visible spectrum. A similar effect was seen in bone tissue (data not shown). Overlaying normalized emission spectra from antibody-conjugated fluorophores with that of cartilage tissue further illustrates the decreased contribution of autofluorescence to overall signal at long wavelengths relative to visible wavelengths (Figure 1b).

Immunostaining of cartilage tissue sections
with collagen type II was used in an attempt to directly compare the autofluorescent emission intensities of cartilage with quantities of antibody-conjugated fluorophores relevant to immunohistochemistry. Collagen type II contributes >50% of total protein in articular cartilage providing an abundant source of antigen for antibody binding and a correspondingly large number of bound fluorophores per tissue unit area. However, the immunostained tissue produced emission spectra characteristic only of autofluorescence. Contributions to the emission spectra from the Alexa Fluor molecules could not be isolated by the fluorometer at any wavelength (data not shown). This is likely a limitation of instrument sensitivity but demonstrates the significance of the autofluorescent signal in relation to that of the antibody conjugates.

To investigate the effects of excitation wavelength on tissue observed using widefield epifluorescent microscopy we examined the autofluorescence of untreated articular cartilage and subchondral bone sections (Figure 2a). An exposure time of 100 ms was used as this is typical of the exposure times needed for routine immunohistochemistry in our hands. Short wavelength filter sets in the visible spectrum gave a strong autofluorescent signal clearly delineating the tissue, a signal that decreased with increasing wavelength. The use of far-red filter sets prevented detection of autofluorescence over background.

Vinculin staining of undifferentiated hESC-MP cells cultured on bone discs was used to highlight the effect of autofluorescence on image clarity and resolution. Cells stained with antibodies conjugated to Alexa Fluor 647 could be imaged with minimal contribution from substrate autofluorescence. In contrast the use of fluorophores emitting shorter wavelengths in the visible spectrum reduced the signal-to-noise ratio and significantly (p < 0.01, n = 9) decreased the dynamic range of the images such that resolution was lost (Figure 2b).

**DISCUSSION**

Using bone and cartilage as model autofluorescent tissues we have demonstrated a dramatic reduction in detectable autofluorescence when replacing the excitation light in the visible spectrum with that of light in the far-red region. Furthermore, utilization of long-wavelength fluorophores significantly improved antibody detection in autofluorescent tissues. The use of far-red wavelengths increased the signal to noise ratio and increased the dynamic range of images, improving image resolution. This ratiometric method of analysis reduced the influence of inherent differences in fluorophore emission intensities, efficiencies of filter sets and camera sensitivity to different wavelengths.

In this study we have worked with cells seeded onto bone discs as a model system, however the results and techniques are also relevant to tissue sections. Whilst cartilage and bone have been used as model tissues in this study the benefits of far-red fluorophore detection are predicted to be beneficial in other autofluorescent tissues such as the brain.

We chose to investigate the use of Alexa Fluor 647 (comparable to Cy5) as the emissions of this fluorophore can be imaged on standard microscopy systems without specialized equipment. The majority of fluorescent microscopes can be retrofitted with bandpass filters spe-
Far-red fluorophores allow improved resolution of antibody detection in autofluorescent cultures.

(A) Untreated cryosections of articular cartilage and subchondral bone were illuminated with light passed through blue (excitation 365 nm; emission 445-450), green (excitation 450-490; emission: 515-565), red (excitation 560-540; emission 630-675) and far-red filters (excitation 640-630; emission 690-650). Exposure time was limited to 100 ms. Scale bars = 200 µm.

(B) Vinculin staining of hES-MP cells seeded onto bone discs and detected with different fluorophores. Exposure times optimized for each fluorophore independently. Scale bars = 20 µm

(C) Standard deviation of pixel intensity calculated for each fluorophore shows a significant increase in Alexa Fluor 647 relative to all other fluorophores (*** p < 0.01, n = 9).

**CONCLUSIONS**

In summary, use of far-red fluorophores in fluorescence microscopy techniques significantly reduces the effect of sample autofluorescence on image quality when compared to visible wavelength fluorophores. The use of the fluorophores detailed here is within the capability of most current microscopy systems, and requires little adaptation of immunohistochemistry protocols currently in use.

**REFERENCES**


©2011 John Wiley & Sons, Ltd