Abstract

Background: Immunofluorescence (IF) is one of the most important techniques where fluorochromes conjugated to antibodies are used to detect specific proteins or antigens. In tissue sections, autofluorescence (AF) can lead to poor quality images that impair assessment. The placenta is a pivotal extra-embryonic organ in embryo development, where trophoblasts make up a large proportion of the cells. Teratoma formation is one of the critical assays for pluripotent stem cells.

Methods: We tested whether ultraviolet (UV), ammonia (NH3), copper (II) sulfate (CuSO4), Trypan Blue (TB), Sudan Black B (SB), TrueBlack™ Lipofuscin Autofluorescence Quencher (TLAQ) and combinations of these treatments could reduce AF in paraffin and frozen sections of placenta and teratoma in FITC, Texas Red and Cy5.5 channels.

Results: We found that UV, NH3, TB and CuSO4 quenched AF to some extent in different tissue and filters, but increased AF in Texas Red or Cy5.5 channels in some cases. SB and TLAQ exhibited the most consistent effects on decreasing AF, though TLAQ reduced the overall IF signal in placenta sections. Not all combined treatments further reduced AF in both placenta and teratoma sections.

Conclusions: SB and TLAQ can effectively quench AF in placenta and teratoma IF.
Introduction

Immunofluorescence (IF) is an important assay used in stem cell research. By incubating cells or tissues with fluorescence-conjugated antibodies, the identity of cells and their descendants to tissues and organs can be identified and quantified by fluorescence microscopy or flow cytometry (Manos et al., 2011; Stack et al., 2014). Though this method is widely used and well developed, so far, autofluorescence (AF) can present a serious issue to obtain clear and conclusive data for some tissue samples (Abuduwali et al., 2013; Yang & Honaramooz, 2012). AF is the natural and fixative induced fluorescence occurring in cells and tissues with a wide spectrum between 450nm and 650nm, which overlaps with the commonly used fluorophores in IF (Monici, 2005; Viegas et al., 2007). Many factors are known to contribute to AF, such as sample fixation, tissue components, lipofuscin, elastin and collagen (Banerjee et al., 1999; Del Castillo et al., 1989; Monici, 2005). Various protocols have been developed to tackle this issue in order to improve imaging quality (Baschong et al., 2001; Davis et al., 2014; Viegas et al., 2007).

The placenta is a vital extra-embryonic organ that serves as the interface between foetal and maternal environments to exchange gas, nutrients and carry away wastes. It also secretes related hormones and growth factors, and provides immune-protection for the foetus. The placenta has various types of cells, including trophoblasts, vascular endothelial cells and blood cells; among which, trophoblasts are the structural and functional components of the placenta, and are the most important cell type. Trophoblasts are the progeny of cells in the trophectoderm. Defects in maternal and foetal components and trophoblasts in the placenta affect its functions and can cause severe pregnancy complications (Maltepe & Fisher, 2015; Rossant & Cross, 2001).

One functional assay used to demonstrate the differentiation potential of human pluripotent stem cells is teratoma formation. When pluripotent stem cells are implanted at ectopic sites in immunocompromised mice, for example skin or kidney capsule, the cells differentiate into various cell types (Cunningham et al., 2012; Lensch et al., 2007; Mullet et al., 2010).

Both placenta and teratoma are important tissues for stem cell and developmental biology. In this study, we employed both physical and chemical approaches to test their effects on quenching AF in placenta and teratoma IF assays.

Methods

Animal ethics statement

All experimental procedures were carried out in accordance to Home Office UK regulations and the Animals (Scientific Procedures) Act 1986 (licence No. 70/8387 and 80/2597). All experimental protocols were approved by the Animal Welfare and Ethical Review Body (AWERB) of the Wellcome Trust Sanger Institute and Wellcome Trust-MRC Cambridge Stem Cell Institute, the University of Cambridge. At the end of the study, mice were euthanized by cervical dislocation, in accordance with stated Home Office UK regulations.

Mice

8-week old pregnant female C57BL/6J, weighed 29–35g and male NSG mice (NOD.Cg-Prkdcscid Il2rgtm1 Wjl/SzJ), weighed 25–28g. All mice were from the Jackson Laboratory. The mice were housed in an environmentally controlled room at 21°C under 7.30AM-7.30PM light/dark cycle. The mice were fed an autoclavable diet (Lab diet -5021); the water was mains supply-chlorinated and filtered to 0.2 microns.

Human embryonic stem cells (hESCs)

H1 human embryonic stem cells (WA01) were imported from WiCell Research Institute. The import and use of human ESCs were approved by The Steering Committee for the UK Stem Cell Bank (Approval number SCSC17-01) and monitored by the human materials and data management committee (HMDMC) of Wellcome Trust Sanger Institute.

Reagents

Ammonia solution (Sigma, 392685); Copper (II) sulfate (Sigma, C1297); Sudan Black B (Sigma, 199664); Trypan Blue (Sigma, T6146); TrueBlack lipofusin autofluorescence quencher (Biotium, 23007); O.C.T compound (VWR International, 361603E); Bovine serum albumin (Sigma, A2153); Donkey serum (Sigma, D9663); Prolong Gold antifade reagent (Life Technologies, P36930); Rabbit anti-AP-2γ (also named as Tfap2c) antibody (Santa Cruz, sc-8977, Antibody ID AB_2286995) used at 1:50 dilution; Goat anti-cytokeratin 7 antibody (Santa Cruz, sc-17116, Antibody ID AB_2134582) used at 1:50 dilution; AlexaFluor 488 donkey anti-rabbit IgG (Abcam, ab15006, Antibody ID AB_2299617) used at 1:500 dilution; AlexaFluor 488 donkey anti-goat IgG (ThermoFisher Scientific, A11055, Antibody ID AB_2534102) used at 1:500 dilution.

Teratoma formation

NSG mice were anesthetised by inhalation with Isoflurane, shaved and cleaned. Analgesia (Carprieve) 0.1ml was administrated by sub-cutaneous injection. A small incision was made on the left flank of the mouse and the kidney was exposed. An injection needle was used to gently pinch the capsule membrane. 1x10⁶ H1 hESCs were transferred into the kidney capsule using a fine glass pipette by mouth pipetting. The kidney was placed back into the cavity and the peritoneum and muscular wall was sutured and the skin stapled. Approximately 8 weeks later, a teratoma grew at the injection site. When the teratoma reached 1.2 mm², the mice were euthanized; kidney and teratomas were dissected, fixed overnight in 10% neutral buffered formalin and embedded in paraffin before sectioning.

The same procedure was applied for the sub-cutaneous teratoma formation assay. 5x10⁶ H1 hESCs were injected subcutaneously into both dorsal flanks of NSG mice. When the size of the teratomas reached 1.2 mm², the mice were euthanized and the teratomas were dissected, fixed overnight in 4% PFA and embedded in O.C.T compound before frozen sectioning.

Tissue section

14.5 days post coitum (d.p.c) mouse embryos placentas (n=6) were dissected from 1 female C57BL/6J mouse; teratomas (n=5) and...
kidneys (n=5) were dissected from 5 male NSG mice. Samples were fixed in 4% PFA and embedded in paraffin. Tissue sections of 5 µm were cut and mounted onto Superfrost Plus glass slides (VWR International, 631-0108). To remove the paraffin, slides were immersed in xylene 2 times, for 3 minutes each, rehydrated with graded ethanol, 100%, 95%, 80%, 70% and 50%, for 3 minutes each, and transferred to tap water.

10.5 d.p.c placentas (n=4) were dissected from 1 female C57BL6/J mouse; teratomas (n=4) from sub-cutaneous injected H1 hESCs were dissected from 2 male NSG mice. Samples were fixed in 4% PFA for 30 minutes, washed with PBS, 3 times, for 15 minutes each, and then placed in 30% sucrose (dissolved in PBS) at 4°C overnight for cryoprotection. For frozen sections, placentas and teratomas were placed in O.C.T. compound in a plastic mould, and frozen on dry ice. 6 µm sections were cut and mounted onto Superfrost Plus glass slides. O.C.T. was removed by washing slides in PBS, 3 times, for 5 minutes each.

Immunofluorescence
For paraffin-embedded sections, antigens were retrieved in sodium citrate buffer (10 mM sodium citrate, 0.05% Tween-20, pH 6.0) by microwave for 15 minutes, followed by rinsing with tap water. The slides (both paraffin embedded sections and frozen sections) were then washed in PBS containing 0.25% Triton X-100 (PBST) twice for 5 minutes each, blocked in PBST/1% BSA/5% donkey serum for at least 30 minutes, then incubated in PBS containing 0.25% donkey serum overnight. The slides were then washed with PBS 3 times for 5 minutes each, blocked in PBST/1% BSA/5% donkey serum for at least 15 minutes, and incubated with AlexaFluor® conjugated secondary antibody for 1 hour at room temperature, then washed with PBST 3 times for 5 minutes each. The sections were mounted in a small drop of Prolong Gold® antifade mountant with DAPI, covered with a coverslip and sealed with nail varnish.

Table 1. Summary of treatments for immunofluorescence.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Abbreviation and Chemical Formula</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ultraviolet (UV)</td>
<td>UV</td>
</tr>
<tr>
<td>Ammonia</td>
<td>NH3</td>
</tr>
<tr>
<td>Copper (II) Sulfate</td>
<td>CuSO4</td>
</tr>
<tr>
<td>After immunofluorescence, Copper (II) Sulfate</td>
<td>IF/CuSO4</td>
</tr>
<tr>
<td>Trypan Blue (TB)</td>
<td>TB</td>
</tr>
<tr>
<td>Sudan Black B (SB)</td>
<td>SB</td>
</tr>
<tr>
<td>TrueBlack lipofusin autofluorescence quencher</td>
<td>TLAQ</td>
</tr>
<tr>
<td>Ammonia, Sudan black B</td>
<td>NH3/SB</td>
</tr>
<tr>
<td>Trypan blue, Sudan black B</td>
<td>TB/SB</td>
</tr>
<tr>
<td>Copper (II) Sulfate, Sudan black B</td>
<td>CuSO4/SB</td>
</tr>
<tr>
<td>Sudan black B, Copper (II) Sulfate</td>
<td>SB/CuSO4</td>
</tr>
<tr>
<td>Sudan black B, autofluorescence quencher</td>
<td>SB/TLAQ</td>
</tr>
</tbody>
</table>

Copper (II) Sulfate (CuSO4): Before or after IF, sections were treated with CuSO4 5 mM in 50 mM ammonia acetate for 90 minutes, then washed with PBST 3 times, for 15 minutes each, then mounted for imaging and analysis.

Combined treatment: Different combinations of chemical treatment are listed in Table 1.

Digital imaging evaluation. The sections were examined under a Leica DMS5000B microscope equipped with narrow bandpass filters for FITC (488nm), Texas Red (594nm), Cy5.5 (630nm) and DAPI (405nm) fluorescence. Images were captured via a monochrome digital camera (ORCA-03G, Hamamatsu) and processed with the SmartCapture software (Digital Scientific UK). The same settings for acquisition mode and exposure time were applied to all samples that had undergone different treatments.

The intensity of AF was graded as reported before with modifications (Davis et al., 2014): -1 (increase), 0 (no visible difference), 1 (general reduction, but increase in blood cells or basement membrane), 2 (reduction, but also the antibody signal), 3 (reduction), 4 (marked reduction).

Results
Paraffin sections of 14.5 d.p.c placenta
Mouse 14.5 d.p.c placenta sections were treated with UV, NH3, SB, TLAQ or NH3/SB. The sections were incubated with the primary antibody against a trophoblast specific marker Tfap2c (Kuckenberg et al., 2010), detected with AlexFluo488 conjugated secondary antibody. The fluorescence signals at 488 nm (FITC),
594 nm (Texas Red) and 630 nm (Cy5.5) were evaluated and images were captured with the same setting. The mouse placenta organizes in three layers, i.e. labyrinth, junctional zone, and decidua (Favaron et al., 2013). We mainly analysed AF in labyrinth and junctional zone. Results were summarised in Table 2.

UV: Compared with untreated control, UV treatment (in FITC channel) reduced AF in junctional zone, but no visible changes were observed in labyrinth. In Texas Red channel, both junctional zone and labyrinth showed reduced AF. In Cy5.5 channel, there was no difference in junctional zone, but AF was reduced in the labyrinth, although it was enhanced in blood cells (Figure 1A and B).

NH3: In FITC channel, AF was reduced in junctional zone, but no visible difference was seen in the labyrinth. In Texas Red channel, AF was reduced in both junctional zone and labyrinth. In Cy5.5 channel, there was marked reduction in junctional zone AF; the same occurred in the labyrinth, but here AF was enhanced in blood cells (Figure 1D).

SB: In FITC channel, AF was reduced in both junctional zone and labyrinth. In Texas Red channel, there was marked reduction in junctional zone AF and in labyrinth there was also a reduction in AF, but strong AF was seen in blood cells. In Cy5.5 channel, there was a reduction in AF in junctional zone, but higher AF was seen in blood cells in labyrinth (Figure 1D).

Sequential treatment with NH3 and SB: In FITC channel, AF was reduced both in junctional zone and in labyrinth. In Texas Red channel, there was marked reduction in junctional zone AF and in labyrinth there was also a reduction in AF, but strong AF was seen in blood cells. In Cy5.5 channel, there was a reduction in AF in junctional zone, but higher AF was seen in blood cells (Figure 1D).

TLAQ: In FITC channel, AF was markedly reduced, but the Tfap2c specific signals were also affected in both junctional zone and labyrinth. In Texas Red channel, both junctional zone and labyrinth showed reduced AF. In Cy5.5 channel, there was a reduction in AF in junctional zone and labyrinth, but strong AF was seen in blood cells (Figure 1F).

Paraffin sections of kidney and teratoma

Next, we applied the above mentioned UV, NH3, SB, TLAQ and NH3/SB treatments to teratoma sections of hESCs, together with TB, CuSO4 and their combination with SB. The results were listed in Table 3. As the hESCs were injected into the kidney capsule, AF in the kidney background was analysed as well. Cytokeratin 7 was used to detect epithelia components in teratoma (Chu et al., 2000), and the signal was picked up using an AlexaFluor 488 conjugated secondary antibody.

UV: Unlike the placenta, no visible difference was found in AF between treated and control tissues in both kidney and teratoma, in all three channels (Figures 2A and B, Figures 3A and B).

NH3: Both tissues showed reduced AF in FITC channel, but no reduction in Texas Red channel. In Cy5.5 channel reduced AF was observed only in kidney (Figure 2C, Figure 3C).

SB: In FITC channel, AF was reduced in both kidney and teratoma. In Texas Red channel, AF was reduced in kidney; but in teratoma, blood cells and basement membranes showed enhanced AF with decreased background. In Cy5.5 channel, AF was generally reduced in both kidney and teratoma, but increased in blood cells and basement membranes (Figure 2D, Figure 3D).

TB: In FITC channel, AF was reduced in both kidney and teratoma. In Texas Red channel, kidney showed reduced AF, but in teratoma AF increased. In Cy5.5 channel, higher AF was observed in both kidney and teratoma (Figure 2E, Figure 3E).

CuSO4: When tissues were treated with CuSO4 after blocking, AF was reduced in both kidney and teratoma in FITC channel; in Texas Red channel, low AF was observed in teratoma, whereas in Cy5.5 channel, kidney showed low AF. When the sections were treated with CuSO4 after IF, AF was reduced in FITC channel.

![Table 2. 14.5 d.p.c placenta paraffin sections.](image)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FITC Junctional Zone</th>
<th>Labyrinth</th>
<th>Texas Red Junctional Zone</th>
<th>Labyrinth</th>
<th>Cy5.5 Junctional Zone</th>
<th>Labyrinth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>UV</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>NH3</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>4</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>SB</td>
<td>3</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>NH3/SB</td>
<td>3</td>
<td>3</td>
<td>4</td>
<td>1</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>TLAQ</td>
<td>2</td>
<td>2</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>
Figure 1. Immunofluorescence of 14 d.p.c mouse placenta paraffin sections. Sections were incubated with anti Tlpa2c antibody and AlexaFluor 488 2nd antibody with different treatments to reduce autofluorescence. Images were captured in FITC, Texas Red and Cy5.5 channels with the same settings and 2 seconds exposure time. Cell nucleus was stained with DAPI. (A) Untreated control (n=2); (B) UV (n=2); (C) NH3 (n=1); (D) SB (n=1); (E) NH3/SB (n=1); (F) TLQA (n=1). 1, 2, 3 Junctional zone of placenta; 4, 5, 6 Labyrinth. Arrow points to blood cells.
Table 3. Kidney and teratoma paraffin sections.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FITC Kidney</th>
<th>FITC Teratoma</th>
<th>Texas Red Kidney</th>
<th>Texas Red Teratoma</th>
<th>Cy5.5 Kidney</th>
<th>Cy5.5 Teratoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>UV</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>NH3</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>SB</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TB</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>CuSO4</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>iF/CuSO4</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TLAQ</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>NH3/SB</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>TB/SB</td>
<td>3</td>
<td>3</td>
<td>0</td>
<td>-1</td>
<td>-1</td>
<td>-1</td>
</tr>
<tr>
<td>CuSO4/SB</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>-1</td>
<td>0</td>
</tr>
<tr>
<td>SB/CuSO4</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>3</td>
<td>-1</td>
<td>0</td>
</tr>
<tr>
<td>SB/iF/CuSO4</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>-1</td>
<td>0</td>
</tr>
<tr>
<td>SB/TLAQ</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>

in both kidney and teratoma; but no difference was observed in Texas Red and Cy5.5 channels (Figure 2F and G, Figure 3F and G).

**TLAQ**: In FITC and Texas Red channels, both kidney and teratoma showed reduced AF. In Cy5.5 channel, AF was reduced only in kidney and no difference was observed in teratoma (Figure 2H, Figure 3H).

**Combined treatment. NH3/SB**: In FITC and Texas Red channels, there was reduced AF in kidney and teratoma. In Cy5.5 channel, AF was reduced in teratoma, and in kidney, there was reduced AF except in the basement membranes where AF was increased (Figure 2I, Figure 3I).

**TB/SB**: In FITC channel, AF was reduced in both kidney and teratoma. In Texas Red channel, no difference in kidney, but increased AF was detected in teratoma. In Cy5.5 channel, increased AF was observed in both kidney and teratoma (Figure 2J, Figure 3J).

**CuSO4/SB**: CuSO4 treatment followed by SB reduced AF in kidney and teratoma in FITC and Texas Red channels, but in Cy5.5 channel there was increased AF in kidney (Figure 2K, Figure 3K).

**SB/CuSO4**: SB treatment followed by CuSO4 reduced AF in kidney and teratoma in FITC and Texas Red channels, no difference was observed in Cy5.5 channel in teratoma, whereas in kidney, AF was increased (Figure 2L, Figure 3L).

**SB/iF/CuSO4**: SB treatment during IF, then CuSO4 was treated after IF can markedly reduce AF in FITC channel, with reduction in Texas Red channel as well. However, in Cy5.5 channel, there was increase of AF in kidney basement membranes with no difference in teratoma (Figure 2M, Figure 3M).

**SB/TLAQ**: Marked AF reduction was observed in both kidney and teratoma in all three channels (Figure 2N, Figure 3N).

**SB and TLAQ** showed consistent effects on all three tissues, so we tested these two reagents on frozen sections as well.

**Frozen sections of 10.5 d.p.c placenta**
Sections were stained with Tfap2c antibody and signals were detected in FITC. The results were summarized in Table 4.

**SB**: Compared with untreated control, AF was reduced in both junctional zone and labyrinth in FITC channel. In Texas Red channel, only labyrinth showed reduced AF. In Cy5.5 channel, general AF reduction was observed with increased AF in blood cells (Figure 4A and B).

**TLAQ**: AF was markedly reduced in both FITC and Texas Red channels. In Cy5.5 channel, AF was reduced in junctional zone and labyrinth, except in blood cells where AF was increased (Figure 4C).

**SB/TLAQ**: In FITC channel, AF was markedly reduced, but was accompanied by reduction in the Tfap2c signal as well. In Texas Red channel, both junctional zone and labyrinth showed reduced AF. In Cy5.5 channel, the junctional zone showed reduced AF, whereas in labyrinth general AF reduction was accompanied by increased AF in blood cells (Figure 4D).
Figure 2. Immunofluorescence of mouse kidney paraffin sections. Sections were incubated with anti CK7 antibody and AlexaFluor 488 2nd antibody with different treatments to reduce autofluorescence. Images were captured in FITC, Texas Red and Cy5.5 channels with the same settings and 2 seconds exposure time. Cell nucleus was stained with DAPI. No CK7+ cells were detected in kidney. (A) Untreated control (n=4); (B) UV (n=2); (C) NH3 (n=1); (D) SB (n=3); (E) TB (n=1); (F) CuSO4 (n=1); (G) IF/CuSO4 (n=1); (H) TLAQ (n=2); (I) NH3/SB (n=1); (J) TB/SB (n=1); (K) CuSO4/SB (n=3); (L) SB/CuSO4 (n=1); (M) SB/IF/CuSO4 (n=1); (N) SB/TLAQ (n=1). Arrow points to basement membranes.
Figure 3. Immunofluorescence of teratoma paraffin sections. Sections were incubated with anti CK7 antibody and AlexaFluor 488 2nd antibody with different treatments to reduce autofluorescence. Images were captured in FITC, Texas Red and Cy5.5 channels with the same settings and 2 seconds exposure time. Cell nucleus was stained with DAPI. (A) Untreated control (n=5); (B) UV (n=1); (C) NH3 (n=1); (D) SB (n=5); (E) TB (n=1); (F) CuSO4 (n=1); (G) IF/CuSO4 (n=1); (H) TLAQ (n=2); (I) NH3/SB (n=1); (J) TB/SB (n=1); (K) CuSO4/SB (n=1); (L) SB/CuSO4 (n=1); (M) SB/IF/CuSO4 (n=1); (N) SB/TLAQ (n=1).
Table 4. 10.5 d.p.c placenta frozen sections.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FITC Junctional Zone</th>
<th>FITC Labyrinth</th>
<th>Texas Red Junctional Zone</th>
<th>Texas Red Labyrinth</th>
<th>Cy5.5 Junctional Zone</th>
<th>Cy5.5 Labyrinth</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SB</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>TLQA</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>3</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>SB/TLQA</td>
<td>2</td>
<td>2</td>
<td>4</td>
<td>4</td>
<td>3</td>
<td>1</td>
</tr>
</tbody>
</table>

Figure 4. Immunofluorescence of 10.5 d.p.c mouse placenta frozen sections. Sections were incubated with anti Tfap2c antibody and AlexaFluor 488 2nd antibody with different treatments to reduce autofluorescence. Images were captured in FITC, Texas Red and Cy5.5 channels with the same settings and 2 seconds exposure time. Cell nucleus was stained with DAPI. (A) Untreated control (n=2); (B) SB (n=2); (C) TLQA (n=1); (D) SB/TLQA (n=1). 1, 2, 3 Junctional zone of placenta; 4, 5, 6 Labyrinth. Arrow points to blood cells.
Frozen sections of teratoma
Teratoma sections were incubated with the Cytokeratin 7 antibody and signals were observed in FITC channel. The results were summarized in Table 5.

SB: AF was reduced in FITC and Texas Red channels; but no difference from untreated control was observed in Cy5.5 channel (Figure 5A and B).

Figure 5. Immunofluorescence of teratoma paraffin sections. Sections were incubated with anti CK7 antibody and AlexaFluor 488 2nd antibody with different treatments to reduce autofluorescence. Images were captured in FITC, Texas Red and Cy5.5 channels with the same settings and 2 seconds exposure time. Cell nucleus was stained with DAPI. (A) Untreated control (n=2); (B) SB (n=2); (C) TLQA (n=2); (D) SB/TLQA (n=2).

Table 5. Teratoma frozen sections.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>FITC</th>
<th>Texas Red</th>
<th>Cy5.5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SB</td>
<td>3</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>TLQA</td>
<td>3</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>SB/TLQA</td>
<td>3</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>
**TLAQ:** Similar to SB-treated ones, reduced AF was observed in FITC and Texas Red channels but not in Cy5.5 channel (Figure 5C).

**SB/TLAQ:** No further AF reduction was observed compared to SB or TLAQ treatment alone in FITC and Texas Red channels. In Cy5.5 channel, no difference was observed either (Figure 5D).

**Discussion**

In this technical report, we presented analysis results of performing immunofluorescence to detect proteins in the placenta and teratoma sections. Due to the complexity of tissues and the process of tissue section preparation, AF from cells or basement membranes can affect antibody signals detected by fluorochromes (Abduwali et al., 2013; Banerjee et al., 1999; Baschong et al., 2001; Davis et al., 2014; Duong & Han, 2013; Viegas et al., 2007). Here, we analysed effects of UV and several chemicals, individually or in combinations, on AF in IF.

UV is a photo-bleaching method, which can change the structure of a fluorophore so that it loses its ability to fluoresce (Billinton & Knight, 2001). Quenching with UV has different effects on tissue AF; some authors reported obvious reduction, while others did not observe noticeable differences (Davis et al., 2014; Viegas et al., 2007). Similarly, in the placenta in the present study, UV treatment reduced AF in Texas Red channel in both junctional zone and labyrinth, but had no effects on kidney and teratoma sections in all three channels.

Previously, NH3, TB and CuSO4 were reported to reduce AF in some tissues (Baschong et al., 2001; Loike & Silverstein, 1983; Srivastava et al., 2011; Zhang et al., 2010). NH3 reacts with free formaldehyde to suppress AF in paraffin sections of formaldehyde-fixed, decalcified bone marrow, and may also dissolve negatively charged lipid derivatives, phenols or polyphenols, and hydrolyse weak esters (Baschong et al., 2001). TB can rapidly diffuse into cells after permeabilization, resulting in uniform distribution in the cytoplasm and nucleus, which results in reduced AF when TB molecules are at a proper distance and orientation to autofluorescence or nonspecifically bound to fluorescent molecules (Srivastava et al., 2011). The mechanism of Cu²⁺ in AF quenching is not clear. Since it’s an excellent electron scavenger, electrons from excited lipofuscin can be transferred to Cu²⁺, or Cu²⁺ and lipofuscin can form nonfluorescent complex (Schnell et al., 1999).

All three chemicals showed heterogeneous effects in the present study; in placenta, NH3 reduced AF mainly in junctional zone; in kidney, all three channels showed reduced AF; in teratoma, similar effects were achieved with no visible difference in Cy5.5 channel. In kidney, TB reduced AF in FITC and Texas Red channels, but increased AF in Cy5.5 channel, whereas in teratoma sections, AF increased in Texas Red and Cy5.5 channels, but not in FITC channel. The effect of CuSO4 depends on when it is applied during IF; we found that only CuSO4 treatment after IF can reduce AF on FITC in both kidney and teratoma.

SB absorbs at 580nm, so it may absorb a small amount of AF or directly interact with AF species (Davis et al., 2014). We found SB can reduce AF in all three tissues tested in both paraffin and frozen sections, but we detected some increased AF from blood cells and basement membranes in Texas Red and Cy5.5 channels, which is consistent with other reports showing AF was quenched most in FITC channel; in Texas Red channel, the effect is much less, which may be due to the specific excitation wavelength or interference of the high transmission value of this filter with SB treated surface (Erben et al., 2016; Viegas et al., 2007). TLAQ is a commercialized AF quencher, Chan et al. used it to reduce AF in human eye tissue section IF (Chan et al., 2015). In this study, we showed that TLAQ treatment resulted in a reduction in AF, similar to the level produced by SB treatment in all three tissues, but TLAQ treatment was less labouring than SB. However, in placenta, TLAQ treatment reduced the specific signals from the antibody as well.

We also analysed some combined chemical treatments. NH3/SB combination did not further reduce AF in placenta, but in kidney and teratoma, AF in FITC channel was markedly reduced compared to NH3 and SB alone. SB/IF/CuSO4 also further reduced AF in FITC in both kidney and teratoma sections. In Texas Red channel, AF was reduced in teratomas, but AF increased in kidney in Cy5.5 channel. SB/TLAQ treated sections showed marked reduction in all three tissues in both paraffin and frozen sections, but in placenta frozen sections, in FITC, the antibody signal was reduced too.

In summary, we showed that SB is a common quencher that can be used in various tissues. TLAQ is also a good quencher, but it may also quench the real signal from the antibody. For UV and other chemical methods, AF in tissues can be reduced to some extent. However, when choosing the chemicals for AF reduction, the property of the tissues, an adequate fluorochrome, and the channel of acquisition to use all need be considered in order to accurately detect antibody signals. Combinations of chemicals do not always further reduce AF.

**Data availability**

Raw data that support the findings of this study are available in OSF: DOI, 10.17605/OSF.IO/3E4KV (Yang, 2017).

**Competing interests**

No competing interests were disclosed.

**Grant information**

This work was supported by the Wellcome Trust [206194].

*The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.*

**Acknowledgements**

We thank colleagues from the Research Support Facility at the Sanger Institute, and the animal facility at Stem Cell Institute, University of Cambridge for mice husbandry.
References


Wellcome Open Research 2017, 2:79 Last updated: 02 JAN 2018

Data Source


Open Peer Review

Current Referee Status: ? ? ❌

Version 1

Referee Report 02 January 2018
doi:10.21956/wellcomeopenres.13263.r29189

Martin W. Wessendorf
Department of Neuroscience, University of Minnesota, Minneapolis, MN, USA

Autofluorescence in tissue can result in false-positive staining and make it difficult to interpret results using fluorescence microscopy. This article examines the effects of thirteen different treatments (or combinations of treatments) for autofluorescence. These treatments were tested in mouse placenta and in teratomas derived from human embryonic stem cells.

A major issue with the experimental design is that the authors attempted to perform these experiments in tissue that was stained using immunofluorescence. The immunofluorescence staining confounds the results, since it is unclear to what extent a treatment reduces specific labeling as reduced as opposed to reducing autofluorescence. I strongly suggest testing treatments for autofluorescence on unstained sections—this would allow the effects of the various treatments to be determined unequivocally. (In addition, it would allow their images to be analyzed quantitatively using densitometry, e.g., with ImageJ, rather than using the subjective rating system that was employed.) Once the authors identify efficacious treatments, they could then test whether or not those treatments are compatible with immunofluorescence.

Another issue is that the authors treat autofluorescence as a single phenomenon. As they note, there are several distinct sources of autofluorescence. It follows that treatments that reduce one type would not necessarily affect another. I suggest that the authors work to identify which type or types of autofluorescence they are dealing with in each tissue. (It would be beneficial if the unstained tissue were imaged using a spectral imaging system, e.g., a spectral confocal device. Doing so might make it possible to determine whether different autofluorescent components were affected differentially by the different treatments.) I also suggest that they review the literature on the chemistry of autofluorescence, since it's unclear whether or not they are aware of the mechanism by which formaldehyde results in autofluorescence. Formaldehyde itself is not significantly fluorescent but when it reacts with amines it can form Schiff bases that result in autofluorescence. Treatment with ammonia will eliminate the formaldehyde and quench the fixation reaction, but will probably do nothing to eliminate Schiff bases that have already formed. However, these Schiff bases can be reduced by treatment with sodium borohydride.

The Methods omit a few important details. First, the authors refer to treating sections 0.25% ammonia. Is this percentage weight/volume or volume/volume? If the latter, what was their stock solution—NH₄OH? If so, how fresh was it? Second, how long was tissue fixed in formaldehyde prior to embedding in paraffin? Third, what were the commercial sources for the reagents used—in particular for the TLAQ? Fourth, was the UV illuminator based on a mercury source or something else? What wavelengths did it provide, and what was the power of the illumination at the surface of the tissue? Fifth, was the formaldehyde freshly
made from paraformaldehyde? If not, what was the source, how long was it stored and at what
temperature? Sixth, what were the bandpasses for the excitation and emission filters and dichromatic
mirrors used in the filter sets? Was there any reason for not evaluating autofluorescence using the DAPI
filter? Were all the different fluorescence channels imaged simultaneously, or one-at-a-time? Finally, the
micrographs would be easier to interpret if all they included higher magnification insets, so that the
intracellular distribution of the autofluorescence could be seen--doing so might give clues regarding the
type of autofluorescence being observed. In addition, displaying all the images as grayscale would make
it easier to compare the effects of treatments across filters.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
No

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
No

Are the conclusions drawn adequately supported by the results?
Partly

*Competing Interests:* No competing interests were disclosed.

*Referee Expertise:* Fluorescence microscopy methods, neuroanatomy, immunohistochemistry

I have read this submission. I believe that I have an appropriate level of expertise to state that I
do not consider it to be of an acceptable scientific standard, for reasons outlined above.

doi:10.21956/wellcomeopenres.13263.r27532

A. Sally Davis

College of Veterinary Medicine, Kansas State University, Manhattan, KS, USA

Thank you for inviting me to review this research. I apologize for the delay in my review, hampered by
unrelated life events.

This work fills a gap in the autofluorescence literature regarding the tissues examined,
particularly placenta and teratoma. While very practical it could do with some reorganization particularly of
its methods and results sections in order to improve its reading. This is primarily an observational report
and a useful starting point. To truly state that X reagent diminished AF to Y extent in a certain tissue
location many more replicates than n=1-2 would be needed. That given it is still useful information and a
good starting point.

Note that many of my Partly responses have to do with the need to better organize the presentation of the methods and results as well as discussion the study limitations. I think that everything is present but needs this rearrangement to be truly useful.

Minor

The **title** does not align with the manuscript content. Alt rec: "Quenching autofluorescence in placental tissues and teratomas" or something similar in which the tissues of focus are called out. You might even consider Preliminary exploration or Survey as terms to put in the title to set the reader up for the low sample size per treatment.

The **intro** is well written but missing kidney (see below).

The methods:

- Please ensure your Reagents section is complete. For e.g. it is lacking paraformaldehyde.
- Header "tissue section" should be "Tissue Sectioning"
- kidney comes out of nowhere in the methods section with no prior introduction or presence in the abstract. The title, abstract and intro should be changed to include it.
- There are also some other minor grammatical issues in this section, such as sub-cutaneous injected should be subcutaneously injected. These are minor but distracting.
- Immunofluorescence - in general this section is a bit difficult to follow. All the treatments are there but the fact that some are done ahead of, in the middle or after immunofluorescence is a bit hard to follow. An introductory paragraph where this is explained or even a figure summarizing this would be very helpful to the reader. You might even consider reversing the order of presentation by presenting the autofluorescence treatments, the focus of your paper first. The table works somewhat but should be called from some sort of intro paragraph wherein you state the overall treatments. As it is there is a summary table that gets introduced last and then the reader has to piece it back together by walking backwards through the text. And this table is only used for combined treatments.
- Treatments section: Should be named "Autofluorescence Quenching Treatments" or similar
- Please define what a "jet wash" is
- Digital image evaluation? How about "Image Analysis" as the section header
- Please provide full info for the filter sets, vendor ID if standard ordered as well as Ex and Em range.
- Not sure what is meant by "2 (reduction, but also the antibody signal)" - AF and immunofluorescence signal reduction?
- While a tissues n is provided there is little sense as to n for each treatment, whether replicates were done, etc. This might be provided in a revised treatments section.

Results:

- In general, this section contains methods that were missed in the methods section and should be moved to it in addition to results. The tables and figures work nicely.
- Primary antibodies used should be introduced in the methods section, not mentioned first in the results section. The first paragraph of the results should be in the methods for example.
- Numbers of sections examined by each treatment are lacking as well. Likewise the first paragraph that details the immunofluorescence methods for the kidney/teratoma tissue should be moved to the methods section.
- Some logic as to why there are less treatments applied to placental tissues should be elaborated somewhere in the manuscript
Combined treatments not treatment for the header in the results section.

Discussion
In general this is quite informative, organized in a logical manner and well referenced. However, the opening sentence doesn't really portray the main focus of this work:
"In this technical report, we presented analysis results of performing immunofluorescence to detect proteins in the placenta and teratoma sections." Instead it should focus on this within the context of different AF treatments to improve immunofluorescence.

- The limitations of this study should be discussed. The low sample number is a good place to start.
- Again there are a few grammatical things, e.g. "laboring" would be better written as "labor intensive"
- The last sentence should be earlier in the paragraph and the second to last sentence is the take home message to end the manuscript.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

**Competing Interests:** No competing interests were disclosed.

**Referee Expertise:** Biosample methods, veterinary pathology, high containment emerging and zoonotic virus work, experimental/investigative pathology

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Referee Report 21 September 2017

doi:10.21956/wellcomeopenres.13263.r25668

Girish K. Srivastava , Ivan Fernandez Bueno
Instituto Universitario de Oftalmobiología Aplicada (IOBA), University of Valladolid, Valladolid, Spain
The reviewers congratulate the authors for performing this research study and presenting the data as a scientific paper.

The reviewers present few serious concerns regarding this paper;

1. The paper lacks a new and innovative idea; however, the studied methodologies and research findings support to advancement in scientific research field.

2. The paper is completely based to develop methodology. Hence, it is considered as a “Method Articles” or “Technical Reports”, not as a “Research Articles”.

3. The critical issue is that nowhere in the manuscript the authors have discussed how they are going to apply these research findings in human clinical pathology.

4. This research needs to apply human tissue samples to extrapolate the research findings application in clinical use.

5. It needs to be clarified the findings application in case of the in vitro cell cultures. Do the authors consider it will work?

6. The experimental animals have been used to obtain tissues of placenta and teratoma for experimental purpose. However, it can be avoided by contacting a bio-bank for tissue samples. The experimental animals, strictly, must be used only for developing or reproducing a pathogenesis model which must be used for final prevention and treatment study.

7. The reasons behind selection of placenta and teratoma tissues for study purpose, it needs to be discussed in details. Why it overruled other tissues?

8. Total number of animals used in this study, it needs to be written in section “Mice”.

9. Number of samples evaluated in each case, it requires to be increased to a minimum number to n=3 for statistically analyze the data. The figures are showing n=1, n=2 in most of the analyzed tissue samples.

10. It requires marking well by arrows the findings mentioned in the paper in the figures.

**Is the work clearly and accurately presented and does it cite the current literature?**
Yes

**Is the study design appropriate and is the work technically sound?**
Yes

**Are sufficient details of methods and analysis provided to allow replication by others?**
Partly

**If applicable, is the statistical analysis and its interpretation appropriate?**
Not applicable

**Are all the source data underlying the results available to ensure full reproducibility?**
Yes

**Are the conclusions drawn adequately supported by the results?**  
Yes

**Competing Interests:** No competing interests were disclosed.

**Referee Expertise:** The reviewers have expertise in handling retinal tissues and cells, and published similar articles describing methodologies for quenching autofluorescence.

We have read this submission. We believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.