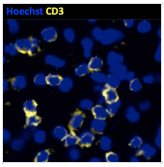


Jul 01, 2021

Immunofluorescence on FFPE tissue sections from inflamed and non-inflamed human gut

Ross J Porter[1], Katie J Smith[1], Gwo-Tzer Ho[1], Emily Gwyer Findlay[1]

[1]Centre for Inflammation Research, Queen's Medical Research Institute, University of Edinburgh, Scotland UK

 dx.doi.org/10.17504/protocols.io.bsqendte

ross.porter

DISCLAIMER

Tissue was provided by Lothian NRS Bioresource [TGU-LAB-1415(IBD)].

We would like to thank the Scientific Twitter Community for their contribution to our protocol, and important suggestion to use Sudan Black / TrueBlack®. We would like to thank Caoimhe Kirby and Lucas Lefevre (McColl Group, University of Edinburgh, Scotland UK) for their kind donation of *TrueBlack®* for this experiment.

Note: Autofluorescence can be reduced using various commercial quenchers - *TrueBlack®* was the quencher that we had available and we have not comprehensively tested this against other similar brands.

ABSTRACT

Analysis of immunofluorescence is commonly confounded by autofluorescence and non-specific staining in the colorectum, particularly within highly inflamed samples. There is no consensus on how to definitively prevent this, and study protocols are variable. Our laboratory identified strong immunofluorescence within negative control samples stained for numerous target antigens, including CD3. Following methodical attempts to ensure there was no reagent contamination, we concluded that this was autofluorescence/ non-specific staining. Significant optimisation was subsequently performed. In this protocol, we report our optimised methodology for immunofluorescence experiments on formalin fixed paraffin embedded (FFPE) tissue sections, stained for identification of CD3+ lymphocytes. We hope this protocol acts as a template to help other laboratories optimise their own immunofluorescence protocols for similar antibodies.

DOI

dx.doi.org/10.17504/protocols.io.bsqendte

PROTOCOL CITATION

Ross J Porter, Katie J Smith, Gwo-Tzer Ho, Emily Gwyer Findlay 2021. Immunofluorescence on FFPE tissue sections from inflamed and non-inflamed human gut . **protocols.io** https://dx.doi.org/10.17504/protocols.io.bsqendte

KEYWORDS

immunofluorescence, autofluorescence, colon, biopsy, immunohistochemistry, IBD, T-cell, CD3, inflammation

LICENSE

IMAGE ATTRIBUTION

Slides were digitally scanned using the ZEISS Axio Scan.Z1 slide scanner. QuPath-0.2.3 software was used for visualisation and quantification of immunofluorescence images.

Citation: Ross J Porter, Katie J Smith, Gwo-Tzer Ho, Emily Gwyer Findlay (07/01/2021). Immunofluorescence on FFPE tissue sections from inflamed and non-inflamed human gut . https://dx.doi.org/10.17504/protocols.io.bsqendte

CREATED

Feb 24, 2021

LAST MODIFIED

Jul 01, 2021

PROTOCOL INTEGER ID

47590

MATERIALS TEXT

Reagents

- Deparaffinisation and Tissue Rehydration: xylene, 100% alcohol, 90% alcohol, 80% alcohol and 70% alcohol
- Phosphate Buffered Saline (PBS) (1-2L) - example recipe https://www.aatbio.com/resources/buffer-preparations-and-recipes/pbs-phosphate-buffered-saline
- dH2O
- 1N hydrochloric acid
- Tween 20
- Triton X-100
- 2.94g tri-sodium citrate
- *TrueBlack®* Lipofuscin Autofluorescence Quencher 20X in DFM (Cat 23007, Biotium)
- Blocking serum (e.g. Donkey serum)
- Foetal calf serum
- Primary antibody
  Mouse monoclonal anti-human CD3 Ab (ab669 [GR3295418-1])
- Secondary antibody
  Donkey anti-mouse AF555 (histology number 1178)
- Parafilm*®M (Sigma-Aldrich)*
- Hoechst
- Anti-fade Floromount-Go® (SouthernBiotech 0100-01, L3220-ZD30) mounting medium

Equipment

- Fume hood (for use of xylene +/- applying mounting medium)
- Thermosetting/microwavable slide rack and container that can hold buffer
- Humidity/ moisture chamber (e.g. slide box with wet-to-damp paper towel underneath slides)
- Microwave (800W)
- Soft tissues
- Refrigerator
- Slide tray
- Aluminium foil
- Digital slide scanner (e.g. ZEISS Axio Scan.Z1)

Tips for efficiency:

- Make sodium citrate buffer (step 4) while slides are in xylene (step 2)
- Make 0.1% Triton X-100 (step 8), block (step 14) and the primary antibody solution (step 18) while sections are undergoing epitope/antigen retrieval (step 6)
- Make *TrueBlack®* (step 10) near the end of step 8
- Only stain 2-3 slides with TrueBlack® at one time to ensure consistent timing (essential for analysis) - use a stopwatch/timer
- Do not cut off time during washing steps - use a stopwatch/ timer

SAFETY WARNINGS

Ensure you are familiar with the specific safety data sheets and recommended precautions for all reagents you are using for this protocol. Disposal of reagents and waste varies between laboratories - please ensure you are familiar with your recommended protocols before starting. For all steps of this experiment, wear a full-length laboratory coat, protective gloves +/- eye protection, and ensure adequate ventilation. The below advice is not fully comprehensive and should only act as an initial guide.

Citation: Ross J Porter, Katie J Smith, Gwo-Tzer Ho, Emily Gwyer Findlay (07/01/2021). Immunofluorescence on FFPE tissue sections from inflamed and non-inflamed human gut . https://dx.doi.org/10.17504/protocols.io.bsqendte

- Xylene is flammable and significantly harmful if inhaled, aspirated, ingested, or comes into contact with the skin or eyes. It can be fatal in case of aspiration. Xylene can also cause specific target organ toxicity. Xylene should be handled with care wearing protective gloves, eye protection, and should be handled exclusively in a fume hood. Care should be taken to prevent against static discharges. Xylene is harmful to aquatic life with long-lasting effects - waste is hazardous. Pregnant and breast-feeding women should not work with xylene.
- Alcohol is highly flammable and causes eye irritation so should be handled with gloves and eye protection. This should not be released into the environment - waste is hazardous.
- HCl is corrosive, including to metals, can cause severe damage and irritation to eyes, skin, the gastrointestinal and the respiratory tract, and has specific target organ toxicities following single exposure. This should be handled carefully with appropriate gloves, goggles and a face shield. This should not be released into the environment - waste is hazardous.
- Citrate buffer - Please be aware that the citrate buffer heated in the microwave will be boiling hot and thermal gloves should be used. The citrate buffer will contain HCl - extra caution is required for respiratory protection given the vapour produced during this process - please see HCl. In the very rare case that good ventilation cannot be ensured, respirator masks may be required.
- Triton X-100 is harmful if ingested, and can cause acute oral toxicity and serious eye damage/irritation. Gloves and eye protection are advised. This should not be released into the environment - waste is hazardous.
- *TrueBlack®* Lipofuscin Autofluorescence Quencher 20X in DFM should not be handled by pregnant women. It is harmful to skin, causes serious eye irritation, and is harmful if inhaled. Protective gloves and eye protection is advised. This should not be directly released into the environment - waste is hazardous.
- Hoechst is associated with acute oral toxicity, is corrosive to the skin, is harmful if ingested, and causes serious eye damage and irritation. Use of gloves and eye protection are advised. Dispose of as per local policy (e.g. in organic waste container).

DISCLAIMER:

Tissue was provided by Lothian NRS Bioresource [TGU-LAB-1415(IBD)].

We would like to thank the Scientific Twitter Community for their contribution to our protocol, and important suggestion to use Sudan Black / TrueBlack®. We would like to thank Caoimhe Kirby and Lucas Lefevre (McColl Group, University of Edinburgh, Scotland UK) for their kind donation of *TrueBlack®* for this experiment.

Note: Autofluorescence can be reduced using various commercial quenchers - *TrueBlack®* was the quencher that we had available and we have not comprehensively tested this against other similar brands.

BEFORE STARTING

Use 4-5µm tissue sections cut by microtomy from FFPE blocks, prepared for immunohistochemistry/ immunofluorescence staining. Sections should be cut relatively fresh (within the past few months). Ensure you have a positive and negative control for every optimisation and experimental run.

This protocol will need to be further optimised for different antibodies and tissues. Please see the final step of this protocol for further optimisation suggestions.

Pre-Protocol Tissue Section Acquisition 2m

1 Slides should be clearly labeled with pencil and placed into a slide-rack. 2m

Deparaffinisation & Tissue Rehydration 10m 20s

2 Sequentially immerse the slides, in the slide rack, into the following solutions: 30m

1. Xylene **00:10:00**
2. Xylene **00:10:00**

Citation: Ross J Porter, Katie J Smith, Gwo-Tzer Ho, Emily Gwyer Findlay (07/01/2021). Immunofluorescence on FFPE tissue sections from inflamed and non-inflamed human gut . https://dx.doi.org/10.17504/protocols.io.bsqendte

3. 100% alcohol **00:02:00**
4. 100% alcohol **00:02:00**
5. 90% alcohol **00:02:00**
6. 80% alcohol **00:02:00**
7. 70% alcohol **00:02:00**

See image in Step 6 for the benefits of these enhanced timings.

3 Place the slides immediately into tap water to prevent tissue dehydration. Never allow the tissue to become dehydrated. 5s

Heat-Induced Epitope Retrieval (HIER) 29m

4 5m

Make sodium citrate retrieval buffer as below (adapted from *Abcam*):

1. Dissolve **2.94 g** tri-sodium citrate (dihydrate) in **1 L** dH2O
2. Titrate to **pH 6.4** using 1N hydrochloric acid
3. Add **0.5 µl** Tween®20 (Sigma-Aldrich, 9005-64-5)

For our experiments, HIER buffer was made fresh each time (as 0.5-1L total was needed). However, Abcam suggest buffer is stable for 3 months at room temperature, or longer at 4°C.

5 Heat the retrieval buffer, in a thermosetting plastic container, in the microwave (800W) at full power for **00:03:00 or until boiling/bubbling** . 3m

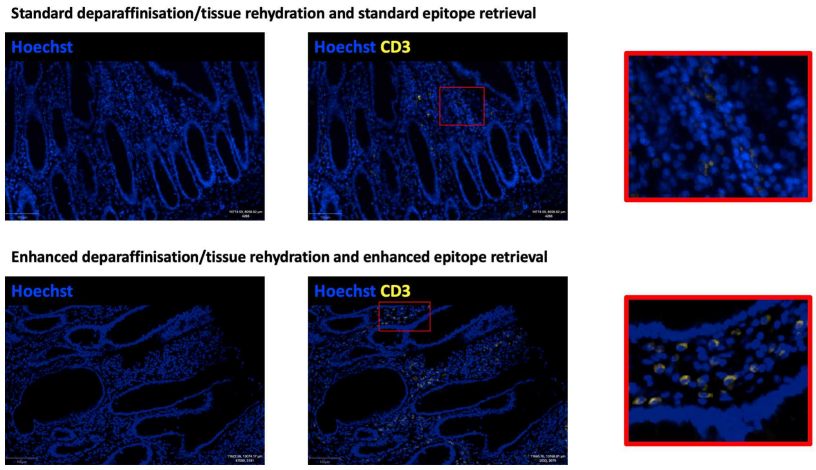
6 ⚠ 20m

Immerse slides, in a slide rack, into the retrieval buffer. Microwave on full power for **00:20:00** .

At 7 minutes and 14 minutes add extra retrieval buffer to **ensure slides remain completely immersed in liquid at all times.**

The benefits of enhanced deparaffinisation/tissue rehydration (Step 2) and enhanced HIER (Step 6), compared with a standard protocol (4 minutes in xylene / 10 minutes in the microwave), are illustrated below.

Citation: Ross J Porter, Katie J Smith, Gwo-Tzer Ho, Emily Gwyer Findlay (07/01/2021). Immunofluorescence on FFPE tissue sections from inflamed and non-inflamed human gut . https://dx.doi.org/10.17504/protocols.io.bsqendte



Enhanced deparaffinisation/tissue rehydration and heat-induced epitope retrieval (HIER) improves immunofluorescence. Standard deparaffinisation/tissue rehydration involved sequential immersion in xylene (2 x 2 minutes) 100% alcohol (2 x 20s), 90% alcohol (20s), 80% alcohol (20s) and 70% ethanol (20s). Standard HIER involved microwaving (800W medium power) for 10 minutes in sodium citrate buffer (pH 6-6.5). Enhanced deparaffinisation/tissue rehydration involved sequential immersion in xylene (2 x 10 minutes) 100% alcohol (2 x 2 minutes), 90% alcohol (2 minutes), 80% alcohol (2 minutes) and 70% ethanol (2 minutes). Enhanced HIER involved microwaving (800W full power) for 20 minutes in sodium citrate buffer pH6.4. Please note these are not serial sections.

7 Cool slides in cold running tap water. 2m

Tissue Permeabilisation 30m

8 Immerse slides in 0.1% Triton X-100 (dissolved in PBS) for **00:15:00** . 15m

9 15m

Wash with PBS **00:05:00**

Wash with PBS **00:05:00**

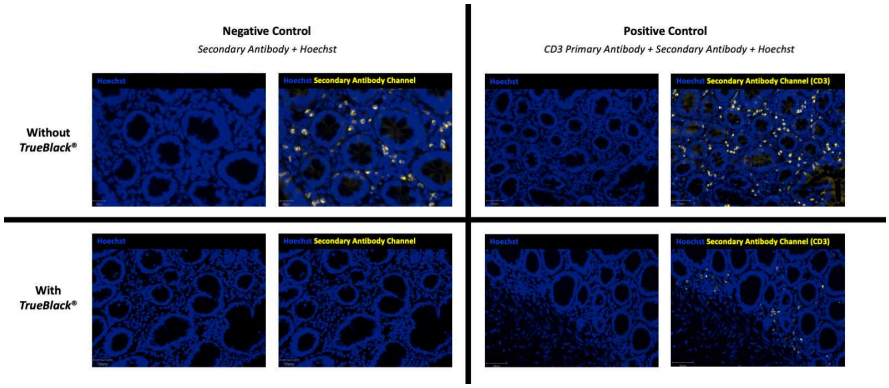
Wash with PBS **00:05:00**

Reduce Autofluorescence 11m 4s

10 Make-up *TrueBlack®* Lipofuscin Autofluorescence Quencher 20X in DFM (Cat 23007, Biotium); make enough for **250 µl per slide of 1:20 dilution as below** . 2m

1. Vortex *TrueBlack®* **00:00:05**
2. Dilute 1:20 in 70% ethanol
3. Vortex to mix well **00:00:05**

Citation: Ross J Porter, Katie J Smith, Gwo-Tzer Ho, Emily Gwyer Findlay (07/01/2021). Immunofluorescence on FFPE tissue sections from inflamed and non-inflamed human gut . https://dx.doi.org/10.17504/protocols.io.bsqendte



This figure illustrates the importance of using *TrueBlack®* in our protocol. The negative control slide without *TrueBlack®* treatment appears to have specific CD3+ immunopositivity; however, this slide had no primary CD3+ antibody applied. The negative control slide treated with *TrueBlack®* was negative. All slides were stained during the same experimental run, using this optimised protocol. This finding was validated on several experimental runs, and contamination excluded systematically over several experimental runs. This figure also illustrates the importance of **always** having a negative control slide for **every** experimental run (and every set of conditions).

Autofluorescence can be reduced using various commercial quenchers - *TrueBlack®* was the quencher that we had available to us in the laboratory, and we have not comprehensively tested this against other chemicals.

11 Lay slides horizontally in a humidity/moisture chamber (see image at Step 15), wiping away any excess PBS with soft tissue. Do not dry the tissue. 20s

12 ⚠ 30s

Apply diluted **250 µl** *TrueBlack®* solution to each slide for **00:00:30** .

Apply *TrueBlack®* to a maximum of 2-3 slides at a time, and use a stopwatch, to ensure accurate and consistent timing.

DO NOT USE ANY DETERGENTS HEREAFTER; if absolutely essential then you should instead apply *TrueBlack®* before mounting - after step 31.

13 15m 2s

Wash with PBS **00:00:02**

Wash with PBS **00:05:00**

Wash with PBS **00:05:00**

Wash with PBS **00:05:00**

Blocking 1h

14 Make the blocking solution. This should be PBS consisting of 25% donkey serum and 10% foetal calf serum. Make enough for **300 µl per slide** . We noted a significant decrease in background autofluorescence when both foetal calf serum and donkey serum were used in the blocking step compared with donkey serum alone. 2m

Donkey serum was used in this experiment because the secondary antibody was raised in donkey.

15 Lay slides horizontally in a humidity/moisture chamber, wiping away any excess PBS with soft tissue. 20s

Citation: Ross J Porter, Katie J Smith, Gwo-Tzer Ho, Emily Gwyer Findlay (07/01/2021). Immunofluorescence on FFPE tissue sections from inflamed and non-inflamed human gut . https://dx.doi.org/10.17504/protocols.io.bsqendte

Slides lying horizontally in a common DIY-humidity/moisture chamber with damp tissue paper underneath, but not touching, slides. Labels with identifiable data have been removed digitally for confidentiality.

16 Apply **300 µl** of blocking solution to each slide and cover the whole slide gently with Parafilm®*M*, cut to shape, to ensure equal distribution of antibody and prevent tissue dehydration. 1m

17 Leave in a in a closed humidity/ moisture chamber, at **Room temperature** for **01:00:00** . 1h

Primary Antibody Application 1h

18 ⚠ 2m

Dilute the primary antibody/antibodies in 10% donkey serum diluted in 1x PBS - make enough for **300 µl per slide** .

DO NOT USE ANY DETERGENTS (e.g. Tween®), as this will remove *TrueBlack®* from the tissue.

Example: Mouse monoclonal anti-human CD3 antibody (ab699 [GR3295418-1]) at 1:50

19 Tip excess block off each slide and lay horizontally in a humidity/moisture chamber. 20s

20 Apply **300 µl** of primary antibody solution to each slide and cover the whole slide gently with Parafilm®*M*, to ensure equal distribution of antibody and prevent tissue dehydration. 1m

21 🌊 20h

Leave in a in a closed humidity/ moisture chamber, at **4 °C overnight** .

Citation: Ross J Porter, Katie J Smith, Gwo-Tzer Ho, Emily Gwyer Findlay (07/01/2021). Immunofluorescence on FFPE tissue sections from inflamed and non-inflamed human gut . https://dx.doi.org/10.17504/protocols.io.bsqendte

Secondary Antibody Application 1h

22 15m

Wash with PBS **00:05:00**

Wash with PBS **00:05:00**

Wash with PBS **00:05:00**

23 2m

Dilute the secondary antibody/antibodies in 10% donkey serum diluted in PBS - make enough for **300 µl per slide** .

Example: Donkey anti-mouse ab150108 at 1:500 (Alexa Fluor® 594)

DO NOT USE ANY DETERGENTS (e.g. Tween®), as this will remove *TrueBlack®*.

24 20s
Lay slides horizontally in a humidity/moisture chamber, wiping away any excess block with soft tissue. Do not dry the tissue.

25 1m
Apply **300 µl** of secondary antibody solution to each slide and cover gently with Parafilm*®M*, to ensure equal distribution of antibody and prevent tissue dehydration.

26 1h
Leave in a in a closed humidity/ moisture chamber, for **01:00:00** at **Room temperature** .

Herein, protect the slides from light.

DNA Staining 1h

27 1m
Make-up hoechst solution 1:1000, diluted in dH2O - make enough for **300 µl per slide** . Protect this solution from light.

28 15m

Wash with PBS **00:05:00**

Wash with PBS **00:05:00**

Wash with PBS **00:05:00**

29 20s
Lay slides horizontally in a humidity/moisture chamber, wiping away any excess PBS with soft tissue. Do not dry the tissue.

30 5m
Apply **300 µl** of hoechst solution to each slide for **00:05:00** at **Room temperature** .

31 15m

Citation: Ross J Porter, Katie J Smith, Gwo-Tzer Ho, Emily Gwyer Findlay (07/01/2021). Immunofluorescence on FFPE tissue sections from inflamed and non-inflamed human gut . https://dx.doi.org/10.17504/protocols.io.bsqendte

Wash with PBS **00:05:00**

Wash with PBS **00:05:00**

Wash with PBS **00:05:00**

Mounting 2m

32 Sequentially for each slide, wipe off excess PBS and mount a glass coverslip using **150 µl** of anti-fade Floromount-Go® (SouthernBiotech 0100-01, L3220-ZD30) mounting medium. 2m

33 Leave slides to dry on a slide tray, covered with aluminium foil (i.e. in complete darkness). 20m

Scanning & Analysis 1h

34

Digitally scan slides soon after staining (digital scanning profiles will need separate optimisation).

35 Ensure the negative control is negative and positive control is positive (see image in Step 10).

36

Analyse objectively with a digital image-analysis software. QuPath (an open-source software) analysis works well for this application in our laboratory. Ensure you use the negative control to 'gate' out residual background staining/ autofluorescence.

Bankhead P, Loughrey MB, Fernández JA, Dombrowski Y, McArt DG, Dunne PD, McQuaid S, Gray RT, Murray LJ, Coleman HG, James JA, Salto-Tellez M, Hamilton PW (2017). QuPath: Open source software for digital pathology image analysis.. Scientific reports. https://doi.org/10.1038/s41598-017-17204-5

Further Optimisation Suggestions

37 This protocol has been optimised using reagents in our laboratory to stain full thickness FFPE biopsies from inflamed and non-inflamed human gut specimens. We encourage further optimisation experiments in other laboratories, with different tissue sections and antibodies.

Specific optimisation suggestions are listed below:

Check there is no inadvertent damage occurring to tissue sections

- Ensure slides are not drying out, especially during antigen-retrieval in the microwave or during antibody incubation
- Ensure all timings and temperatures are accurate, especially during antigen retrieval
- Beware of chemical reagents that make tissue distortion or damage more likely (e.g. EDTA, Tween)

Issues with antibody-epitope binding

- Alter deparaffinisation and rehydration times
- Increase or decrease heat-induced epitope retrieval (HIER) time
- Trial higher temperatures for HIER; consider use of a pressure cooker or autoclave

Citation: Ross J Porter, Katie J Smith, Gwo-Tzer Ho, Emily Gwyer Findlay (07/01/2021). Immunofluorescence on FFPE tissue sections from inflamed and non-inflamed human gut . https://dx.doi.org/10.17504/protocols.io.bsqendte

- Use a more accurate time and temperature controlled microwave (professional rather than household)
- Trial different reagents for HIER e.g. Tris-EDTA
- Trial different pH levels for HIER buffers
- Reduce the time of primary antibody application (e.g. from overnight to 1 hour)
- Increase or decrease concentration of Tween (beware of increased tissue distortion at higher concentrations)
- Increase or decrease antibody concentrations (primary and secondary)
- If necessary, consider different fixation methods for your tissue

To optimise fluorescence and reduce autofluorescence/ non-specific background staining

- Ensure you have robust positive and negative controls for every experiment
- Increase the blocking time
- Increase the number and duration of washing steps
- Protect slides from light during staining and imaging
- Attempt multiple staining steps (i.e. stain for each antibody sequentially)
- Alter the time slides are exposed to *TrueBlack® (e.g. decrease to 15s or increase to 1 minute)*
- If there are issues with both antigen retrieval and fluorescence, try applying *TrueBlack®* at the end of this protocol before mounting (this will also allow increased use of detergents before this step) - see data sheet for TrueBlack®.
- If *TrueBlack®* is not effective for your tissues, consider other agents such as *Vector®TrueVIEW®* Autofluorescence Quenching Kit (SP-8400-15).
- Ensure the cover slip and mounting medium are optimal for your detection (and compatible with immunofluorescence)
- Ensure the best fluorophores are selected for imaging, and ensure there is good separation between fluorophores for co-stains
- Ensure there are no dust or bubbles on the side at time of mounting

If the final staining results are inconsistent, consider use of an autostainer or optimising slide scanner settings.

Citation: Ross J Porter, Katie J Smith, Gwo-Tzer Ho, Emily Gwyer Findlay (07/01/2021). Immunofluorescence on FFPE tissue sections from inflamed and non-inflamed human gut . https://dx.doi.org/10.17504/protocols.io.bsqendte