Imaging cell signalling in tissues using the IBEX method

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i. Chapter Title

Imaging cell signalling in tissues using the IBEX method

ii. Abstract

The study of cell signalling within tissues can be enhanced using highly multiplexed immunohistochemistry to localise the presence and spatial distribution of numerous pathways of interest simultaneously. Additional data can also be gained by placing the identified proteins into the context of adjacent structures, stroma and interacting partners. Here we outline a protocol for using the recently described IBEX method on tissues. This is an open and simple cyclic immunohistochemistry approach suited to this application. We describe a simplified protocol and provide guidance on the method, using a 12-marker panel on human retina to demonstrate the approach.

iii. Key words

Cell signalling
IBEX
Immunohistochemistry
Retina
Immunofluorescence

1. Introduction

Classical cell signalling imparts the ability to cells to detect and respond to internal and external environmental stimuli, modulating biological processes to effect a change in function and behaviour (1,2). Dysregulation of cell signalling pathways is also frequently implicated in various pathological states including autoimmune disorders and cancer (3). Therefore, studying cell signalling is vital for understanding the molecular mechanisms that control cellular responses and identifying potential therapeutic targets. A huge amount of information can be gained by the *in vitro* study of cell signalling, however examining responses at an *ex vivo* tissue level stands to supplement the integration of signalling across a wider and systems level of understanding. Furthermore, the use of emerging spatial biology techniques will provide additional insights through the ability to localise signalling across tissues.

The Iterative Bleaching Extends Multiplexity (IBEX) method is a recently described imaging approach that can be applied to enable highly multiplexed analysis of signalling proteins in tissue sections (4,5). Multiplexed immunohistochemistry on tissue cryosections can be performed with existing, validated and commercially available signalling pathway antibodies using IBEX. Only standard fluorescence confocal microscopes are required improving the accessibility to most research groups. IBEX works through registration of iterative cycles of fluorescent antibody staining with bleaching to achieve fluorophore inactivation between each round.

In this chapter, we demonstrate the technique on human retina using selected proteins in the p16, NF-κB and cGAS-STING (6) signalling pathways that have been studied in our lab in relation to inflammation and neuronal senescence. In addition we can combine other structural and cellular antibody markers to place the cells with detected signalling proteins in spatial context of the surrounding tissue environment (**Figure 1**). IBEX provides a powerful tool to study cell signalling by allowing for the simultaneous detection of multiple targets simultaneously and in tissue to provide a more comprehensive and spatially resolved view of cell signalling networks.

2. Materials

2.1 Biological materials

Ensure the tissue sourced is covered by appropriate research ethics clearance. Human retina for this protocol was obtained through Moorfields Eye Hospital Biobank, (NHS REC approval: 20/SW/0031) with consent and eye retrieval provided by NHS Blood and Transplant tissue services. The retina used in this study was obtained from a 50-year-old male donor who died of decompensated alcoholic cirrhosis.

2.2 Solutions

1. Sucrose: Prepare 15% and 30% Sucrose solution (w/v) dissolved in PBS. Keep the solution sterile by passing through a 0.22um filter and it can be stored at 4°C for several months. Dilute 30% Sucrose in PBS to get 15% Sucrose.
2. Fixative: 4% Paraformaldehyde prepared from 16% methanol-free formaldehyde solution in ampules (for example Thermo Fisher Scientific, Cat. No. 28908)
3. Blocking solution: 1:20 Normal Donkey Serum 1:100 Human Fc Block (or species equivalent) diluted in 1x BD Perm Wash (BD Biosciences, Cat. No. 554722, made from the 10x stock with PBS).
4. Antibody-staining solution: antibodies diluted in 1x BD Perm Wash and a 1:1000 dilution of DAPI to stain nuclei to be used as a fiducial marker.
5. $LiBH_4$ solution: 5mg in 5ml of distilled water. Leave to rest for 20 minutes until small bubbles are seen forming. Note hydrogen gas can be generated in an exothermic reaction, so perform this in a chemical hood with care. $LiBH_4$ (e.g. STREM Chemicals, Cat. No. 93-0397) is best purchased in 1 g aliquots to minimize degradation.

2.3 Data analysis software

1. Python (version 3.7.0 or higher)
2. Imaris and Imaris Converter (Oxford instruments, version 9.5.0 or higher)
3. The free registration algorithm is available from https://github.com/niaid/sitk-ibex and the Imaris extension code is available from https://github.com/niaid/imaris_extensions

3. Methods

3.1 Tissue grossing and processing.

1. Use standard fixation conditions and tissue preparation for the tissue and organ of interest. Here we freshly prepare a 4% PFA solution and incubate posterior segments supplied by the eye bank in this at 4°C for 48 hours. This is performed in an appropriate biological safety cabinet.
2. Exchange the fixative on the posterior segments for a solution of a 1:4 dilution of BD Cytofix/Cytoperm (BD Biosciences, Cat. No. 554722) in PBS and incubate at 4°C for 16 hours.
3. Wash the samples twice at room temperature using 1xPBS for 5 minutes each to remove excess fixative.
4. Prepare dissecting tools and place the sample on a dissecting mat or other fibre-free clean surface. We use a dissection microscope. Dissect the posterior segment, removing lens, sclera and vitreous to isolate the retina. Dissect this into pieces around 10mm by 10mm.
5. Place the samples in completely dissolved 15% sucrose solution for a few hours at 4°C until the sample has sunk to the bottom of the container.
6. Transfer the sample to a completely dissolved 30% sucrose solution at 4°C overnight.
7. Fill a Histomould with optimal cutting temperature (OCT) medium (CellPath, SKU: KMA-0100-00A) and embed the tissue. Use forceps to adjust the tissue orientation for subsequent frozen sectioning. Ensure the tissue is located approximately at the centre of the mould. Place the Histomould on dry ice and allow to slowly freeze.
8. Label the mould and store in a -80°C freezer until required.

3.2 Tissue Preparation

1. Coat microscope slides with chrome alum gelatin (Newcomer Supply, Cat. No. 1033B) by adding 5µl to one side and use a second microscope slide to smear the gelatine evenly along the entire slide in one smooth motion. This is akin to a blood smear. Then dry the coated slide for 1 hour in a 60°C oven.
2. Section the OCT-embedded tissue with a cryostat at 12-15µm thickness on to the centre of the coated slides.
3. Outline the tissue with ImmEdge hydrophobic pen and leave to dry overnight at room temperature.

3.3 Permeabilization, Blocking, Staining and Mounting

1. Wash tissue with PBS for 5 minutes to remove OCT medium and aspirate this away.
2. Cover the tissue with blocking solution and leave for 1-2 hours at room temperature in a humidified chamber.
3. Prepare the panel of antibodies diluted in antibody-staining solution.
4. Aspirate the blocking solution and then apply the first designed primary antibody panel (see Notes 1-6 and **Table 1** for details). Incubate overnight at 4 °C in a humidified chamber.
5. Wash with 1xPBS three times then mount the sample with a drop of Fluoromount G mounting media (Southern Biotechnologies, Cat. No. 00-4958-02) and a coverslip.

6. Allow one hour for the slide to settle at room temperature before imaging. Keep the time between mounting and imaging consistent between IBEX rounds.

3.4 Imaging with a confocal microscope

1. Use a confocal microscope according to standard operating instructions (we employ a Leica TCS SP8 X inverted confocal microscope) to image the fluorophores in the panel designed.
2. Place the slide firmly into the top left corner of the stage insert, so the position remains consistent.
3. Record all the parameters, as these will need to be reloaded at the next imaging session, such as objective, zoom, resolution, z-stack size (number of steps and spacing). Set the z-stack clearly within visible tissue where the fiducial marker (e.g. DAPI) is imaged and distinctive.
4. Acquire and save the image.

3.5 Sample Bleaching

1. Remove the slide and place in a 50 ml falcon tube filled with 1xPBS and wait around 20 minutes until the coverslip slides off spontaneously.
2. Wash three times for 5 minutes in 1xPBS.
3. Apply freshly prepared LiBH4 solution to the tissue at room temperature for 15 mins exposed to standard room lights. Remove and replace with fresh LiBH4 solution for a further 15 minutes.
4. Wash the sample with 1xPBS for 5 minutes three times.
5. Prepare the next panel of antibodies diluted in antibody-staining solution.
6. Aspirate the PBS and then and apply the next antibody panel (see Notes 7-11 and **Table 1** for details). Incubate overnight at 4°C in a humidified chamber.
7. Wash with 1xPBS three times then mount the sample with a drop of Fluoromount G mounting media and a coverslip.
8. Allow one hour for the slide to settle at RT before imaging. Keep the time between mounting and imaging consistent between IBEX rounds.

3.6 Realignment on confocal microscope

1. Replace the slide into the top left corner of the slide insert.
2. Reload the previous parameters to ensure consistent with the last acquisition.
3. Open the image file from Panel 1 and use the fiducial marker (e.g. DAPI) to realign the start and end of the z-stack of the current sample to this using the distinctive nuclear structures manually. This needs to be close, but the registration software will refine the fit in post-processing.
4. Acquire and save the image.
5. Repeat from step 4.5.1 multiple times, until all the desired panels have been imaged.

3.7 Post-processing and registration

1. Transfer all the confocal imaging files for the multiple rounds to an image processing workstation. Imaris software is recommended for this workflow.
2. Perform initial channel labelling and any pre-processing such as gaussian smoothing, brightness and contrast correction required.
3. Label the channels as required by the SITK-IBEX package (e.g. P1_GFAP_AF647) and save the files.
4. Launch and run the SITK-IBEX Python package to align the rounds as per the included standard instructions.
5. The final combined file can then be reviewed in Imaris and taken forward for image analysis as dictated by the scientific aims.

4. Notes

1. Careful planning is required to design panels of markers (antibodies or lectins) to be used with the IBEX technique. The goal is to minimise the overlap of signal between one round to the next and optimise the fit of available fluorophore conjugated antibodies into the minimum number of rounds. The microscope or imager setup will determine how many fluorophores can be detected in a single panel.
2. Ensure planned markers are compatible with IBEX and wherever possible use antibodies directly conjugated to fluorophores.
3. Unconjugated primary antibodies which require a fluorescent secondary antibody can be used but they must be used in rounds in which there are no other antibodies that were raised in the same species, and where there have been none in the preceding rounds. This is because IBEX only inactivates fluorophores, it does not strip antibodies unlike other approaches. Alternatively purified antibodies can be labelled with commercial antibody conjugation kits.
4. It is advisable to avoid using antibodies which target the same cell type, or tissue region in the same round as this makes it harder to distinguish if there is spectral overlap of fluorophores.
5. Ensure you use antibodies conjugated to fluorophores which have been validated to bleach in the original protocol (5) or that you have tested the bleaching on.
6. Include a common marker which does not bleach as a fiducial marker throughout all the rounds for the SITK-IBEX registration software. Usually, a reliable nuclear marker such as DAPI is ideal, or use an antibody conjugated to Alexa Fluor 594.
7. Use the smallest number of rounds possible by fitting as many antibodies and different fluorophores into each panel as the microscope setup allows.
8. Place the most abundant and therefore brightest markers, for instance lectins or highly efficient antibodies in later rounds to minimize leftover signal that can arise from less efficient bleaching due to shear abundance.
9. Place the weakest markers or challenging antibodies (e.g. transcription factor or phosphoprotein directed antibodies) in early panels to increase the likelihood of a detectable signal.
10. Before starting the full IBEX protocol, test each planned panel of antibodies on separate tissue slides to confirm they work individually, titrate the concentrations and ensure the markers are distinguishable in each round.

11. For additional information and help see the IBEX Knowledge Base and Forum at https://doi.org/10.5281/zenodo.7693278

5. References

1. Booth LA, Tavallai S, Hamed HA et al (2014) The role of cell signalling in the crosstalk between autophagy and apoptosis. Cell Signal. 26(3):549-55.

2. Borst J, Ahrends T, Bąbała N et al (2018) CD4+ T cell help in cancer immunology and immunotherapy. Nat Rev Immunol. 18(10):635-47.

3. Kuchitsu Y, Mukai K, Uematsu R et al (2023) STING signalling is terminated through ESCRT-dependent microautophagy of vesicles originating from recycling endosomes. Nat Cell Biol. 25(3):453-66.

4. Radtke AJ, Chu CJ, Yaniv Z et al (2022) IBEX: an iterative immunolabeling and chemical bleaching method for high-content imaging of diverse tissues. Nature Protoc. 17(2):378-401.

5. Radtke AJ, Kandov E, Lowekamp B et al (2020) IBEX: A versatile multiplex optical imaging approach for deep phenotyping and spatial analysis of cells in complex tissues. PNAS. 117(52):33455-65.

6. Valls PO, Esposito A. (2022) Signalling dynamics, cell decisions, and homeostatic control in health and disease. Curr Opin Cell Biol. 75: 102066.

Table 1. List of antibodies used in the study

Target	Host	Fluorophore	Manufacturer	Catalog number	RRID	Dilution
$p16^{INK4a}$	Rabbit	AF546	Santa Cruz	sc-1661	AB_628067	1:50
STING (D2P2F)	Rabbit	AF647	Cell Signaling Technology	13647	AB_2732796	Conjugation kit then 1:25
NF-κB p65	Rabbit	AF546	Santa Cruz	sc-8008	AB_628017	1:25
Iba1	Goat	Purified	Abcam	ab5076	AB_2224402	1:50
Goat IgG (H+L)	Donkey	Secondary AF546	Thermo Fisher	A-11056	AB_2534103	1:1000
PDGFR	Rabbit	AF488	Abcam	ab196376	AB_2936929	1:100
GFAP	Rabbit	AF647	Biolegend	837511	AB_2734610	1:200
PKCα	Rabbit	AF680	Santa Cruz	sc-8393	AB_628142	1:100
Glutathione Synthetase	Rabbit	AF488	Proteintech	CL488-66323	AB_2883309	1:100
Calbindin D-28K	Rabbit	AF550	Millipore Merck	ABN2192	AB_2935805	Conjugation kit then 1:25
CD45	Rabbit	iFluor 594	Caprico Biotechnologies	1016136	AB_2936930	1:25
Collagen IV	Rabbit	AF647	Abcam	ab19808	AB_445160	1:50

AF: Alexa Fluor™

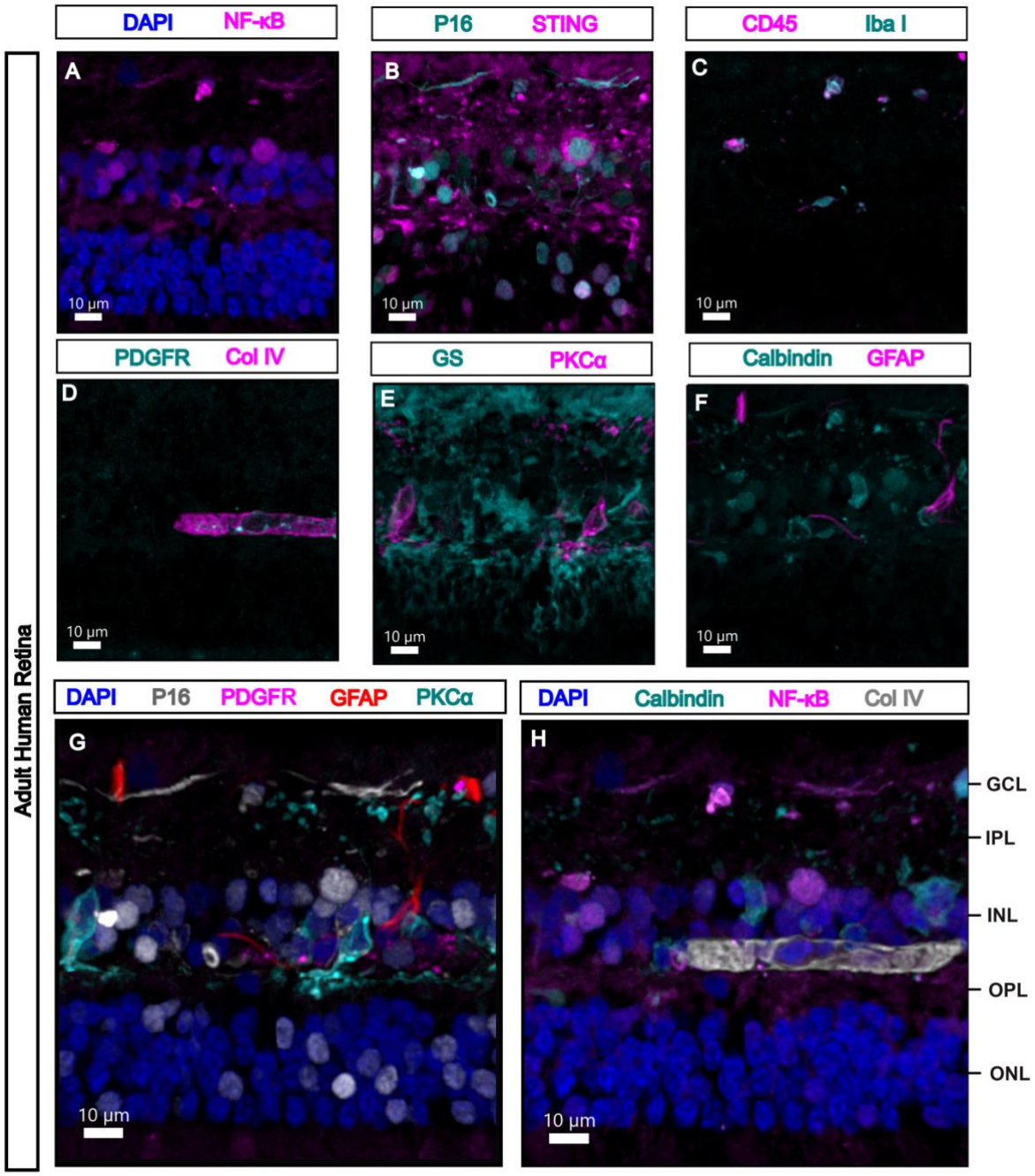

Figure 1. An exemplar 12-marker IBEX dataset performed on adult human retina. The same location of the sample is shown in all panels. For clarity two markers per panel are shown in **A-F**. Merged panels of five markers illustrate the combined panel from multiple rounds in **G-H**. White arrows indicate a neuronal cell where three different signalling markers are detectable. Other markers identify surrounding retinal structures and cell types that may be interacting. GCL: ganglion cell layer, IPL: Inner plexiform layer, INL: inner nuclear layer, OPL: outer plexiform layer, ONL: outer nuclear layer.