**Protocol for Iterative Bleaching Extends Multiplexity (IBEX) with Zebrafish retina (multiple rounds of immunohistochemistry on the same sample)**

**Day 1**

Grow embryos to desired stage

1. Add tricaine.
2. Fix in Eppendorf in 4% PFA overnight
3. Wash with PBS and replace with 30% sucrose solution
4. Embed embryos in OCT in moulds facing the same way
5. Freeze in -80°C for 5 minutes then store in -20°C for up to a week
6. When ready to section, coat microscope slides with chrome alum gelatine; Add 4uL to one end of the slide and smear it across in one motion with a coverslip. (this makes sure the tissue remains adhered to the slide over the course of many rounds)
7. Dry the slides for 1 hour at 60°C in an incubator/oven
8. Cryosection tissue 10-14 um onto dried coated slides (in the centre of the slide ideally, avoid the end furthest from the frosting for easy coverslip removal in the future)
9. Leave covered on the bench to dry overnight at room temperature (RT).
10. Freeze slides the next morning **if not** using immediately

If using immediately:

1. Place slides in Coplin jar with PBS to rehydrate and wash off OCT 2x5 minutes
2. Boil slides in rice cooker in a Coplin jar filled with sodium citrate (or other desired antigen retrieval depending on the antibodies/tissue being used) for 20 minutes
3. Leave to cool in Coplin jar on the bench until around RT (45 minutes)
4. Wash with PBS 3x5 minutes either in Coplin jar or use PAP pen (instruction from now assumes Coplin jar is used for washes)
5. Place slide in a humidified chamber and add 200 uL block solution (10% goat serum, 1% BSA, 0.8% Triton X, 0.1% Tween made up with PBS) and gently cover with parafilm, leave at RT for 1 hour
6. Make up primary antibody solution as per usual dilutions in block solution (if using FlexAble kits see section “FlexAble” below) with DAPI 1:500
7. Remove parafilm gently and pour/wipe off excess block solution, immediately add 150 uL of the antibody solution to the slide and reapply the parafilm
8. Leave in 4°C overnight in humidified chamber

**Day 2**

1. Remove parafilm and wash in PBS 3x20 minutes.
2. If using secondary antibodies (see section “panel design” below), add 1:1000 in PBS and leave for 1-2 hours at RT in humidified chamber then wash 3x10 minutes in PBS
3. Remove slide from PBS and remove excess liquid with an aspirator or tissue
4. Add 2 drops of mounting media with a pipette tip.
5. Place coverslip over tissue (ideally in the centre of the slide) by placing down on one end at a 45-degree angle then lowering the other end slowly with forceps to ensure no air bubbles form
6. Leave to dry at RT in the dark for at least an hour before imaging. (note: this should be a consistent time period for each round)
7. Image on confocal/imager making sure to record:
   1. Objective used
   2. Optical zoom
   3. Rotation
   4. Resolution
   5. Line averaging
   6. Fudicial for alignment
   7. Z-stack direction
   8. Z-stack step size (um)
   9. Total z-stack size (um)
   10. Number of steps
   11. Galvo or wide
   12. Number of Tiles
   13. Position on slide of the sample imaged (will need to be located again every time)

**Note**:

* All of these recorded parameters must stay the same for every imaging round
* Make sure the top and bottom of the z-stack has distinguishable structure/landmarks which can be recognised in following imaging rounds

1. After imaging, gently wipe of any oil on the coverslip then place slide into a 50 mL falcon tube and wait for around 20 minutes for the coverslip to slide off the tissue
2. Remove slide from tube slightly to allow the coverslip to slide off slowly and dispose of the coverslip
3. Wash slide 3x5 minutes with PBS to remove mounting media
4. Make up Lithium borohydride solution (in a fume cupboard add 5 mL purified water from a 15 mL falcon to a weighing boat with 5 ug lithium borohydride powder then pour solution back into 15 mL falcon and replace lid LOOSLY as hydrogen gas will be released through the reaction. Make sure no water comes in contact with the Lithium borohydride powder stock and keep it uncovered for the minimum amount of time and store appropriately in for example with drierite
5. Leave the solution in the fume cupboard for 20 minutes until there are visible bubbles in the solution. This can now be removed and should be used within 2 hours
6. Add 200uL of the lithium borohydride solution to the slide and leave on the benchtop in direct light for 15 minutes replacing with fresh lithium borohydride for a second 15 minutes
7. Wash 3 x 10 minutes in PBS (important: as skipping this wash means the next round of antibodies may be deactivated as soon as they are added)
8. Add the next round of primary antibodies and put in 4°C overnight
9. Continue from day two for as many rounds as possible/desired (until tissue quality degrades or all desired markers have been used)

**Note**:

* another antigen retrieval step can be carried out between rounds if some makers work better with Tris-HCL for example
* when imaging after the first round, make sure all parameters are the same as those recorded on the first imaging session, adjust channels for optimal imaging an ensure the area of imaging is the same by opening the round 1 image file and comparing. Importantly, if imaging a z-stack, make sure that the top and bottom of the stack are in the same place as in round 1. Always align to round 1 when imaging for consistency.

**Image processing**

1. Process all image files individually and import into Imaris or Imaris Viewer (Oxford Instruments).
2. Name channels in the format RoundNumber\_Marker\_Fluorophore using only letters and underscores, for example the channel representing GS AF488 signal in the fourth round may be called R4\_GS\_AF488
3. Use open-source registration software (<https://github.com/niaid/sitk-ibex> ) (Radtke *et al,* 2020) to register files together
4. Place files named after their corresponding round in the same folder
5. Select the files and input \_ as the separator and DAPI as the alignment channel and run
6. Resample and save combined image
7. Select DAPI for input output correlations which shows how well the alignment/registration has worked
8. The single Imaris image file containing all rounds aligned will be called ‘output.ims’
9. This can be imported into other image analysis software or exported as a TIFF for example

**Proteintech FlexAble Antibody Labelling Kits**

1. Calculate amounts depending on individual antibody concentrations (check suppliers' website)
2. If making enough for a single slide (150 uL) we recommend:
   1. 2 FlexAble reactions
   2. 1 ug Antibody
3. Complete conjugations as per FlexAble protocol
4. Test if FlexAble kits work with chosen antibodies before adding into an IBEX procedure.

**Notes on panel design**

The following guidance will help produce the most effective IBEX panels:

* Ensure planned markers are compatible with IBEX
* Use primary conjugated antibodies where possible – secondary antibodies can be used in the first round as long as none of the other antibodies are raised in the same species
* Ensure you use antibodies conjugated to fluorophores which have been validated to bleach in the original protocol (Radtke *et al.,* 2020) or that you have successfully tested the bleaching process on
* Include a common marker in every round for alignment, we use DAPI
* Place bright or unbleachable markers in the last rounds to minimize leftover signal and place weaker markers in early panels
* Test antibody rounds individually before committing to the full IBEX

**Reference**

A. J. Radtke, E. F. Kandov, B. C. Lowekamp, E. Speranza, C. Chu, A. Gola, N. Thakur, R. Shih, L. Yao, Z. R. Yaniv, R. Beuschel, J. Kabat, J. Croteau, J. Davis, J. M. Hernandez, R. N. Germain "IBEX - A versatile multi-plex optical imaging approach for deep phenotyping and spatial analysis of cells in complex tissues", Proc Natl Acad Sci, 117(52):33455-33465, 2020