



## Standard operating procedure: IMC – Staining, Ablation and Analysis

### HEALTH AND SAFETY HAZARDS AND CONTROL MEASURES



#### Hazardous chemical - Xylene

**Xylene** is a Flammable. Irritating to the skin, eyes, mucous membranes, and respiratory tract. A carcinogen that may damage the liver and kidneys through prolonged/repeated exposure. Acute toxicity. Wear either nitrile gloves or xylene resistant gloves. Always use in a chemical fume hood. Do not wash into drains, dispose via chemical waste route. Store in fume cabinet separate to flammable solvents. Note: if nitrile gloves are used, use forceps to minimise exposure to xylene.



#### Hazardous chemical - Ethanol

**Ethanol** is a highly flammable liquid and vapour. It causes serious eye irritation. Wear nitrile gloves. Minimal risk of eye splash as ethanol steps are performed in a fume hood. Avoid use near flames, heat, or sparks.



#### Hazardous chemical – Triton X-100

**Triton X-100** is a corrosive liquid. It can cause mild skin irritation and serious eye damage. Wear either nitrile gloves or xylene resistant gloves. Use concentrated Triton X-100 in fume hood/micro cabinet or wear safety goggles to prevent eye splash. Do not wash into drains, dispose via chemical waste route.



#### Hazardous gases – Argon

Argon is an inert noble gas, with an expansion rate of 696 times its liquid volume when it evaporates. Any leaks in the vessel could displace the oxygen in the room, leading to asphyxiation. The Hyperion suite is equipped with an O<sub>2</sub> monitor, the area must be immediately evacuated in the event the O<sub>2</sub> levels deplete below 20%.



#### Hazardous chemical – PAP pen

PAP pen contains a flammable liquid, known to cause irritation to the skin and lungs upon exposure. It is also a known carcinogen, can damage fertility and may cause damage to an unborn child. Gloves should be always used when using the PAP pen, any pregnant persons should contact OH regarding the use of a PAP pen.

**Steamer** – Hot steam may cause burns. Heat may cause glass containers to break. Staff/students are trained in the safe use of the steamer. Only use plastic containers in the steamer.

### PROTOCOL

#### Imaging Mass Cytometry

- All tissue stained for the IMC should be between 5-10µm thick to ensure optimal results.



- Fluidigm recommends conjugation of antibodies with their MaxPar X8 Kit.
- Regions of Interest on the tissue are drawn and then ablated using a laser at a frequency of 200Hz at a 1 $\mu$ m resolution.
- Ensure all channel '.tiffs' files and their associated masks per sample are stored in a distinct folder or the HistoCAT software will not open the images as needed.

### Staining

1. In a fume hood: Dewax slides in a Xylene (Honeywell) bath for 5 minutes, then move the slides into a second Xylene bath for another 5 minutes.
2. In a fume hood: Rehydrate the slides through an ethanol (VWR) series, 100%, 100%, 90%, 70% for five minutes each, before cleansing in dH<sub>2</sub>O for five minutes.
3. Perform an antigen retrieval in a pH=9 1xEDTA (30ml 10XEDTA/270ml dH<sub>2</sub>O) buffer, using a steamer (CookWorks) for 20 minutes.
4. Cool the slides on ice for 5 minutes, then cleanse in dH<sub>2</sub>O followed by 1xPBS for 5 minutes each.
5. Draw a hydrophobic barrier around the tissue with a PAP pen (reproductive toxicity hazard) (Abcam) and treat with 10% normal horse serum (Vector) in primary diluent for 60 minutes.
6. Treat the slides with a pre-prepared antibody cocktail, made of your conjugates diluted in primary diluent, then incubate at 4°C overnight.
7. Treat the slides in 0.02% Triton X-100 (Sigma-Aldrich) in 1xPBS for 8 minutes, the slides are then cleansed in dH<sub>2</sub>O twice for 5 minutes each.
8. Treat the tissue with Ir-Intercalator (Fluidigm) at a concentration of 1:400 in 1xPBS for 30 minutes.
9. Cleanse the slides in dH<sub>2</sub>O for 5 minutes, leave to dry at least 20 minutes before running on the Hyperion.

### Ablation

1. Ensure that the argon and helium gases are open and that the pressure is optimal (80kPa argon/50kPa helium).
2. Start the 'CyTOF' software (Login: admin / no password), allow to initialise.
3. Click 'Start' in the top left corner, ensure the Plasma Start Up Sequence finishes successfully in the log manager (in the bottom left corner).
4. Once the sequence is complete, leave for 30 minutes for the plasma to stabilise.
5. Load a manufacturer's 3-Element Full Coverage Tuning Slide (Fluidigm) and run an 'autotune' 100 $\mu$ m wide and 500 $\mu$ m high, ensure all sub-calibrations are ticked before starting.
6. Ensure that the 'AutoTune' has been successful by comparing the results to the word document 'AutoTune for Calibration' in the Desktop.
7. Click on the 'Data Acquisition' tab and open a new .mcd file matching your slides specifications
8. Take a picture of your slide, then right click on the template, and select import image. Ensure that the image is oriented with the slide label on the right-hand side.
  - a. We recommend taking a picture on your mobile device and emailing the image to your college email account, which can then be accessed via the IMC PC
9. Use the panorama tool to draw an area you wish visualised, click on the panorama tab at the bottom of the screen and click 'Start' to acquire your image.
10. Once visualised draw an area you wish ablated using the ROI (region of interest) tool, click on the ROI tab at the bottom of the screen.
11. Ensure your ROI has a suitable template by right clicking the template box, either select a template or create a new one.



- a. A 'template' is a list of Ln-isotopes you wish to collate data for from the IMC detector
- b. Select the isotopes required using the 'Period Table' tool
- c. ROI will not start without a template, including 'energy test' ROI
12. Ensure you have selected an energy level that cuts through the tissue but does not damage the glass below it.
  - a. You may wish to run an 'energy test,' draw several ROI (100µmx100µm)
  - b. Give them sequentially larger energy levels and click 'Start.'
  - c. The ROI that cuts through the tissue but does not damage the glass is the best energy level for your tissue.
    - i. Damage to the glass will appear as grid-like grooves in the slide.
    - ii. The laser should cut through as much tissue as possible, ideally leaving a clear ablation area with no obvious damage to the glass slide.
13. Once the ROI have been setup click 'Start' to begin ablation.
14. Once all tissue has been ablated shut-down the system by clicking 'Stop' in the top left corner of the screen.
  - a. You may also tick the 'Shutdown' box in the 'Data Acquisition' tab to shut down the device when your final ROI completes.
15. Wait for the 'Plasma Stop Sequence' to finish successfully.
16. Turn off the argon and helium gas supply.

### Masking and Segmentation

1. The IMC data will be saved as a .mcd file.
2. Open the data using the MCDViewer software available from Fluidigm.
3. Export your data by selecting File->Export, then select to export the data as either a single stacked 'ome.tiff' or several individual 'ome.tiff's for each channel.
  - a. Either a single 'ome.tiff' stack or individual 'ome.tiff' files are viable options and can be opened in ImageJ.
4. Open these 'ome.tiff' using ImageJ (1.53c), then use the Despeckle function to remove single pixels using Process -> Noise -> Despeckle.
5. Select Image -> Adjust -> Brightness and Contrast and adjust to remove any background.
6. Save each individual channel as a 'tiff,' keeping all .tiff files in sample specific folders.
7. Select 6 channels, plus 1 DNA channel, to create your mask. The channels are ideally cell markers denoting unique cell types and phenotypes.
8. Change the colour of the channels, red for the cell markers and green for the nuclei, then save as a JPEG.
9. Open the Ilastik software (1.1.3post3) and start a 'pixel classification', then follow the steps of the Ilastik protocol, detailed below.
  - a. Click 'Add New.' and import your JPEG.
  - b. Click 'Feature Selection' -> 'Select Features...' then tick every box in the 'Features' window.
  - c. Click 'Training,' then 'add label.' Rename your labels nuclei, signal, and background, then start identifying each group. Only do a small section, then check your labelling by selecting 'Live Update' then 'Segmentation' and get an update on segmentation process.
  - d. Click 'Prediction Export,' then 'Choose Export Image Settings...' and ensure the format is set to 'tiff.' Your JPEG can now be exported as a probability map for masking.
10. Open the CellProfiler software (4.2.1) and open the 'CellProfiler' pipeline (details below), select 'Analyse Images.' Masks will now be generated for your samples.



- a. IdentifyPrimaryObjects will use the nuclei signal to create 'objects' each object representing a single nucleated cell.
- b. IdentifySecondaryObjects will expand around all primary objects (nuclei) with surrounding signal, associating all cell signals with a nucleus.
- c. IdentifyTertiaryObjects fills in the space between the primary and secondary objects, effectively representing the cytoplasm.
- d. MaskObjects will turn these objects into a 'mask' which can then be overlaid on the individual '.tiff' channels to infer each cells characteristic.
- e. Each mask should be named after its corresponding sample and ROI.

### Data Analysis

1. Ensure all individual channel '.tiff' files and their associated masks in a sample are stored in distinct folders.
2. Open the HistoCAT software (1.76) then select Load -> Load Samples and import in the case folder containing all individual. tiffs and the mask.
3. Accept the pixel expansion and do not transform data, then select a folder to save the 'gates. Please note this is where your data will be exported to.
4. Once your files have loaded select 'Save -> Export gates as CSV,' your data will now be accessible in a .csv format.
5. You may infer your 'minimum positive value,' the lowest your cell signal can be in each channel that can be considered a positive signal, from your ImageJ edits previously the minimum set using the Brightness and Contrast tool.
  - a. Open the B&C tool then open the .tiff image, the minimum value will be represented on the B&C tool.
  - b. You may also choose to record the minimum values when you are first processing the images in ImageJ.
6. Colocalisation analysis can be done in one of three ways.
  - a. Pull the channel data from your .csv file, if both channels are positive there is colocalisation if one is not there is no colocalisation. This result can either be presented as a total number of colocalised cells or as a percentage of colocalised cells in each population.
  - b. Open the two channels on the ImageJ program, then use the 'Analysis -> Colocalization -> Colocalization Threshold,' the level of colocalisation is generated as Rcoloc with a scale between 0 (no colocalisation) to 1 (complete colocalisation).
  - c. Merge the two channels together on the Image program, using 'Image -> Color -> Merge Channels,' then manually count all the colocalised cells. This result can either be presented as a total number of colocalised cells or as a percentage of colocalised cells in each population.
7. Proximity analysis can be performed using the 'Create ring regions new' macro made by the PM Group.
  - a. Set the scale via 'Analysis -> Set Scale' and set 'Distance in pixels' to 1 and 'Known distance' to 1 and 'Unit of Length' to  $\mu\text{m}$ .
  - b. Then use the macro via 'Plugin -> Macros -> Create ring regions New,' open your channels and draw an ROI around the object being investigated. Add to the ROI manager (which should open when you start the macro).
  - c. Then open the channel of the signal you are investigating and run the macro and create 1 ring region  $10\mu\text{m}$  thick. The average signal of the area around the object will now be recorded.
8. Correlation analysis can be done by copying your channel data from the .csv file, then import the data into GraphPad prism and do a 'Correlation Analysis' to get an r value and a p value



