ScopeM Tasks

Wednesday, June 25, 2025 7:37 PM

1.

- Nd2 file compatibility (old dataset were denoised tif files, these are the raw nd2 files) (adapt the deep learning model to recognize annotated cells from the old data set) (high)
- Flatfielding for non-uniform illumination correction / background subtraction (high)
- Same pipeline but without excluding the membrane signal (quantify all the signal) (high)
- Automated/manual removal of dead cells (incomplete membrane staining in channel 2) (medium)
  - Might also be possible by excluding results of cells that show very high uptake values (dead cells usually show a lot of antigen signal), probably easier to implement?
- Include 4th channel: quantify co-localization of antigen (C1) channel and new channel (would probably be C2) (low, maybe in future)
- Identify single cells -> crop -> new tif file for every single cell --> downstream processing (reslicing, max projection etc.) for visualization easier (low, but very nice to have if easily doable)
- For the plots: ability to plot more than 2 groups next to each other. Ideally: 2 conditions: PEG12 / PEG34 and in each condition the 3 timepoints (0,15,30min) (low, can also do it myself with the csv results files)

2.

- User-defined threshold parameter from which z plane upwards the quantification happens (e.g. Quantify slices 20-100, but exclude 1-20) (high)
- We need this because there is a homogenous distribution of the antigen at the synapse plane which we dont want to include since it has not been taken up by the cell yet
  - Ideally with identification of synaptic plane (determined as brightest plane in channel 3) and then going 1-2 um (user-specified distance) above
    - This would help in case the images have differing start/end positions (Z-wise) --> normalize by using the synaptic plane as starting point

- Identify antigen clusters inside cell --> generate mask (discrete object segmentation) (medium)

Similar to this:

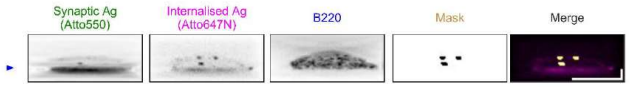

Figure 43: Antigens are considered internalised only 1.5 µm above the synapse. Example image from Matlab analysis showing the mask (yellow) for internalised antigens. In this example, only Atto647N+ endosomes (magenta) are considered internalised if they reside 1.5 µm above the synaptic plane (blue arrow), determined as the sharpest plane by the Matlab script. The fluorescence signal from Atto550 (green) on the DNA sensor, as well as B220 (blue) staining are also shown. Scale bars, 5 µm.

3.

Extract these metrics from images:

- **Total extracted antigen method 1: total antigen signal in each cell mask (like before, but exluding the synaptic signal as descibed in 2.) (high)**
- **Total extracted antigen method 2: sum of pixel intensities inside antigen cluster mask (C1) divided by total antigen signal in each cell mask (if this makes sense in your opinion) (medium)**

- Mean synaptic antigen intensity (at the contact point of the cell with the synapse): (low)
- Percentage of cells extracting at least one antigen cluster (low)
- Number of clusters extracted per cell (low)
- Mean antigen intensity per cluster (low)

Similar to this:

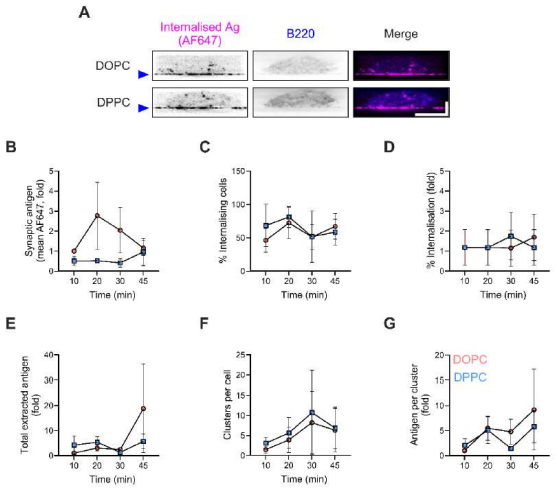

Figure 41: Antigen internalisation in A20 B cells. A20 B cells were plated onto DOPC and DPPC PLBs coated with anti-IgG at a density of 120 ± 26 molecules/µm^2 across all independent experiments. Cells were allowed to interact with the bilayer for the desired time and fixed. Lateral views of example cells are shown in panel **(A)**. The blue arrow highlights the synaptic plane. Horizontal scale bar - 5 µm and vertical scale bar - 10 µm. Image quantification provided **(B)** the mean synaptic antigen intensity, **(C)** the percentage of cells extracting at least one antigen cluster, **(D)** the percent antigen internalisation, **(E)** the total extracted antigen, **(F)** the number of clusters extracted per cell, and **(G)** the mean antigen intensity per cluster. N = 3 independent experiments; across all experiments n = 181 cells for DOPC and 132 cells for DPPC. Data normalised to DOPC 10 min where stated, and plotted as mean ± SEM.

This is from the methods section of this thesis: https://kclpure.kcl.ac.uk/portal/en/studentTheses/characterising-the-effects-of-antigen-mobility-and-valency-on-b-c

2.11.4 Z-stack image processing

Prior to z-stack image processing and quantitative image analysis, **images were cropped to remove poorly illuminated areas at the edge**s, background-subtracted to remove camera dark counts, and flatfield-corrected to account for the Gaussian profile of the illuminating laser. Flatfielding is described 289 and ImageJ macros for flatfield corrections can be found in Section 7.5.2 of the Appendix, Chapter 7. In short, flatfield images were background-subtracted and then normalised to the mean of their pixel values. Each channel of a background-subtracted raw image was then divided by the corresponding normalised flatfield image.

Analysis of multi-colour z-stack images then was performed with a user-guided pipeline in Matlab (Mathworks) tethered to ImageJ 298 via the MU plugin (http://bigwww.epfl.ch/sage/soft/mij/). The Matlab script was developed by Prof. Pavel Tolar and has been described in detail previously 81,147,148. Briefly, cells were detected by initial object detection in 2D and edge refinement in 3D using both the B220 and brightfield channels. 3D cell masks were then stored as image stacks for subsequent analysis, where cells touching the edge of the image were excluded. The **synapse was identified as the sharpest image plane**. **Antigen extraction was then determined for each cell by bandpass filtering each plane and identifying antigen positive clusters (either Atto647N+ or AF647+) above the synapse using a user-defined global threshold. Antigens were only considered as internalised if they were at least 3 planes (1.5 µm) above the synapse plane. Local background due to fluorescence scattered from the substrate was removed from each identified antigen cluster by local background subtraction using a 3-pixel wide rim around the cluster in each plane**. Data were inspected and manually gated using a custom-written visualisation tool in Matlab to **remove any non-cellular debris, incorrectly segmented cells, and poorly B220-stained cells from the analysis.**

The mean and total antigen fluorescence intensity of the pixels in the synapse plane was used as a measure of synaptic antigen binding. The total extracted antigen was calculated as the sum of pixel intensities in all extracted antigen clusters. The percentage antigen internalisation was calculated as the total extracted antigen divided by total extracted antigen plus the antigen in the synapse. The mean antigen intensity of extracted clusters was used as a measure of antigen per cluster. Masks of the extracted antigen clusters, synapse, and total cell were used to quantify fluorescence in other channels. Cell area was calculated from the area of the cell mask at the synapse plane. Cells were considered as failing to extract antigen if no antigen clusters were detected in the cell.

Main goal: quantify antigen uptake difference between timepoints and conditions (later timepoints --> more signal, unknown how conditons affect it)

0 min timepoint 30 min timepoint --> more antigen uptake and in higher z planes