**TCR triggering toolbox**

Using 2-colour PALM/STORM or STORM/STORM, it is now possible to study TCR triggering efficiency at the receptor level. TCR triggering efficiency is defined as the ability of an external stimulus to lead to sustained TCR-CD3 phosphorylation.

To stimulate T cells, you can use glass coated with activating antibodies (anti-CD3 + anti-CD28) or with pMHC, or you can use supported lipid bilayers presenting ICAM-1 and pMHC. Different stimulations can lead to different cellular outcomes, and to investigate the mechanism of TCR triggering, we are using a single-molecule degree of colocalisation (DoC) analysis to correlate the **spatial distribution of TCR** with the **degree of triggering** (or degree of colocalisation with other proteins). Our approach is to image the localisation of total CD3ζ and of phosphorylated CD3ζ (or pCD3ζ). For example, we are imaging CD3ζ-PSCFP2 by PALM and pCD3ζ-AlexaFluor647 by STORM.

In this toolbox, you will find protocols and explanations for the following steps:

1. Making bilayers (or coating glass with antibodies or pMHC)
2. Sample preparation (cell transfection, stimulation and immunostaining)
3. Two-colour PALM/STORM imaging
4. Data analysis
5. Summary protocol
6. Annexes
7. **Supported lipid bilayers**

This section describes the preparation of surfaces – such as glass coated with PLL, activating antibodies, or pMHC; or supported lipid bilayers.

For SMLM, the glass needs to be very clean to reduce the background. Bilayers are prepared in chambered Labtek dishes (8-well Labteks, Nunc). Coating with PLL, antibodies or pMHC can be done on high precision coverslips (round or square, 18 x 18 mm; Zeiss) or in Labteks. Depending on your application, you may also want to try low-background precision cover glasses (Marienfeld, Australian Scientific).

*Note: if using Labteks, do not clean chambers with acetone, as this will dissolve the plastic!!*

* **Coating glass with PLL, antibodies or pMHC**

**Cleaning coverslips**

There are many different protocols for cleaning coverslips. Depending on your samples, you may have to adapt the cleaning procedure. Here is a simple/general one that works quite well.

* Wipe coverslips with ethanol wipe, then with a clean wipe, then hold with tweezers.
* Using 10ml pipette, squirt MilliQ H2O on each side.
* Squirt acetone on each side.
* Squirt 70% ethanol on each side.
* Dry with nitrogen stream (use filter to avoid blowing particles onto the coverslip).
* Optional: draw ‘circle of grease’ with Dako pen to confine the sample.

Use small Petri dishes (from TC) or 6-well plates to store coverslips once cleaned.

**Coating with PLL**

* Add 0.01% (w/v) poly-L-lysine (PLL) and incubate for 30 min at room temperature.
* Wash 3x in MilliQ H2O.
* Air dry for 30 min (preferably in a TC hood) – has to be dry before adding the cells.

**Coating with antibodies**

* Add anti-CD3 and CD28 antibodies (10 µg/ml each) and incubate for 1h at 37°C (or overnight at 4°C).
* Wash 3x in PBS before adding cells, but don’t let them get dry. If you need to store them, keep them at 4°C in PBS.

**Coating with pMHC**

Here again there are different protocols for coating with pMHC (or pMHC and ICAM-1). You can start with this one (all steps at room temperature).

* Clean coverslip (as described above) and plasma clean for 5 min.
* Coat with PLL (as described above).
* Incubate with 5% BSA for 30 min.
* Wash 3x with PBS.
* Incubate with streptavidin (1 µg/ml) + NiCl2 (100 mM; if planning on adding ICAM-1) for 30 min.
* Wash 3x in PBS.
* Incubate with pMHC (and ICAM-1) for 60-90 min.
* Wash 3x in PBS.
* **Preparing supported lipid bilayers**

**Cleaning Labtek chambers (if using fresh ones)**

* Clean the coverglasses with 10 M NaOH for 15 min.
* Then 1 M HCl in 70% ethanol for 10 min.
* Rinse chambers thoroughly with MilliQ water.

**Cleaning Labtek chambers (if separating plastic chambers from coverglass and reusing them)**

* Clean the coverglass with MilliQ H2O.
* Sonicate in 1M KOH for 20 min.
* Wash 3x in MilliQ.
* Sonicate in MilliQ for 15 min.
* Sonicate in 100% ethanol for 15 min.
* Dry cover glass in 70°C oven.
* Plasma clean for 10 min.
* Use dental glue to glue the cover glass onto the 8-well Labtek chambers.
* Dry for 1 hour.
* If not using them straightaway, keep them under vacuum.

**Preparing liposomes**

* Mix lipids in glass tube – composition can be variable but final concentration at 1 mg/ml (use **Lipid mix calculations spreadsheet** to calculate desired volumes, see **Annexe 1**). Lipids are purchased as powder or in chloroform. If dissolving lipids yourself, use glass pipettes. Small glass bottles for aliquoting lipids are in white cupboard (blue boxes). Overlay lipids with argon before freezing at -80°C. Once thawed, store lipids under argon at -20°C for up to 2 months.
* Go to the fume hood and evaporate chloroform under nitrogen flow (5-10 min). Dried lipids at the bottom of the tube have white misty appearance.
* Place dried lipids under vacuum overnight (in desiccator). Alternatively, continue drying with a gentle stream of nitrogen for about 30 min.
* Rehydrate lipids in 1 ml PBS or MilliQ H2O.
* Vortex 30s or more until lipids have detached from glass.
* Extrude (at least 15x through extruder) and clean extruder with isopropanol and water (a more detailed protocol of extrusion is available if needed). After extrusion, the liposome solution should be clear and no longer cloudy.
* Store liposomes at 4°C. Do not freeze them. Liposomes are good for 1-2 weeks under nitrogen.

Alternatively, liposomes can also be made using the tip sonicator:

* After rehydrating and vortexing the lipids as above, sonicate on ice for 2-3 cycles of 2 min sonication with output control of 2 and duty cycle of 80%.
* Check the clarity of the lipid mix and repeat if necessary.
* Filter liposomes through 0.2 µm filter and store at 4°C.

*Note: when working with lipids, do everything in glassware – not plastic!*

**Making bilayers**

* Dilute liposome solution to 0.2 mg/ml in PBS (1 ml total volume) and apply 0.25 ml of liposome mixture to each well (in the presence of 5 mM calcium chloride).
* After 30 min, rinse away excess liposomes with about 8 ml of PBS per well (e.g. using syringe and thin needle). Always leave about 0.25 ml at the bottom of the well. Never expose the bilayer to air – this will destroy it!
* Incubate bilayer with 100 mM NiCl2 for 10 min, RT, then wash away excess Ni2+ ions with PBS.
* Block with 0.1% BSA in PBS for 30 min, RT.
* Addstreptavidin (2 μg/ml) in 1% BSA for 10 min, RT, then wash away excess with 8 ml of PBS per well.
* Add biotinylated protein(s) (e.g. pMHC monomers) and/or His-tagged-ICAM-1/B7 over bilayer & allow to bind for 100 min, RT (or O/N, 4°C) then wash away excess (use at least 20 ml to reduce protein aggregation).
* Store protein-integrated bilayer at 4°C & use within 24 hours, but preferably the same day.

**Checking bilayer mobility by FRAP**

* Check the mobility of your bilayer by Fluorescence Recovery After Photobleaching (FRAP).
* Check both the mobility of your lipids (by using fluorescently-tagged lipids, e.g. rhodamine-tagged DOPE) and the mobility of your attached proteins (by using fluorescent streptavidin or fluorescently-labelled proteins). For example, I use fluorescent lipid at 1:1000 dilution for FRAP validation.
* Principle behind FRAP: when you bleach a region of your bilayer, the bleached region will be repopulated by non-bleached fluorescent lipids/proteins if the bilayer is mobile, whereas the bleached area will stay dark if the bilayer is not mobile. The speed and fraction of the fluorescence recovery will provide information about the diffusion coefficient and mobile fraction of the sample.
* For FRAP, use Olympus FV1000 or Leica SP5 (BMIF). For a detailed explanation of doing FRAP on the Olympus FV1000, see **Annexe 2**.
* For data analysis, use **FRAP analysis template** to extract diffusion coefficient and mobile fraction (see **Annexe 2** for the theory behind the analysis and how to use the template).
* Mobility can also be checked by fluorescence correlation spectroscopy (FCS).

1. **Sample preparation**

This section describes the cell transfection (e.g. with CD3ζ-PSCFP2), stimulation on bilayers/coated glass, fixation, permeabilisation and immunostaining (e.g. with anti-pCD3ζ antibody directly conjugated to AlexaFluor647).

* **T cell transient transfection (NEON)**

*Notes:* Before starting, take buffers out of the fridge (Buffers “E” and “R” at RT; FBS and medium without Pen/Strep at 37°C) at least 20 minutes before the transfection.

Book Hood 1 (GMO work).

**Prepare recovery medium**: RPMI medium (without Pen/Strep) + 10% extra FBS

* In 12-well plate, add 1.5 ml/well (counting 200,000 cells per transfection and 4 transfections per well, so we want 800,000 cells/well)
* Incubate at 37°C (at least 20 min i.e. the duration of the cell preparation and transfection).

**Count cells** (Casy counter)

* Cell density should be between 0.5-0.8 x 106 cells/ml
* Take appropriate number of cells in 15 ml falcon and wash cells in PBS 2x (spin 4 min, 37°C, 100 RCF/g)
* After centrifugation, carefully remove supernatant without disturbing the pellet.

**Prepare microporator** (Neon kit, Invitrogen)

* Switch on microporator at the back, take out the pipette and put chamber into the hood
* ‘Database’ 🡪 select program (for Jurkat, chose Program 1 if using commercial R buffer, Program 2 if using homemade R buffer) 🡪 load
* Put tube into chamber and get Neon tips from cupboard
* Add 3 ml buffer “E” into tube in chamber.

**Prepare DNA**

* In an Eppendorf tube, add 1 µg DNA/transfection i.e. 4 µg per condition.

**Prepare cells**

* After washing the cells, resuspend cells in buffer “R” (10 µl per transfection i.e. 40 µl per condition)
* Add cells to DNA.

**Transfection**

* Get 12-well plate with recovery medium from incubator
* 10 µl cells+DNA into pipette tip – no bubbles!!
* Click pipette into place and press ‘Start’.
* Once complete, pipette cells into medium (can re-use same pipette tip for same condition or wash in PBS in between conditions).
* Incubate cells overnight at 37°C.

**Finishing off**

* Clean tube, dry, and keep (leave in hood to UV)
* Turn off the microporator and put it away.
* **T cell stimulation on bilayers or antibody-coated glass, fixation and staining**
* Incubate bilayers at 37°C for around 10 min prior to cell activation so that they are nice and warm.
* Centrifuge transfected cells for 5 min, 1200 rpm, and resuspend pellet in desired volume.
* Drop cells onto the bilayer and incubate at 37°C for desired time.
* Fix cells by adding 4% PFA in PBS for 20 min, RT, then wash with PBS. [Stop here if doing one-colour PALM].
* Permeabilise with 0.1% Triton X-100 in PBS for 4 min, RT, then wash 2x in PBS.
* Block in 5% BSA for 10 min, RT, then wash 2x in PBS.
* Incubate with primary Ab in 1% BSA for 1 hour, RT, then wash 3x in PBS.
* Incubate with secondary Ab for 1 hour, RT, then wash 3x in PBS.
* For directly-conjugated pCD3ζ-AF647 antibody, staining overnight at 4°C is better (1:5 dilution). See **Annexe 3** for antibodies.
* Optional: pre-wash with cysteamine (35 mM in PBS) – see STORM buffers section.
* Store in excess buffer, 4°C.

1. **Two-colour PALM/STORM imaging**

This section contains tips for single-colour and dual-colour 2D imaging (PALM, STORM, and two-colour PALM/STORM). We currently have access to two SMLM (Zeiss Elyra) microscopes, one in the BMIF (Lower Ground) and one on Level 3.

If using beads as fiducials, add just before imaging (e.g. add 10 μl of 250 nm beads directly on the sample for 10 min, then rinse 3x with saline).

* **PALM imaging**

**Microscope**

* Turn on system about 1h before imaging.
* Open Zen software > Start system.
* Macro > 3D PALM > 2D/2D (Elyra downstairs). Macro > tick filter position 2 (Elyra L3).
* Open microscope chamber and add immersion oil (obj. 100x, NA 1.46). Put on sample carefully and close chamber.

**Software**

* Locate > Online (eye piece).
* Turn on lamp (BF) and find cells using the eye piece.
* Acquisition 🡪 Tick Time series 🡪 20,000 cycles.
* Turn on lasers. Light path 🡪 Laser WF (= TIRF). Chose filter (e.g. green for PSCFP2; red for mEos2).
* Channels: select laser and laser power (e.g. 488 for PSCFP2; 561 for mEos2); select TIRF and TIRF angle (~66-67); collimator (~2000); exposure time (e.g. 30 ms); gain (~100).
* “Continuous” 🡪 check cells and adjust focus and TIRF angle. Put cell of interest in the middle. Manually crop 256 x 256.
* Adjust laser power (e.g. 30-50%) > Continuous. Adjust TIRF angle. Tick 405 (~0.01-0.5).
* Start experiment (no need to stop continuous) – this will acquire 20,000 frames.
* File > Save As (.czi format) in Data Drive (transfer to Users server at end of session). Regularly clean up your data from the Data drive to avoid clogging up the computer.

**End of session**

* Turn off lasers. Laser levels back to 0.
* Clean objective with isopropanol and lens tissue.
* Turn off microscope if you are the last user of the day.
* **STORM imaging**

**Pre-treatment with cysteamine (to remove background)**

* Fix and stain cells as usual.
* Removal of “floating background”: incubate in PBS + 35 mM cysteamine for 15 min, RT.
* Rinse 2x 3 min with PBS on rotating shaker at 80 rpm. Or if using Labteks, rinse profusely with PBS.
* Let soak in PBS overnight (4°C).

**Buffers**

* Base buffer: PBS 90 ml

Hepes 0.651 g

Glucose 0.451 g

Glycerol 5 ml

* Place on shaker until well mixed.
* Adjust pH to pH 8 (using NaOH).
* Filter with 0.2 μm filter and 60 ml syringe – into 2x 50 ml falcon tubes.
* Store at RT.
* Cysteamine: prepare 1M solution: in 1 ml H­2O use 77 mg cysteamine (measure out and adjust volume). [Stock: keep under N­2 and seal with parafilm]. Once prepared, 1M stock can be stored at 4°C for a couple of days if kept under N2.
* Enzymes: Glucose oxidase (GO) and Horseradish Peroxidase (HRP) – stored in -20°C freezer (STORM aliquots). Return aliquots to freezer if not used up during your session.

**Imaging**

* Just before imaging, wash your sample in PBS and add ~500 µl Base STORM buffer.
* Add 2.5 μl GO, 1.25 μl HRP, 30 μl cysteamine (the latter is variable – see what works...)
* Use high laser power (~60-100%) to deplete fluorescence (to push fluorophores into dark state). Switch to EPI mode to deplete inside the cell.
* Acquisition in 256 x 256.
* For AlexaFluor647, use low levels of 405 or 488 to improve blinking.
* Can add more cysteamine if there is not enough blinking.
* **Dual-colour PALM/STORM imaging**

**Sequential acquisitions**

* The first option is to acquire the PALM and STORM channels sequentially.
* For two-colour sequential acquisitions of PSCFP2 and AlexaFluor647, the AlexaFluor647 channel is acquired first, followed by PSCFP2, similarly to one-colour imaging.
* Beads (as fiducials) or model-based drift correction are used for the channel alignment in ZEN.

**Simultaneous acquisitions**

* The second option is to acquire the PALM and STORM channels simultaneously, using two cameras.
* The instructions below are for imaging green and far red (e.g. PSCFP2 and AlexaFluor647 or AlexaFluor488 and AlexaFluor647) on the Elyra on Level 3. For other combinations, just make sure you use the correct filters.
* At the start of your session, change the filter set on the right hand side of the microscope (from filter set 3 to filter set 1).
* Change dichroic in front of the cameras (from filter module 2 to filter module 1 – i.e. 488/647 filter cube).
* In the software: Macro > Dual-camera > select filter position 2.
* In the light path, select correct filter (405/488/642).
* Tick both cameras (TV1 and TV2).
* Adjust settings for acquisition: 30 ms exposure time for both channels.
* Set camera gain very low (e.g. 1) before the alignment, then click live (in 512 x 512 view to get alignment of the entire field).
* To align channels: Macro > Dual-camera > Tick calibration pattern. Align green and red images. The x,y alignment is performed separately from the z alignment. Usually, manual alignment is sufficient; no auto x,y shifting or rotation alignment are needed.
* Adjust laser powers and gain. For example, set camera gain to 100. For finding cells, use 30% of 488 nm laser and 10% of 642 nm laser. You can also turn on the 405 nm laser at very low laser power (e.g. 0.005%) to help find the cells. For acquisition, use 30-60% of 488 nm and 30-50% of 642 nm lasers.
* Acquire your datasets (TV1 is 642 channel; TV2 is 488 channel).
* Make sure you change everything back at the end of the session.

1. **Data analysis**

This section contains information on the theory behind Ripley’s K and the DBSCAN cluster detection method for one-colour data and the Degree of Colocalisation (DoC) analysis for two-colour data, as well as a quick guide on the routines and how to use them.

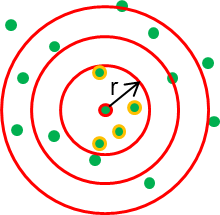
* **Image processing using ZEN**

To learn how to reconstruct your single-molecule images and export the table of coordinates and region coordinates, see **Cluster Analysis Manual**. For each cell several non-overlapping representative regions of 4 x 4 μm2 are selected for analysis. The format of your input data is the same for the following routines as for the previously used Cluster mapping routine.

* **Ripley’s K function (one-colour analysis)**

**Theory**

We use Ripley’s K function to determine the extent of clustering of a population of molecules compared to a randomly distributed set of molecules. This can be calculated using SpPack, an add-in for Microsoft Excel (for info on how to use SpPack, see **Cluster Analysis Manual**), or using a custom MATLAB version optimised for larger datasets (much faster than SpPack). In short, for each molecule the Ripley’s K function calculates the number of neighbour molecules within a given radius r corrected by the total density; finally, for each radius the average is calculated over all molecules. The Ripley’s K function provides ensemble information on the whole region of interest; it provides information on the level of clustering of molecules in a region, however no information is available on individual clusters.



**Ripley’s K MATLAB routine**

Input: In the same folder put: (i) text files corresponding to Zen Tables named 1.txt, 2.txt... (with the full coordinates of each cell), and (ii) a coordinates text file (named coordinates.txt).

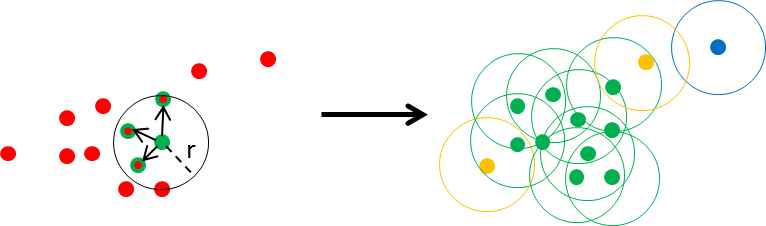
Run: To use the routine: Run "RipleyRoutineV2.mat" and select the coordinates file.

Output: The output of this routine is saved in the folder "Ripley\_Result".

* Ripley’s K plots (L(r)-r curve) for each region and one plot with average curve ±SEM
* Excel file containing calculated L(r)-r data for each region (and pooled data on last sheet), as well as maximum L(r)-r values and r value at which this max occurs for pooled data.
* **DBSCAN method (one-colour analysis)**

**Theory**

To retrieve information on individual clusters, we used DBSCAN analysis (density-based spatial clustering application with noise) to identify individual clusters. The DBSCAN method detects clusters using a propagative method which links points belonging to the same cluster based on two parameters; the minimum number of neighbours ε (ε = 3) in the radius r (r = 20 nm). The DBSCAN routine is implemented in MATLAB and subsequently coded in c++ and compiled in a MEX file (Matlab executable file) to improve the speed of processing as we are working with large data files.

 F:\Imaging data\PALM\Bilayers\OT-I system - Yui\Jurkat-OT-I on bilayers\Two-colour TCRz-pTCRz\Single-colour analysis 2\Ch1\DBSCAN_for_Zen_V_1S\Image from DBSCAN\Cell3_Region3Region_with_Cluster.tif

Outliers are removed if their local density is below a total density threshold set for r = 50 nm. DBSCAN is then applied to the remaining molecules. DBSCAN is a propagative cluster detection method where connectivity between molecules is established if the number of neighbours is above a certain threshold (e.g. 2 in the diagram) within a radius r (e.g. 20 nm). The connection is propagated if the parameters are fulfilled (green dots) and stops when the parameters are no longer fulfilled (yellow dots). This method can also identify any remaining isolated points or noise (blue dots). The routine also draws cluster contours for visualisation purposes (see the example cluster map on the right).

**DBSCAN routine**

Input: In the same folder put: (i) text files corresponding to Zen Tables named 1.txt, 2.txt... (with the full coordinates of each cell), and (ii) a coordinates text file (named coordinates.txt). Use ungrouped data for DBSCAN routine.

Run: To use the routine: Run "Full\_Routine\_DBSCAN\_V2C\_1.mat" and select the coordinates file.

*Note: This routine is able to separate two-colour tables and analyse each channel separately.*

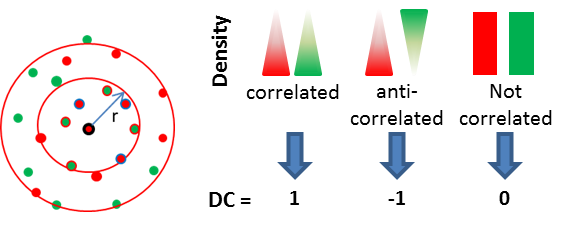
Output: The output of this routine is saved in the folder "DBSCAN\_for\_Zen\_V\_1S".

* Image Cluster Density Map: density maps (normalised and absolute) for each region
* Image from DBSCAN: cluster maps i.e. visual representation of the clusters (green) with contours (red) and excluded particles (blue)
* DBSCAN\_Cluster\_Result.mat: all the results in a Matlab variable, useful if you want to extract more information, run further analysis...
* DBSCAN Results.xls: average for each region of some parameters. More parameters can be added.
* **Degree of Colocalisation (DoC) analysis**

**Theory**

The two-colour data were analysed using a modified version of a coordinated-based colocalisation method. The first step in the analysis is to remove molecules which are isolated, by excluding points with a local density below a random distribution (total density = total number of molecules / total area of region of interest). This is important to reduce the size of the dataset since the subsequent steps are highly computationally demanding. In addition, these outlier molecules do not contribute to the clusters and would anyway get excluded in the DBSCAN step.

Then, for each molecule, the local density of each channel is calculated at increasing radius size (10 to 500 nm), providing the density gradient around that molecule for each channel. The two density gradients are tested for correlation with the Spearman criteria, which score monotonic dependence. This parameter of correlation is corrected with nearest neighbour distance to account for long distance interactions. As a result, each molecule is scored with this parameter, indicating the degree of colocalisation (DoC) ranging from -1 to 1, with -1 characterising anti-colocalisation (or segregation), 0 corresponding to single species and 1 defining high colocalisation. The method favours the molecules in clusters compared to randomly distributed molecules.



From the local density of each molecule taken at a radius of 20 nm and normalised to the total density of the region, a pseudomap is created in which each molecule is colour-coded according to the normalised relative density (= density map). In a similar way, a map showing the degree of colocalisation for each molecule is created (= DoC map; or TCR triggering map when looking at TCR-CD3 phosphorylation). These maps enable us to visualise the morphology of the clusters and their organisation. The threshold value of 0.4 was determined from simulations to discriminate colocalised from non-colocalised molecules. Binary maps show molecules in blue when below the threshold (non-colocalised molecules) and in red when above the threshold (colocalised molecules).

In the next step, we detect the individual clusters using a DBSCAN analysis, similarly to the one-colour analysis. The clusters are subsequently separated into colocalised clusters (containing more than 10 molecules with a DoC score above 0.4) and non-colocalised clusters. These two populations of clusters are then analysed to extract their density, size, circularity and any other type of information.

**DoC analysis routine**

This routine runs in different steps: (i) extracts regions from coordinates file and 1.txt, (ii) calculates Degree of Colocalisation for each channel, (iii) draws maps and extracts statistical values about colocalisation, (iv) detects clusters (by DBSCAN method) and (v) draws maps and extracts parameters and statistical values from DBSCAN/degree of colocalisation.

For two-colour analysis, the coordinate tables need to contain the coordinates for both channels in the same table. If you performed simultaneous acquisitions, you will automatically get both channels in the same table when doing your reconstructions in Zen. If you performed sequential acquisitions, you have to do separate reconstructions for each channel, followed by Channel Alignment. This gives you a table with the coordinates from both channels.

Input: In the same folder put: (i) text files corresponding to Zen Tables named 1.txt, 2.txt... (with the full coordinates of each cell for both channels), and (ii) a coordinates text file (named coordinates.txt).

Run: To use the routine: Run "Full\_Routine\_DofC\_DBSCAN\_Stat\_V3" and select the coordinates file.

Output: The output of this routine is saved in the folder "Degree\_Of\_Colocalisation".

* The folder “Image from DofC” contains DC frequency histograms for each region and for pooled data (top histogram: Channel 1, bottom histogram: Channel 2).
* The folder “Statistic and Plots for Colocalisation” contains maps (raw data, raw data with removed outliers, density and DC maps, binary maps) and an excel file with data about the molecules (separated into Channel 1 and Channel 2). Values are the average for each region.
* The folder “DBSCAN for DoC Ch1\_V3” contains the results from the DBSCAN analysis, i.e. cluster maps and an excel file with data about the clusters (separated into colocalised and non-colocalised clusters). Values are the average for each region.
* **General notes on Matlab**
* Before running a routine in Matlab, set the path to the folder containing all your Matlab routines (go on Home tab > Set path > select your folder and Save).
* Set the ‘current folder’ to the folder containing the data you want to analyse.
* Orange error messages can usually be ignored; red error messages mean something really went wrong.
* Don’t make any changes in the code!

1. **Summary protocol**

**Transfection (Neon):**

* Prepare recovery medium (1.5 ml medium without pen/strep + 150 µl FBS per well) in 12-well plate
* Centrifuge cells (0.8 x 106 cells/condition)
* Prepare DNA: 4 µg per condition
* Resuspend cells in buffer “R” (40 µl per condition) and add to DNA.
* Microporation using 10 µl pipette tip (4x per condition) with 3 ml buffer “E” in tube in cuvette
* Incubate cells overnight at 37°C in recovery medium.

**Making bilayers**

* Clean Labtek chambers
* Dilute liposome solution to 0.2 mg/ml in PBS and apply 0.25 ml of liposome mixture per well (in the presence of 5 mM CaCl2) and incubate at room temperature for 30 min.
* Rinse away excess liposomes with about 8 ml of PBS per well. Never expose bilayer to air!
* Incubate bilayer with 100 mM NiCl2 for 10 min, RT, then wash with PBS.
* Block with 0.1% BSA in PBS for 30 min, RT.
* Addstreptavidin (2 μg/ml) in 1% BSA for 10 min, RT, then wash away excess with 8 ml of PBS per well.
* Add biotinylated pMHC and/or His-tagged-ICAM-1/B7 over bilayer & incubate for 100 min, RT (or O/N, 4°C) then wash away excess with PBS.

**Cell activation and immunostaining**

* Before application of cells, rinse 3x & pre-warm bilayers (in PBS).
* Add desired cell numbers & incubate at 37°C.
* Fix cells at appropriate time-points by adding 4% PFA in PBS for 20 min, RT, then wash 3x with PBS.
* Permeabilise with 0.1% Triton X-100 in PBS for 4 min, RT, then wash 2x in PBS.
* Block in 1-5% BSA for 10 min, RT, then wash 2x in PBS.
* Incubate with primary Ab in 1% BSA for 1 hour, RT, then wash 3x in PBS.
* Incubate with secondary Ab for 1 hour, RT, then wash 3x in PBS.
* Pre-wash with cysteamine (35 mM in PBS).
* Store in excess buffer, 4°C, until your imaging session.

1. **Annexes**

**Annexe 1: Lipids for making bilayers**

**Lipids used for bilayers**

All lipids are purchased from Avanti Polar Lipids. Catalogue numbers listed below are for lipids provided in chloroform.

* DOPC (1,2-dioleoyl-sn-glycero-3-phosphocholine) = provides the structural framework of the supported lipid bilayer (Cat. No. 850457C)
* Biotin-DOPE (1,2-dioleoyl-sn-glycero-3-phosphoethanolamine) = lipid with a biotin headgroup for attachment of streptavidin (and therefore biotinylated pMHC) (Cat. No. 870273C)
* DGS-NTA(Ni) (1,2-dioleoyl-sn-glycero-3-[(N-(5-amino-1-carboxypentyl) iminodiacetic acid) succinyl] (nickel salt) = nickel-headed lipid used for attachment of His-tagged proteins (e.g. ICAM-1 and B7) (Cat. No. 790404C). The association of Ni2+ ions with this lipid is not very stable, which is why we incubate the bilayers with NiCl2 to rebind some Ni2+ to the lipids.
* DOPE-PEG5,000 (polyethylene glycol) = lipid with PEG5,000 polymers covalently attached to the lipid headgroups (Cat. No. 880230C). Used to improve bilayer uniformity and reduce protein aggregation.

*Note: store at -80°C and always overlay with argon!!*

**Lipid mix**

A typical lipid mix to start with would be the following (as calculated with the **Lipid mix calculations spreadsheet** for getting 1 mg/ml final concentration when resuspended in 1 ml PBS):

* 96 µl DOPC (at 10 mg/ml) = 97% mol
* 14 µl biotin-DOPE (at 1 mg/ml) ≈ 2% mol
* 27 µl DGS-NTA(Ni) (at 1 mg/ml) ≈ 1% mol
* 8 µl DOPE-PEG5,000 (at 1 mg/ml)

The concentration of pMHC presented on the bilayer can be controlled precisely by changing the concentration of biotin-DOPE lipid in the lipid mix. For example, 0.1%, 0.01%, 0.001%... The density of pMHC molecules on the bilayer can be measured by FCS (at low concentrations) by using fluorescent streptavidin.

You can also pre-mix your lipids and store the lipid mix at -20°C, taking aliquots of the pre-mixed solution when needed (use within 2 months). Always overlay with argon.

**Annexe 2: FRAP on Olympus FV1000 – FRAP analysis**

**Getting started**

* Turn everything on (X-Cite lamp first, microscope last)
* Log in (password = bmif)
* Start software FV10 and click OK (user; no password)
* Image Acquisition Control > Dye List (green blob) > Chose fluorophore (e.g. rhodamine phalloidin; 543 laser)
* Acquisition Settings > Microscope > 100x oil objective (NA=1.4)
* Use trans lamp (for finding cells)
* Use epi lamp (for finding fluorescence) > Chose appropriate filter on box (e.g. Cy3 for rhodamine)
* Use ‘XY Repeat’ for slow ‘live’ or Focus 4x to be able to focus on the sample.
* Ctl +H changes to ‘high-low’ view.
* Acquisition settings:
  + - scan speed 4 or 8 μs/pixel
    - frame size 256 x 256 pixels
    - zoom 4.6
* Untick ‘Kalman filter mode’
* SU > Pinhole
* Offset = always 0

**FRAP acquisition**

* Use “Stimulus Setting” window
* SIM mode (simultaneously bleach whilst acquiring)
* Fast scan 10 μs/pixel
* 405 laser = 100%
* Chose Clip Tornado and draw ROI
* Main scanner sync:
  + - Image scan > Activation
    - Wait time 4 frames
    - Activation time 20-30
* In main window (Image Acquisition Control): hit ‘Time’ then ’XY’ to start experiment.
* Save time courses as .oif (contains metadata)
* Close software before turning off hardware.

**FRAP analysis**

* Use Fiji.
* Drag and drop .oif file
  + - “Display metadata”
    - “Display OXE-XML metadata”
    - “Display ROI”
* Draw ROI on first bleached frame
* Image > Stacks > Plot z-axis profile
* Copy and paste List into Excel template.
* Draw non-bleached region (control) and do the same.
* This is the raw data over time (FRAP and non-FRAP regions).

**FRAP analysis template: theory**

1. **Corrects for image photobleaching (using non-FRAP data)**

1. **Normalisation**
2. **Plots recovery curve**

Remove pre-bleach frames

t=0 at first bleach frame (intensity = 0 too).

1. **Curve fitting (using different models)**

* Interaction-based (single-exponential fitting) to determine mobile and immobile fractions and t1/2 (time until half-recovery)

Amplitude: corresponds to plateau

Koff: dissociation constant

To perform fit: Developer tab > Add-ins > Solver add-in

Data > Solver

Set objective = select χ2 to Minimum

By changing cells: Amplitude and Koff

Solve > OK.

* Diffusion-based (based on 2D diffusion and assumes circular bleach region and Gaussian bleach)

[= modified Bessel functions]

r = radius

D = diffusion coefficient

You need to set the appropriate radius and time interval.

To find the time interval (in Fiji): Image > Show info > Frame interval (at the bottom of the file).

> Run Solver.

**How to use FRAP analysis template**

* Get data from FRAP and non-FRAP regions in Fiji > paste into FRAP analysis template. Note down radius of ROI and frame interval.
* Check number of pre-bleach frames (for pre-bleach mean), check which frame has lowest bleach intensity and adjust data layout accordingly.
* Interaction-based curve fitting: copy time and recovery data. Adjust all parameters and data ranges (+ mean recovery) > Solver.
* Diffusion-based curve fitting: change ROI radius. Adjust parameters and data ranges > Solver.

**Annexe 3: Antibodies**

**Antibodies used in TCR triggering analysis**

* Anti-pCD3ζ (directly conjugated to AlexaFluor647 or AlexaFluor488, CD3ζ phosphorylated at Tyr142): use 1:5 (558489 and 558486, BD)
* Anti-CD45 (rabbit polyclonal): use 1:100 (ab10559; Abcam)
* Anti-pZap70 (rabbit polyclonal, Zap70 phosphorylated at Tyr319): use 1:50-1:100 (2701S, Cell Signaling)
* Anti-pLAT (rabbit polyclonal, LAT phosphorylated at Tyr171): use 1:50-1:100 (3581S, Cell Signaling)
* Anti-Lck (mouse monoclonal): use 1:50-1:100 (sc-433, Santa Cruz Biotechnology)
* Anti-mouse TCRβ (directly conjugated to AlexaFluor488 or AlexaFluor647): use 1:100 (H57-597; 109215 and 109218, Biolegend)

**Secondary antibodies**

* Anti-rabbit: AlexaFluor647-conjugated goat antibody specific to the rabbit F(ab’)2 IgG fragment: use 1:100 (111-606-047, Jackson ImmunoResearch)
* Anti-mouse: AlexaFluor647-conjugated goat antibody specific to the mouse F(ab’)2 IgG fragment: use 1:100 (115-606-006, Jackson ImmunoResearch)

**Annexe 4: Output Excel files from DBSCAN and DoC analysis**

**DBSCAN**

‘DBSCAN Results’ file contains:

* Coordinate information extracted from coordinates.txt file (including an empty column for notes about the data)
* Percent\_in\_Cluster: fraction of molecules in clusters compared to total number of molecules (need to x100 for percentage)
* Number: number of particles per cluster (average for each region)
* Area: average cluster area in nm2 per region
* Density: density of molecules in clusters (molecules per nm2)
* RelativeDensity: density of molecules in clusters relative to total density in region
* TotalNumber: total number of molecules detected in region
* Circularity: average circularity of clusters per region (circularity is measured as the ratio of perimeter to area such that a perfect circle equals 1)
* NumberOfCluster: total number of clusters in region.

**Degree of Colocalisation (DoC)**

‘Region’ file (in “Statistic and Plots for Colocalisation” folder) contains:

DC Results 1:

* Percent DC>0.4 Ch1: percentage of Channel 1 molecules that have a DoC score higher than 0.4 (i.e. are colocalised)
* Percent DC>0.4 Ch2: percentage of Channel 2 molecules that have a DoC score higher than 0.4 (i.e. are colocalised)
* Remaining columns are average and normalised densities of all molecules (Channel 1 and Channel 2 together) divided into molecules that are colocalised and molecules that are not colocalised and the correlation between density and DoC [these values are not that useful].

DC Results 2:

* AvNormDensityCh1>0.4: average normalised density of Channel 1 molecules that have a DoC score higher than 0.4 (i.e. are colocalised)
* AvNormDensityCh1<0.4: average normalised density of Channel 1 molecules that have a DoC score lower than 0.4 (i.e. are not colocalised)
* AvRelaDensityCh1>0.4: average relative density of Channel 1 molecules that have a DoC score higher than 0.4 (i.e. are colocalised)
* AvRelaDensityCh1<0.4: average relative density of Channel 1 molecules that have a DoC score lower than 0.4 (i.e. are not colocalised)
* Same values for Channel 2.

‘DofC\_DBSCAN’ file (in “DBSCAN for DoC Ch1\_V3” folder) contains:

* DensityCluster>0.4: relative density of Channel 1 molecules in clusters that are colocalised (i.e. have more than 10 molecules with a DoC score higher than 0.4)
* DensityCluster<0.4: relative density of Channel 1 molecules in clusters that are not colocalised (i.e. have less than 10 molecules with a DoC score higher than 0.4)
* Density\_Nb<=10: relative density of Channel 1 molecules in clusters that contain less than 10 molecules
* Same but with area and circularity.