



emulate

Immune Cell Recruitment 3D Analysis User Guide

EP216 Rev A

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Overview

Introduction This user guide gives directions for 3D analysis in Immune Cell Recruitment.

In this protocol This protocol contains the parts listed below.

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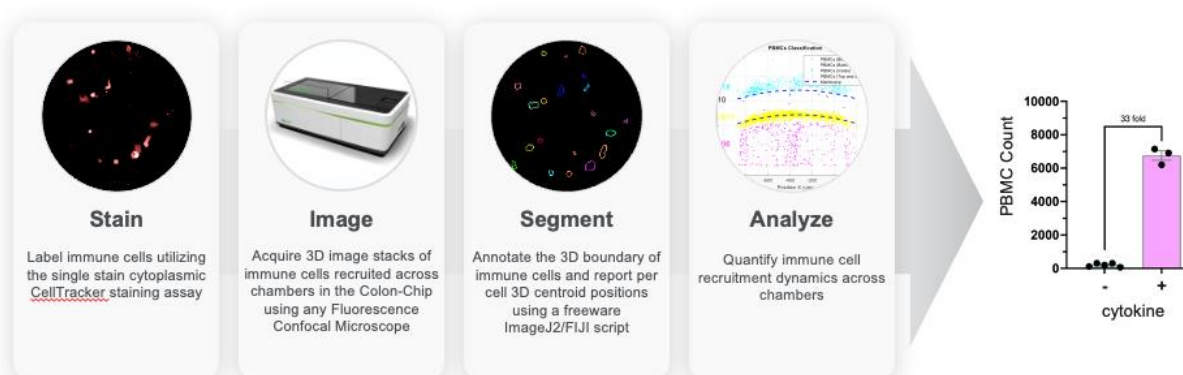
Part I. Overview

Introduction

This user guide was developed to enable the assessment of Immune Cell Recruitment when performing the protocol “Basolateral Recruitment of Peripheral Blood Mononuclear Cells (PBMCs) in Colon Intestine-Chip.” It provides guidance on staining and imaging parameters for acquiring three-dimensional (3D) image stacks with a fluorescence confocal microscope. Additionally, it describes using an analytical package that includes an ImageJ2/Fiji and Python script for segmenting and analyzing migratory immune cells.

Figure 1

Immune Cell Recruitment imaging and analysis workflow supporting the Colon Intestine-Chip model.



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Part II. Immune Cell Recruitment Analytical Package Contents

Overview

Introduction

Describes the contents of the Immune Cell Recruitment (ICR) Analytical Package.

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ICR Analytical Package Contents

Immune Cell Recruitment Analytical Package

Includes both an ImageJ2/Fiji script and a Python script which enable 3D segmentation and spatial analysis of migratory immune cells in the Colon Intestine-Chip, respectively.

ImageJ2/Fiji 3D Image Segmentation Script

Automates visualization and annotation of single-stained immune cells acquired within a 3D image stack. The 3D centroid position is then calculated per segmented cell. Z-stacked imagery can be acquired using any fluorescence confocal microscope.

| | |
|-----------------------------|--|
| Segmentation Script Inputs | <ul style="list-style-type: none"> • 3D image slices in the z-direction (.tiff, .jpeg) • Images expressing fluorescent signal from fixed, single-stained immune cells |
| Segmentation Script Outputs | <ul style="list-style-type: none"> • Excel file containing per cell 3D centroid positions (X, Y, Z) in microns to be inputted into the Python Immune Cell Recruitment Analysis Script • Maps showing segmentation and/or annotation overlays • Text file containing an audit trail ("log") of user inputs and execution steps |

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Python Immune Cell Recruitment Analysis Script

Automates the classification of identified immune cells. Calculated 3D centroid positions are plotted in the xz-plane; membrane positions are estimated based on cell clustering patterns; and immune cells are categorized by location to assess overall recruitment ([Table 1](#)).

| | |
|---------------------------------|--|
| Required Analysis Script Inputs | <ul style="list-style-type: none"> Excel file generated by the ImageJ2/Fiji 3D Image Segmentation Script Images expressing fluorescent signal from fixed, single-stained immune cells |
| Analysis Script Outputs | <ul style="list-style-type: none"> Excel file with the percentage and absolute value of migrated immune cells Migratory plots in the xz-plane depicting immune cell locations within the chip and their recruitment characterization |

Table 1: Characterization of immune cell recruitment within the Colon Intestine-Chip model.

| Location | Biological Characterization | Channel |
|----------------------------------|------------------------------------|----------------|
| Below the bottom of the membrane | Free flowing cells, not adhered | Bottom Channel |
| At the bottom of the membrane | "Recruited PBMCs", | |
| Inside the membrane | "Recruited PBMCs", still migratory | Membrane Layer |
| Top and above the membrane | "Recruited PBMCs", fully migrated | Top Channel |

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Part III. Software Installation

Overview

Introduction

Instructions for downloading and installing the ICR Analytical Package.

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Installation

ICR Analytical package

Designed to be used with freely available software packages.

System Requirements

- Windows XP, Vista, 7, 8, 10, 11, etc.
 - Mac OS X 10.8 “Mountain Lion” or later on x86 architecture (not all python libraries utilized in script support use on Apple Silicon)
 - Linux on AMD64 and x86 architectures
-

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Installing ImageJ2/Fiji 3D Image Segmentation Script

Installation

| Step | Action |
|------|--|
| 1 | Download and install the latest version of the ImageJ2/Fiji application (requires v1.52i or later) |
| 2 | Update ImageJ2/Fiji using the “Help/Update” menu |
| 3 | Download the latest version of the 3D Object Counter plugin |
| 4 | Drag and drop the plugin file on the <i>ImageJ2/Fiji</i> |
| 5 | Save the file in the plugin folder using “Save Dialog” |
| 6 | Download the latest version of the Excel Read and Write plugin |
| 7 | Navigate to Help > Update in ImageJ2/Fiji |
| 8 | Click on “Manage Update Sites” |
| 9 | Find and check the “Results to Excel” checkbox |
| 10 | Click “Close” and then “Apply Changes” |
| 11 | Restart ImageJ2/Fiji before proceeding |

Installation Check

A 3D object counter entry should now appear under the ImageJ2/Fiji plugins menu. It contains 2 entries:

- 3D Object Counter
- Set 3D Measurements

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Installing Python Immune Cell Recruitment Analysis Script

Installation

| Step | Action |
|------|--|
| 1 | Download and install Python version 3.10.0 (the exact version is required) |
| 2 | Download and install the Anaconda Python interpreter |
| 3 | <p>Download and install the following libraries using the associated command in Spyder's Terminal:</p> <ul style="list-style-type: none"> – <i>matplotlib</i> (conda install -c conda-forge matplotlib) – <i>numpy</i> (conda install -c conda-forge numpy) – <i>pandas</i> (conda install -c conda-forge pandas) – <i>tkinter</i> (conda install -c anaconda tk) – <i>PySimpleGUI</i> (conda install -c conda-forge pysimplegui) |
| 4 | Download the most recent version of 'ICR_sClustering.py' script |

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Part IV: Immune Cell Staining Guidance

Overview

Introduction

The ImageJ2/Fiji 3D Image Segmentation Script quantifies the immune cells stained with the cytoplasmic stain CellTracker™ Deep Red Dye (ThermoFisher Scientific, Cat. C34565). Please refer to pages 33-37 in the Emulate protocol EP215 Basolateral Recruitment of Peripheral Blood Mononuclear Cells (PBMCs) in the Colon Intestine-Chip for immune cell staining and administration.

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Part V. Immune Cell Imaging Guidance

Overview

Introduction

How to optimize imaging parameters to best image immune cells.

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Microscope Optimization

Introduction

Confocal fluorescence microscopy is well-suited for 3D imaging within diffuse, thick samples. For this protocol, it is the preferred imaging tool for the quantification of migratory immune cells. Prior to imaging, the chip requires fixation to preserve the migratory state of immune cells. Please refer to page 40 in the Basolateral Recruitment of PBMCs in the Colon Intestine-Chip for details regarding chip fixation.

Characterizing Microscope PSF

To optimize imaging performance, we recommend characterizing your microscope's point spread function (PSF). This can be done by imaging fluorochrome-labelled polystyrene beads having a diameter smaller than the microscope's theoretical resolution. Light diffraction blurs the bead's image to make it appear larger than its true dimensions. This PSF image defines the resolution in the axial and lateral directions. Because optical microscopes have worse resolution in the axial versus lateral direction, the PSF is approximately a cylindrically symmetrical ellipsoid.

Avoiding Sampling Error: Microscope

Fully understanding your microscope's resolution will help you to avoid under- or over-sampling—a mistake frequently made during imaging. Table 2 summarizes these errors' potential effects on imaging as well as their potential consequences when thresholding with the ImageJ2/Fiji 3D Image Segmentation Script.

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Table 2

| Sampling Error | Effect of Imaged Cell Object | Potential Segmentation Errors with Script |
|-----------------------|---|---|
| Under-sampling | <ul style="list-style-type: none"> Partial capture of signal from immune cell | <ul style="list-style-type: none"> Higher rates of false negative results. Partial cell images smaller than the minimum cell size parameter will be missed. Compensation by reducing the acceptable min cell value may increase the rate of false positives Incorrect 3D centroid measure |
| Over-sampling | <ul style="list-style-type: none"> Immune cells looking stretched Lower signal-to-noise ratios (SNR) due to greater signal spread at the detector | <ul style="list-style-type: none"> Higher rates of false negative results. The threshold-based cell segmentation protocol will struggle to identify true signal from background in low SNR images. Background signal removal via the incorrect preprocessing technique may remove true signal |

Avoiding Sampling Error: Sampling Frequency

To avoid sampling errors and ensure the complete capture of cellular objects during imaging, it is important to consider the Nyquist Sampling Theorem, which states that the sampling frequency should be at least twice the resolution of the current dimensions. In other words, the minimum justified pixel size is one-half (1/2) the lateral resolution, and the minimum z-step size should be one-half (1/2) the axial resolution. With this guidance in mind, in the next section, we describe the use of imaging parameters in the Colon Intestine-Chip protocol.

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Recommended System Parameters

Overview

Since imaging is performed through the Colon Intestine-Chip, we recommend using an objective lens with the parameters listed in [Table 3](#). The lens' large working distance enables imaging through the entirety of the chip's thick PDMS layer as well as its upper and lower channels. The low numerical aperture (NA) supports faster imaging speeds and has a resolution that will not hinder downstream quantification. It's important to note why an air immersion objective was selected. Given the closeness in refractive index between water and the chip, it was determined that confocal microscopes using water immersion objective lenses suffer defocusing issues.

Table 3

Recommended objective lens parameters to image immune cells in the Colon Intestine-Chip when imaging with fluorescence confocal microscopy

| | Recommended Parameters |
|-------------------------|------------------------|
| Objective Lens Type | 20x Air |
| Numerical Aperture | 0.4 |
| Working Distance [mm]** | 8.39 |

***Working distance is defined as the distance from lens to focal point without coverslip correction*

Note on Resolution Limit

Importantly, resolution is a function of the objective's NA, the chip system's refractive index, the chip's depth of imaging, and the fluorochrome's emission wavelength. Image acquisition should always be carried out near the resolution limit of the optical system (i.e., justified pixel size and z-step size). Based on the recommended objective lens specifications above and the emission wavelength of the CellTracker™ Deep Red Dye, the minimum justified pixel size is 283 nm, and the z-step size is 2.8 μm ([Table 4](#))

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Table 4 Optical resolution and pixel size in confocal microscopy

| | Lateral resolution dx, y | Axial resolution dx, z |
|--|-------------------------------------|-----------------------------------|
| Resolution Limit | $(0.4\lambda_{em})/NA$ | $(1.4\lambda_{em})/NA^2$ |
| Suggested Parameters: 20x Air, NA = 0.4, $\lambda_{em} = 650 \text{ nm}$ | 650 nm | 5.7 μm |
| Min Pixel Size | 283 nm | 2.8 μm |

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Additional Recommendations to Ensure Imaging Success

Dynamic Range

It is essential to maximize the dynamic range by avoiding overexposure of your sample during imaging. Overexposure occurs due to long scanning dwell times and/or when the excitation laser power is too high and can lead to pixel saturation. When saturated, higher intensity grey levels in the histogram are lost, adversely affecting dynamic range. Pixel saturation paired with incorrect background pre-processing techniques can lead to inability to properly segment cells with thresholding-based protocols.

Laser Power Drift

By regularly imaging fluorochrome-labelled polystyrene beads, it is possible to monitor potential drift in the excitation laser power. Ensuring constant excitation power is critical for consistent sensitivity and specificity of performance if the threshold-based cell segmentation protocol.

System Drift

PSF geometry can be used as an indicator of potential problems with the objective lens, scanning components, and other relay optics within the microscope. It is important to assess consistency in diffraction pattern and to ensure the resolution in the lateral and axial directions remain unchanged.

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Imaging Guidance

Getting Started To ensure successful analysis when setting up the image acquisition, include the parameters listed below

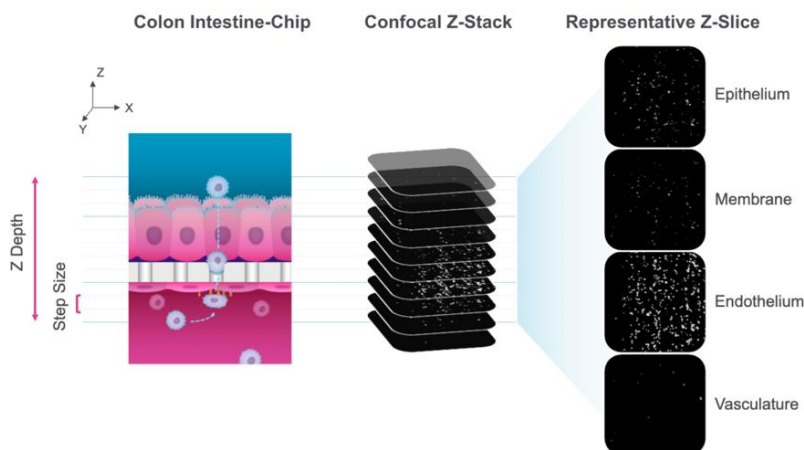
Fluorescence Imaging of CellTracker™-stained immune cells

Choose the appropriate excitation/emission channel to capture immune cells stained with CellTracker™ dye. Excite fluorophore with laser power that maximizes image dynamic range, by preventing pixel oversaturation and maximizing SNR. The ICR Analytical Package analyzes only a single channel image, but this does not preclude you from extending imaging to include additional features, such as nucleic stain or transmitted light. That said, ensure there is no crosstalk between collected channels, else cells will be incorrectly segmented.

Capturing Z-stack range to acquire extant of migratory immune cells

Start the first z-slice slightly (~10 µm) below the membrane to capture attached immune cells and any cell monolayer (if applicable). Extend the z-stack height upwards to include the total membrane height (50 µm) as well as the total top channel cell monolayer height (depending on cell type, from 10-100 µm). If possible, it is recommended to extend an extra 10 µm additional margin on both ends of the z-stack to account for any possible misalignment of samples. Recommendations for the sampling resolution of the z-stack based on objective is outlined in the guidance above.

Figure 2 Representative immune cell recruitment data captured utilizing confocal imaging.



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Part VI: 3D Image Segmentation Protocol

Overview

Introduction

This protocol shows the step-by-step process for detecting immune cells in 3D image stacks.

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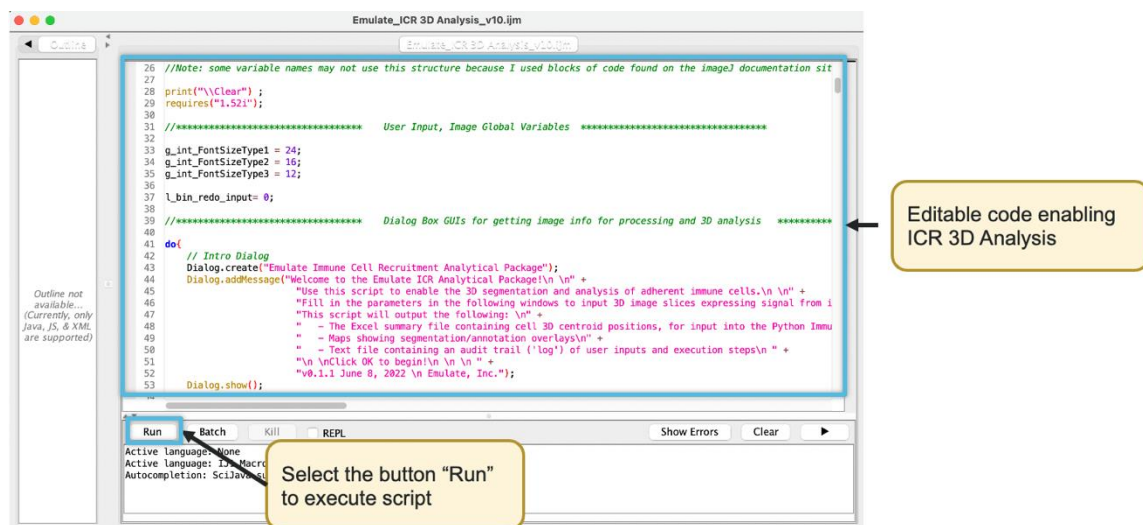
3D Image Segmentation Protocol

3D Image Segmentation Protocol

This protocol shows the step-by-step process to run the **ImageJ2/Fiji 3D Image Segmentation Script** to detect immune cells in 3D image stacks.

| Step | Action |
|------|--|
| 1 | Open ImageJ2/Fiji |
| 2 | Open and load the most recent version of “Emulate_ICR 3D Analysis.ijm” |
| 3 | Create an Output Directory within your project folder <i>Note: The Output Directory cannot reside within the Source Directory</i> |
| 4 | Select the “Run” button in the macro window to run the script |

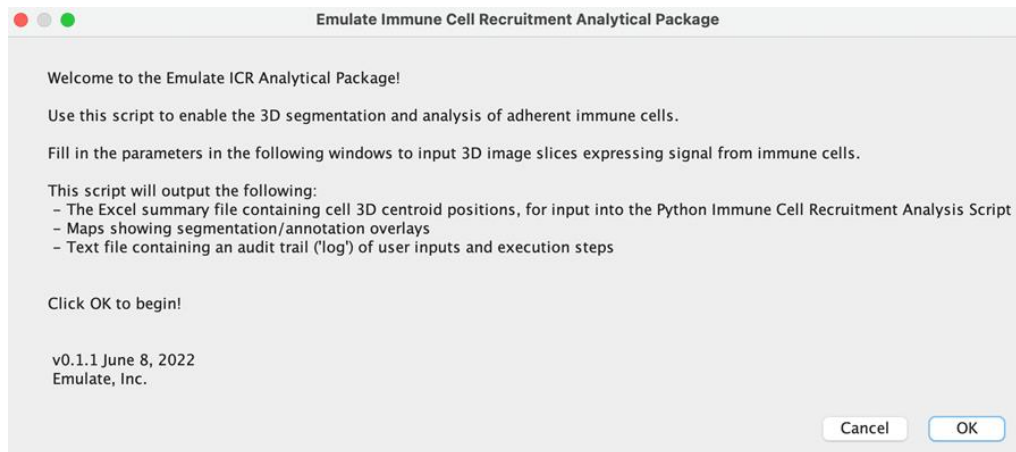
Figure 3: Representative macro window as displayed in ImageJ2/FIJI



| | |
|---|---|
| 5 | A welcome window briefly describing script out will appear. Select the “OK” button to proceed |
|---|---|

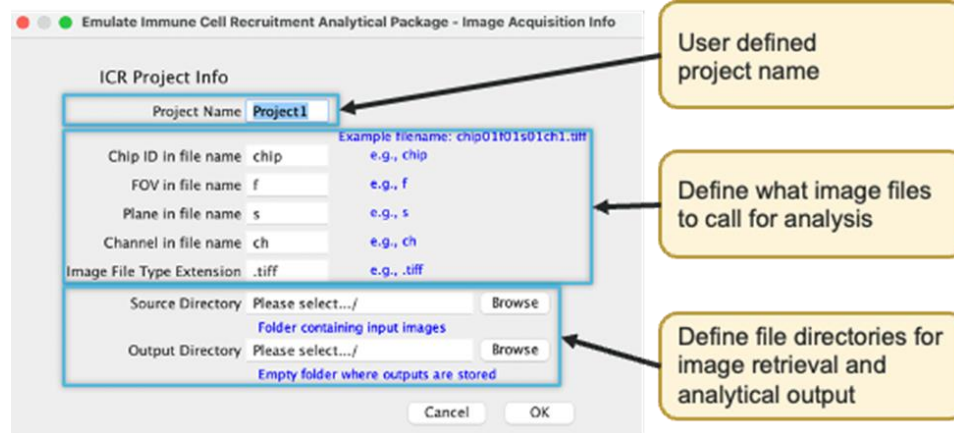
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Figure 4: Emulate Immune Cell Recruitment Analytical Package welcome window displaying script inputs and outputs in addition to version number



6 The interface **Image Acquisition Info** will open

Figure 5: Defining ICR project settings



7 Enter the *Project Name*

8 Enter the following information from the file name. This will enable the script to call image files to be analyzed:

- Chip ID in file name
- FOV in file name
- Plane in file name
- Channel in file name
- Image File Type Extension

For further guidance on proper naming, see [Table 5](#)

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Table 5: Examples of correct and incorrect filename identifiers.

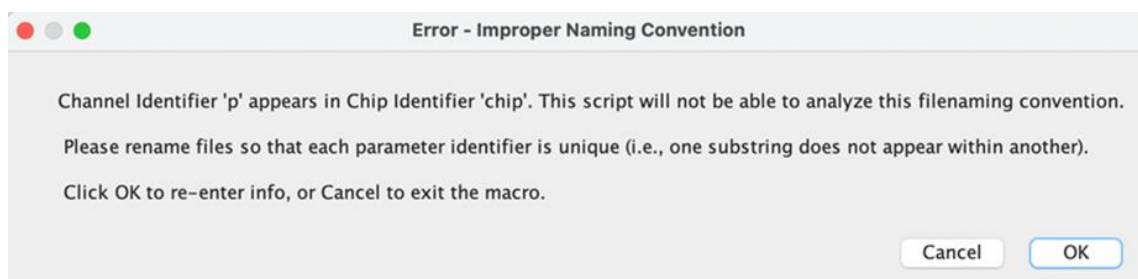
Note: All filename identifiers entered above must be unique. Entering an incorrect file name will cause an error message to appear (see Figure 6). For instance, in the table below, the first example will not work, because the string “p01” appears within the string “chip01”, so the filename identifiers are not unique. The second example will work: even though the identifiers are “c” and “ch”, the string “c01” does not appear in the string “ch01”, so there is no overlap. Please ensure all filenames follow a similarly unique convention.

| IDENTIFIER | INCORRECT INPUT | CORRECT INPUT |
|---------------------------|-----------------------|--------------------|
| Example Filename | chip01f01p01ch01.tiff | c01f01p01ch01.tiff |
| Chip ID | chip | c |
| FOV | f | f |
| Plane | p | p |
| Channel | ch | ch |
| Image File Type Extension | .tiff | .tiff |

Note: If filename identifiers have significant overlap, the script will throw an error prompting you to rename the files as necessary. Choose the Source Directory folder by selecting the “Browse” button. In the folder browser window, select the project folder containing the input images (not the individual measurement folder, but the folder all the measurements were exported too).

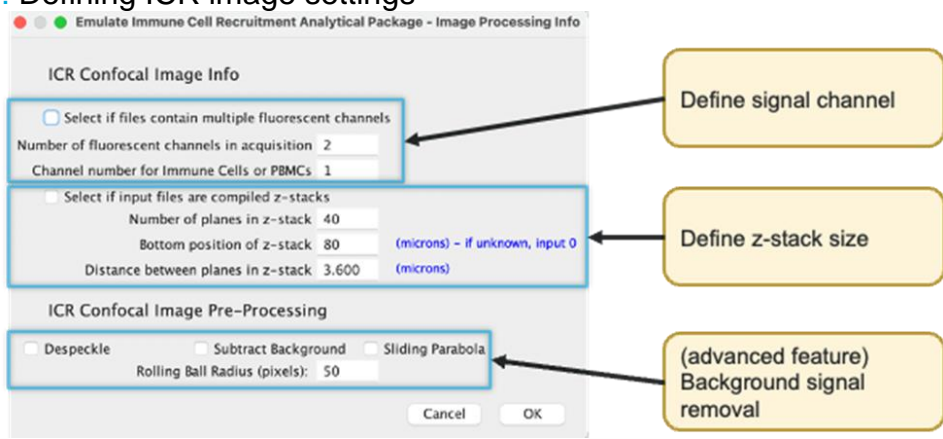
- Choose the Output Directory folder by selecting the “Browse” button. Within the folder browser window, select the empty folder created in step 3. The script will output all annotation maps and data files to this folder.

Figure 6: Error warning displayed when incorrect filename identifiers are used.



- Select the button “OK” when all information has been entered

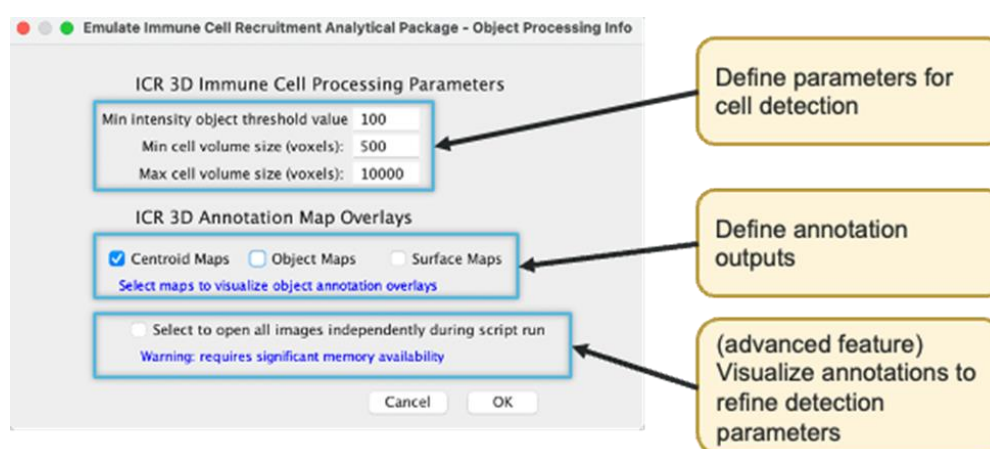
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| | |
|--|---|
| 11 | The Image Processing Info Interface will open |
| <p>Figure 7: Defining ICR image settings</p>  | |
| 12 | If images for analysis contain multiple fluorescence channels, please select the box next to the statement “Select if files contain multiple fluorescent channels” |
| 13 | Enter the number of fluorescent channels acquired |
| 14 | Enter which channel contains the fluorescent signal for stained immune cells |
| 15 | <p>If image files are already compiled into z-stacks, please select the box next to the statement “Select if input files are compiled in z-stacks”</p> <p><i>Note: If each slice/plane within the stack is saved as a separate file, leave this box unchecked – the script will compile all planes within a single FOV and analyze them together. If the images are already compiled into Z-stacks, check this box and the script will skip straight to the 3D object counting step</i></p> |
| 16 | Enter the number of planes acquired in the z-stack |
| 17 | <p>Enter the position in microns of the bottom plane of the z-stack</p> <p><i>Note: If this value is unknown, enter “0”. This will change how immune cells are classified using the Python Immune Cell Recruitment Analysis Script</i></p> |
| 18 | Enter the distance, in microns, between the planes in the z-stack |

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| 19 | <p>(Advanced) We provide four options for pre-processing imagery to filter out background noise. Options include despeckle, subtract background, sliding parabola, and rolling ball radius.</p> <p><i>Note: As fluorescent signal is only generated from the CellTracker dye within a highly diffuse sample, imagery should have a large signal-to-noise ratio. If required, we provide pre-processing algorithms</i></p> |
| 20 | Select the “OK” button when all information has been entered |
| 21 | The Object Processing Info Interface will open |

Figure 8: Defining ICR cell detection settings



| | |
|----|--|
| 22 | <p>Enter the following 3D processing parameter values:</p> <ul style="list-style-type: none"> (a) Min intensity object threshold value (b) Min cell volume (reported in voxels) (c) Max cell volume (reported in voxels) |
| 23 | <p>Select which annotation map overlays (if any) you want saved in Output Directory.</p> <p><i>Note: Selection is not required, but the maps enable you to review segmented cells and adjust processing parameter values to optimize object identification</i></p> |

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| 24 | Select the box next to the statement “Select to open all images independently during script run” to open each image as it is segmented. <i>Note: this will require significant memory and is not recommended for large data sets</i> |
| 25 | Select the button “OK” to run the script. Outputs will populate in the Output Directory folder |

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Example Outputs from ImageJ2/Fiji 3D Image Segmentation Script

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The ImageJ2/Fiji 3D Image Segmentation Script will output the following:

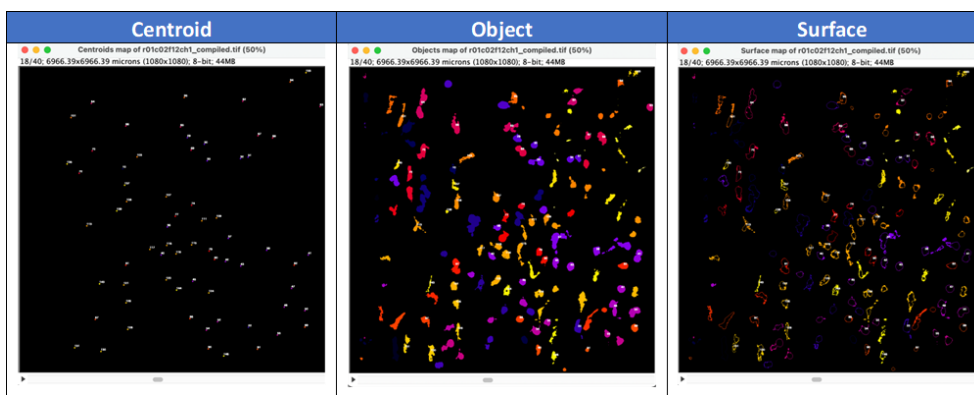
Excel file
containing per
cell 3D
centroid
positions

Chip ID →

| Chip13 | | |
|---------|----------|---------|
| X | Y | Z |
| 623.573 | 971.125 | 149.872 |
| 543.978 | 1057.754 | 150.011 |
| 342.589 | 253.596 | 144.249 |
| 396.173 | 277.38 | 152.391 |
| 1056.53 | 613.864 | 153.748 |
| 897.008 | 855.718 | 155.05 |
| 634.911 | 439.655 | 153.224 |
| 622.248 | 1052.904 | 155.17 |
| 817.76 | 341.445 | 142.728 |
| 216.049 | 960.087 | 136.301 |

Per cell centroid coordinates reported in microns

Maps showing
segmentation
and/or
annotation
overlays



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Text file
containing an
audit trail
("log") of user
inputs and
execution
steps

```

Emulate_ICR_AnalyticalTools_Chip17_AuditTrail.txt — Edited
***** Project Run Info *****
g_str_RunName : Chip17
g_str_extension : .tiff
g_str_chipID : r02c
g_str_fovID : f
g_str_sliceID : p
g_str_channelID : ch
g_bin_debugMode : 0
g_str_dirpath : /Volumes/ScientificArchive/Chip17 Input/
g_str_savepath : /Volumes/ScientificArchive/Chip17 Output/
***** ICR Image and Pre-Processing Info *****
g_bin_multichannel : 0
g_int_channels : 3
g_int_PBMC_channel : 1
g_bin_zstack : 0
g_int_zslices : 35
g_int_zbottom : 80
g_flt_zdistance : 4
g_bin_despeckle : 1
g_bin_subtractBack : 1
g_int_subtractBack_radius : 50
g_bin_subtractBack_sliding : 1
***** ICR 3D Object Processing *****
g_int_minThresh : 75
g_int_minSize : 250
g_int_maxSize : 15000
g_arr_str_outputLabels[0] : Centroid Maps
g_arr_bin_OutputMapChoice[0] : 1
g_arr_str_outputLabels[1] : Object Maps
g_arr_bin_OutputMapChoice[1] : 1
g_arr_str_outputLabels[2] : Surface Maps
g_arr_bin_OutputMapChoice[2] : 1
g_str_chipIdentifier : r02c
g_str_fovIdentifier : f
g_str_sliceIdentifier : p
g_str_channelIdentifier : ch
outside of CUT

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Part VII. Immune Cell Recruitment Analysis

Overview

Introduction

The following protocol shows the step-by-step process for running the Python Immune Cell Recruitment Analysis Script for characterizing immune cell recruitment.

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| Topic | See Page |
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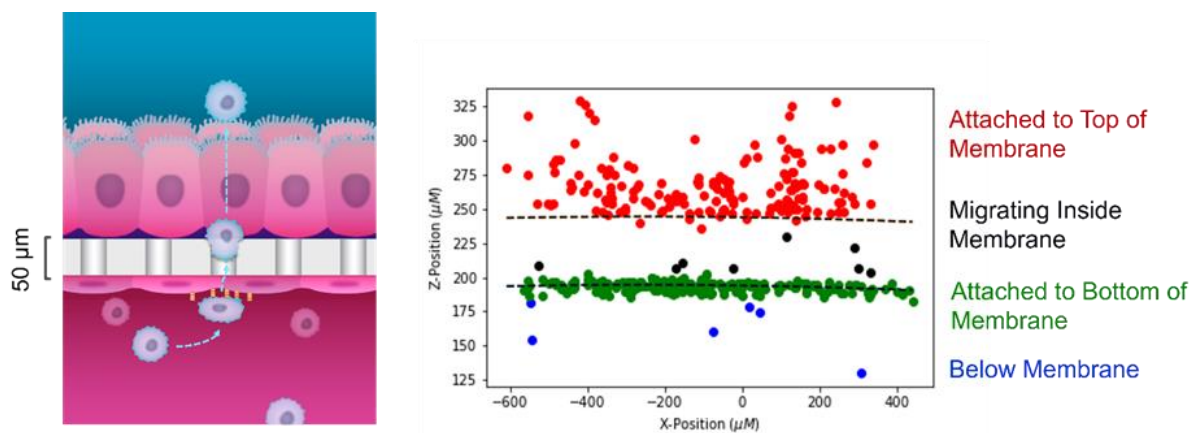
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User Determination of Z-Range to Assist Classification

Location Estimation

Since recruitment status is classified based on immune cell migratory patterns within the Organ-Chip, the Python Immune Cell Recruitment Analysis Script needs to estimate locations corresponding to the membrane edges. Assuming large attachment of immune cells along the bottom of the membrane, the cellular clustering pattern can be utilized to fit a curved line in estimation of the membrane's bottom edge. The upper channel starts + 50 μm above this height, which corresponds to the membrane's upper edge (see [Figure 9](#)).

Figure 9 Visualization of the classification of immune cells by z-position relative to the membrane



Ensuring Python script accuracy

In experiments with lower attachment densities, the accurate detection of the membrane edges can be difficult. To ensure accuracy in membrane edge detection, regardless of recruitment levels, the Python script requires users to input an expected z-range for the membrane. Membrane edge detection can be estimated with the following steps.

| Step | Action |
|------|---|
| 1 | Open a representative z-stack in ImageJ2 or a similar viewer |
| 2 | View the lowest plane, in depth, represented within the z-stack |

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| | |
|---|---|
| 3 | Scroll through the z-planes, such that each plane gets progressively higher, in depth, within the z-stack |
| 4 | <p>Record location (z-depth, in microns) of the bottom edge of the membrane. Depending on image type, the following scenarios will be visible:</p> <ul style="list-style-type: none"> – Fluorescence only: Aggregation of fluorescent signal from immune cells adhered to the bottom of the membrane. Cells will resemble “pancakes”, stretched in the xy plane and short in height, along the z – Brightfield (if image stack includes a reference channel): Membrane pores become visible – Brightfield + Fluorescence: Visualize both membrane pores and fluorescent signal from adhered cells <p><i>Note: For most accurate results, record the value at the edge of the co-culture channel instead of center of the membrane.</i></p> |
| 5 | For the location of the upper membrane, we recommend adding 60 μm to the z-location found for the bottom edge of the membrane. |

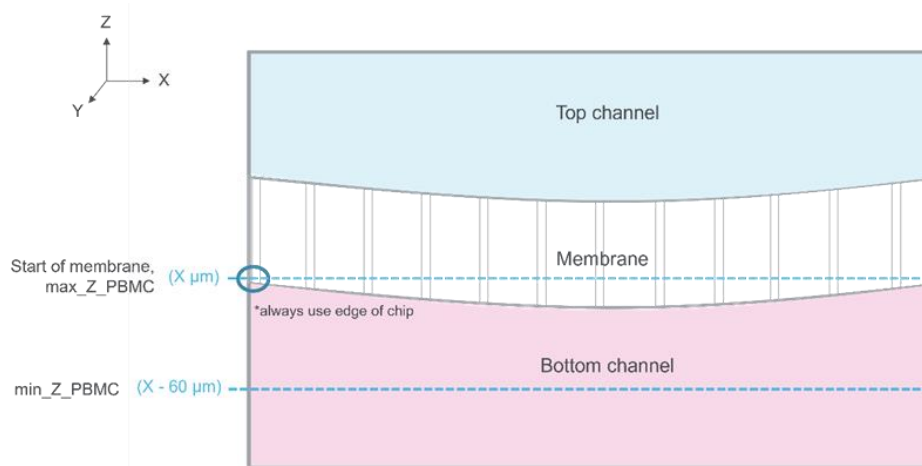
Max and Min z-range

The max and min of the membrane z-range is (Figure 10):

- Membrane Z-range maximum = start of the membrane as determined by user in μm
- Membrane Z-range minimum = start of the membrane – 60 μm

Figure 10

Defining potential Colon Intestine-Chip membrane location range for correct identification within python script

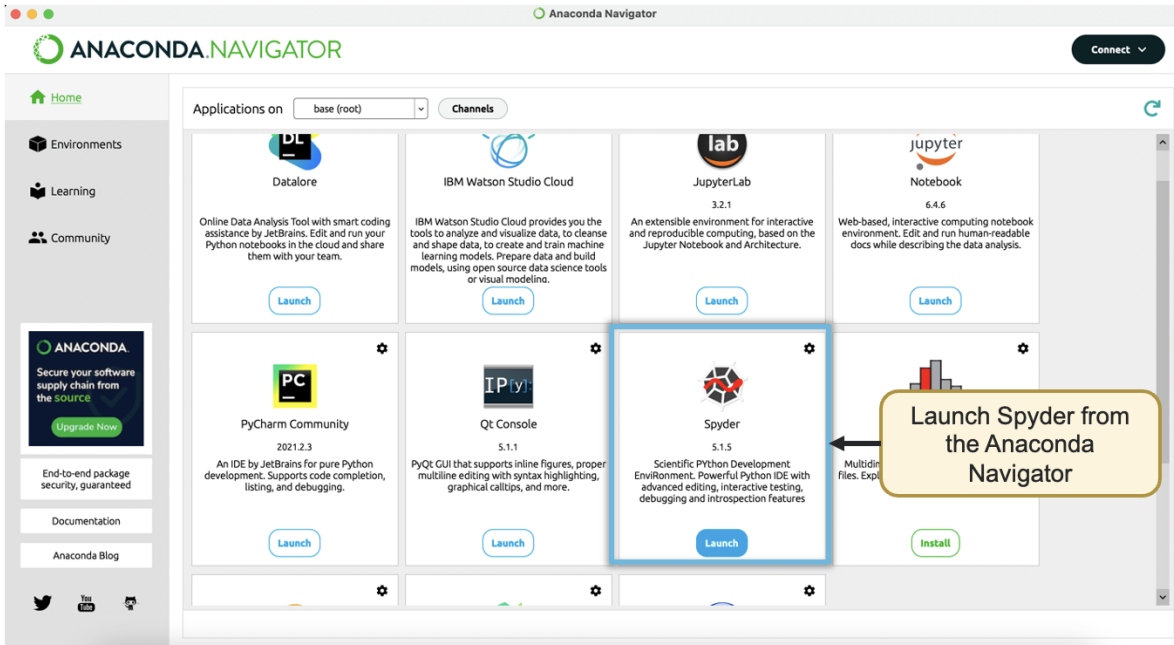


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Running Spyder Integrated Development Environment (IDE)

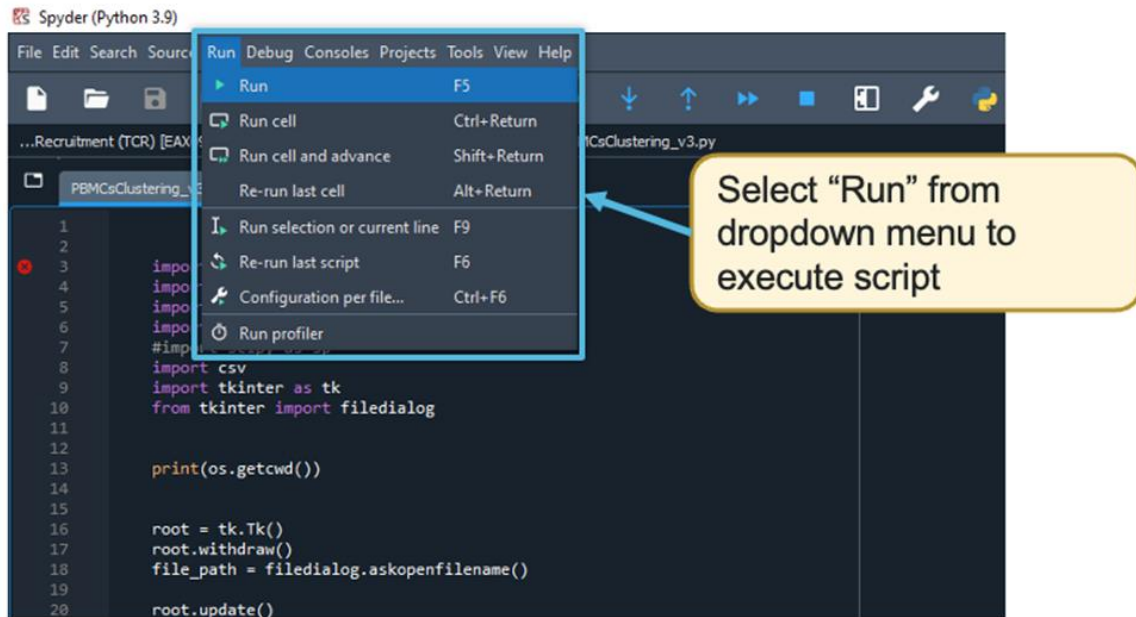
Protocol

The Scientific Python Development Environment (Spyder) IDE permits the visualization of migratory plots that project immune cells and their recruitment status with respect to their location within the xz-plane.

| Step | Action |
|------|--|
| 1 | Open the Anaconda Navigator |
| | <p>Figure 11: The Anaconda Navigator is a desktop application that lets you easily manage open-source Python tools without using the command line</p>  |
| 2 | Select “Launch” button on the Spyder module. |
| 3 | The IDE (console window) will open. |
| 4 | In the Spyder IDE, go to File/Open and select the ‘PBMCSClustering.py’ script |
| 5 | Press the “Run” button to run the script |

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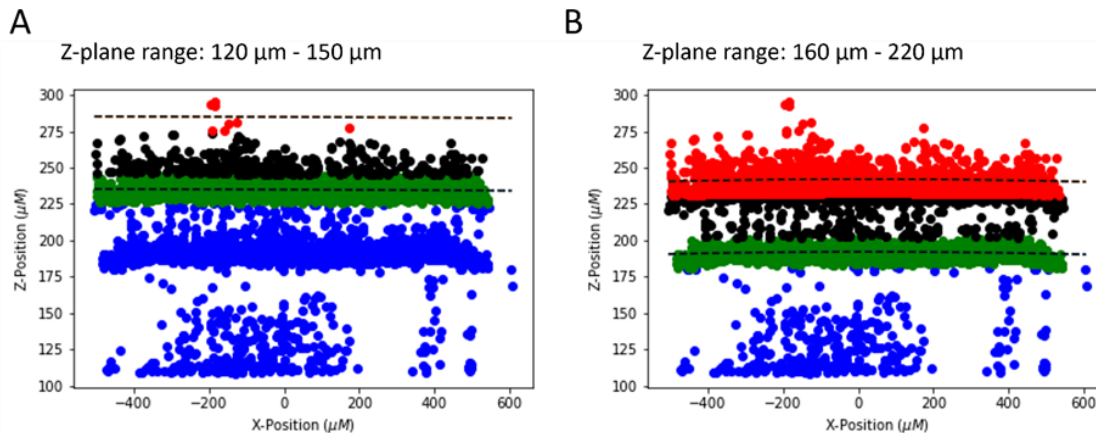
Figure 12: Spyder IDE



| | |
|---|--|
| 6 | <p>A dialogue box will appear. Select the Excel data file to analyze.</p> <p><i>Note: The output directory location will be the same as that of the Python script file</i></p> |
| 7 | <p>When the script prompts, enter the values determined in “User Determination of Z-Range to Assist Classification” section.</p> <p><i>Note: If the output XY categorization plots do not look correct, adjust the z-plane range so that the script more accurately categorizes the immune cell objects (see Figure 13 for example).</i></p> |
| 8 | <p>Outputs will populate in the same folder in which the ‘PBMCsClustering.py’ script was saved (Figure 13)</p> |

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Figure 13: Script output xz plots demonstrating z-plane range effect on immune cell categorization. A) Plot with z-plane range incorrectly set to 120-150 μm , where two distinct layers of cells are visible at $\sim 180 \mu\text{m}$ and $\sim 230 \mu\text{m}$ (50 μm membrane distance apart). These two uniform layers indicate that the membrane is lower than estimated and is better fit using the z-plane range in B) 160-220 μm as the bottom start of the membrane.



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Output Examples from Python Script

Introduction

The Python Immune Cell Recruitment Analysis Script will output the following:

Step 1

Excel file with the percentage and absolute value of migrated immune cells.

| Chip ID | | Characterized Immune Cell Counts | | | | % Migration |
|------------------|--------|--|---|-------------------------------------|--|------------------------------|
| spreadsheet Name | | Number of PBMCs below the bottom of the membrane | Number of PBMCs at the bottom of the membrane | Number of PBMCs inside the membrane | Number of PBMCs at the top and above of the membrane | Percentage of Migrated PBMCs |
| 0 | Chip4 | 1383 | 13855 | 377 | 19 | 2.78 |
| 1 | Chip9 | 2 | 29 | 7 | 58 | 69.15 |
| 2 | Chip10 | 30 | 16 | 1 | 5 | 27.27 |
| 3 | Chip11 | 88 | 8 | 0 | 1 | 11.11 |
| 4 | Chip12 | 51 | 1164 | 34 | 82 | 9.06 |
| 5 | Chip13 | 92 | 1967 | 47 | 86 | 6.33 |
| 6 | Chip14 | 362 | 1631 | 34 | 131 | 9.19 |
| 7 | Chip15 | 87 | 1152 | 13 | 33 | 3.84 |
| 8 | Chip16 | 64 | 174 | 152 | 84 | 57.56 |

The file, named “PBMC_Position_Statistics.xlsx”, contains the following immune cell counts:

- Below membrane
- Attached to bottom of membrane
- Inside membrane
- Top of membrane (epithelium)

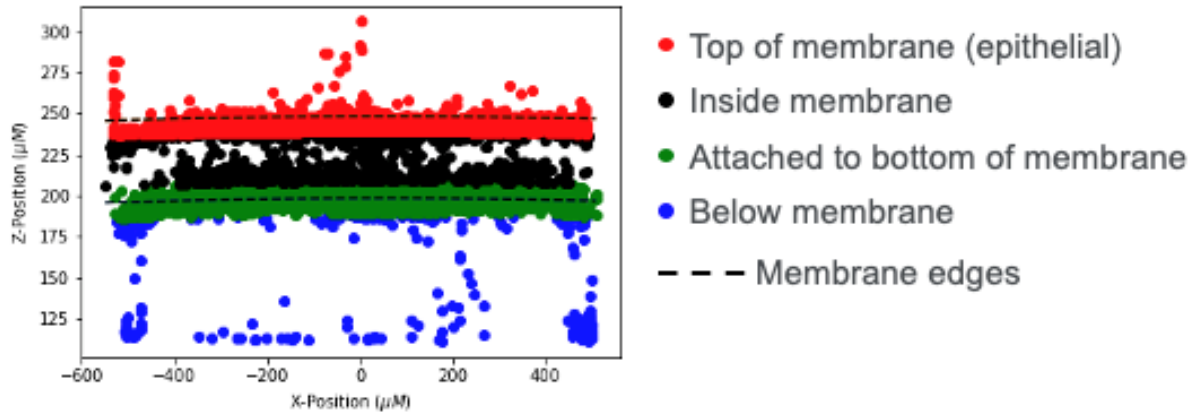
The Excel file also contains a metric of “Percent of Migrated Immune Cells”, calculated from:

$$\frac{(N_{\text{top of membrane}} + N_{\text{inside membrane}})}{(N_{\text{top of membrane}} + N_{\text{inside membrane}} + N_{\text{bottom of membrane}})} \times 100$$

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Step 2

Migratory plots in the xz-plane depicting immune cell locations within the chip and their recruitment characterization.



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Part VIII. Reference

Introduction

| Resource | Reference |
|---|---|
| The Immune Cell Recruitment (ICR) Analytical Package utilizes the ImageJ plugin | <i>Cordelières, F (2006) 3D Objects Counter (Version 2.0.1) [Source code].</i> Link |
| The original plugin | S. Bolte & F. P. Cordelières, A guided tour into subcellular colocalization analysis in light microscopy, Journal of Microscopy, Volume 224, Issue 3: 213-232 |
| 3D Object Counter Plugin Manual | https://imagejdocu.list.lu/lib/exe/fetch.php?media=plugin:analysis:3d_object_counter:3d-oc.pdf |
| Colon Intestine-Chip Culture Protocol | Link |

Microscopy Terms

| Term | Description |
|-----------------------|--|
| Point Spread Function | Measure for the quality of an optical system as it reveals how points are blurred in an image |
| Optical Resolution | Ability of an imaging system to resolve two points. In other words, the shortest distance between two points that can be observed as separate entities |
| Working Distance | Distance from lens surface to focal point |
| Numerical Aperture | Range of angles over which the optical system can accept or emit light |
| Nyquist Frequency | Minimum rate at which a sample can be sampled without introducing error |

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Image Acquisition Parameters

| Term | Description |
|---------------------------|---|
| Project Name | User defined ICR project name |
| Chip ID in File Name | Component of Image file name defining chip ID |
| FOV in File Name | Component of Image file name defining imaged field-of-view |
| Plane in File Name | Component of Image file name defining z-plane in image stack |
| Channel in File Name | Component of Image file name defining fluorescence channel |
| Image File Type Extension | Defines image type for analysis |
| Source Directory | Defines pathway to folder containing imagery for analysis |
| Output Directory | Defines pathway to folder created to accept results (i.e., script output) |

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Image Processing Parameters

| Term | Description |
|--|--|
| Multichannel Image | Fluorescence image containing multiple signals, each acquired on a discrete channel |
| Number of Fluorescence channels in acquisition | Defines how many channels were acquired during imaging |
| Channel Number for Immune Cells or PBMCs | Defines which channel is representative of the fluorescence signal |
| Z-stacks compiled | Fluorescence image containing multiple z-planes within an image stack |
| Bottom Position of z-stack | In microns, the location of the lowest z-plane within an image stack |
| Distance between planes in z-stack | In microns, the distance between each z-plane of an image stack |
| Despeckle | Median filter designed to remove salt and pepper noise by replacing each pixel with the median value of its 3 x 3 neighborhood |
| Subtract Background | Define which intensities on the lower end of histogram represent background and set these pixels as no signal |
| Sliding Parabola | Image processing tool that removes features smaller than defined radius |
| Rolling Ball Radius | Image processing tool to fix uneven background signal. Set radius to at least the size of the largest object NOT part of background. |

For further detail on image processing:

<https://imagej.nih.gov/ij/docs/menus/process.html>

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Object Processing Parameters

| Term | Description |
|--|--|
| Minimum Intensity Object Threshold Value | Define the intensity value separating background from cell |
| Minimum Cell Volume Size | Defines minimum cell size detection limit, reported in voxels (pixels ³) |
| Maximum Cell Volume Size | Defines maximum cell size detection limit, reported in voxels (pixels ³) |
| Centroid Maps | Annotation mask of geometrical centers for all cells detected |
| Object Maps | Annotation mask overlayed detected signal from all cells |
| Surface Maps | Annotation mask overlay of all surface voxels |

Immune Cell Recruitment Parameters

| Term | Description |
|----------------------------------|--|
| XZ Characterization plot | Visualization of all immune cell objects in a chip in an xz view, color-coded by the script classification. The chip membrane is visualized as a dotted black line |
| Membrane (max) | The maximum expected z-plane where the bottom of the membrane lies (default value 220 μm) |
| Membrane (min) | The minimum expected z-plane where the bottom of the membrane lies (default value 100 μm) |
| Percent of Migrated Immune Cells | Calculation of migrated immune cells (inside and above the membrane) as a percentage of the total immune cells on the bottom + inside + top of membrane (excluding those below the membrane) |