DDD
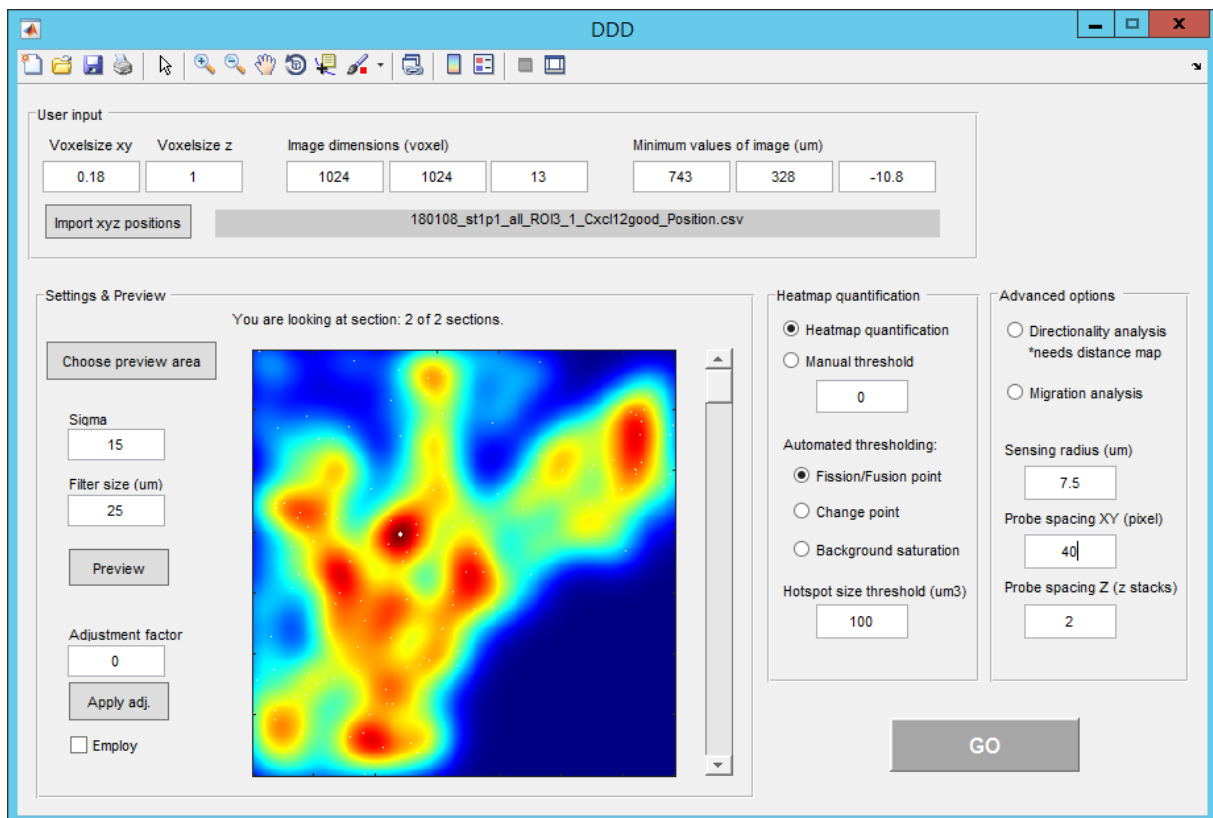
DDD is a compilation of several simple algorithms meant for analyzing 3D discrete data. DDD stands for **D**ensity from **D**iscrete **D**ata.

License
DDD is free software: you can redistribute it and/or modify it under the terms of the 3-Clause BSD License.

This software is provided by the copyright holders and contributors "AS IS" and any EXPRESS or IMPLIED WARRANTIES, including, but not limited to, THE IMPLIED WARRANTIES OF MERCHANTABILITY AND FITNESS FOR A PARTICULAR PURPOSE ARE DISCLAIMED. See the BSD-3-License for more details (https://opensource.org/licenses/BSD-3-Clause).

Table of Contents

Installation guide

In order to install the standalone version of **DDD** the user can download the installer from https://www.bsse.ethz.ch/csd/software/DDD.html.

DDD works on Windows operating systems (developed, tested and run only on Windows 10) and requires the Matlab Runtime Compiler (MRC) 2018a. If the MRC is not yet installed on the machine, the user can download the MRC Version 9.4 from https://www.mathworks.com/products/compiler/matlab-runtime.html.

The installation of the standalone version of **DDD** (administrator rights needed) and if needed the MRC is guided by an installation wizard and will require around 1 minute (if MRC is installed) to complete. If the MRC is not installed it will require more time as indicated by a progress bar in the installation wizard. To start **DDD** double click on the application icon named "**DDD**", either as a shortcut created on your desktop or in e.g. C:/Program Files/ **DDD**.

In order to use the **DDD** version within Matlab, download the appropriate source code version from https://www.bsse.ethz.ch/csd/software/DDD.html. Unzip the downloaded file and add the directory containing the unzipped files into the Matlab path. In order to start the software type "**DDD**" in the command window and press enter.

Hardware & software requirements

DDD was tested and runs reliably on Windows 10 with the software packages pointed out in the Installation guide. Although not a minimum requirement 16 GB of RAM and a Intel i7-6600 CPU (2.6 GHz) was used during testing and was found to yield good runtime results (see below).

Data prerequisites

DDD work with XYZ coordinates of e.g. the centroid of segmented cells or spots. The segmentation has to be carried out in another program e.g. Bitplane's Imaris software package. The import of XYZ coordinates is based on the .csv export function of Imaris, as it is a standard software used by many laboratories. However, coordinates derived from other segmentation software can be adapted to this format easily.

Furthermore, the dimensions of the image analyzed as well as a reference point need to be entered. This data can be found in the images metadata, which can be assessed by e.g. opening the image file in ImageJ or directly from Imaris.

For some advanced analysis options, tissue masks are optional, whereas distance maps are required.

Suggested workflow

- Spot/Cell segmentation in Imaris and subsequent export of statistics as .csv files.

- Open DDD, enter the required data in the upper field of the software and open the "Position" file from the exported. csv files.
- Choose a preview region of your image (not too small to avoid overfitting) and enter/change the parameters for heatmap generation on the left side of the software. Optionally add the adjustment factor.
- Once satisfied with the settings, decide if and if so, which kind of quantification you want and adjust the buttons and settings accordingly.
- Press "Go" and name the heatmap file in the location of your choice. DDD creates a new folder, which later contains heatmap and quantification as well as a log file. Once you pressed "save" the software will run. The cursor (on top of the software) will show an hourglass or equivalent "busy" sign, until the software has finished.
- The heatmap(.tiff) can subsequently be added to the Imaris file of the image analyzed ("Add channels") or opened in ImageJ or other programs for better visualization.

User input

Basic inputs

In order to perform accurate analyses, image metadata such as voxel/pixelsize in xy and z (in micrometer) is required e.g. 0.24 and 1. In addition, image dimensions in pixel (xy) and z-stacks (z) e.g. 1024, 1024, 15, as well as the coordinates of the reference point minimum X/ minimum Y / minimum Z are needed. These coordinates might be 0/0/0 in a standalone image. In a cropped region of interest, they could be -5/234/897. This reference point is the point with the smallest values of each axes. It is needed to accurately interpret the XYZ coordinates imported and also to later add the heatmap via "Add channels" to the original image in Imaris.

Import xyz positions

DDD works with XYZ coordinates. Clicking here lets you navigate your computers folder structure to choose the Imaris exported "Positions" file, which is a csv file of the xyz coordinates of the centroids of the segmented objects. This structure can be easily mimicked when exporting XYZ coordinates from other software.

Settings & Preview

Choose preview area

The coordinates provided are rendered in a binary maximum intensity projection. In this pop-up figure a cross-hair appears with which the user can select a rectangular region of interest (in the xy) on which the parameters for heatmap generation can be tested.

Sigma

Sigma refers to the width of the Gaussian curve (in 3D) with which each dot is blurred. The bigger "Sigma" the more blurred the actual DDD will be.

Filter size

Filter size refers to the size of the Gaussian filter i.e. the voxels affected by the blur, specified by Sigma.

Adjustment factor

DDD saves an adjustment factor for every heatmap it produces. This factor can be used to adjust the values of a subsequently generated heatmap to the level of a heatmap produced before. For example, adjust the level of a treatment condition to no-treatment to get a clear visual difference. If not adjusted, DDD will scale all heatmaps from 0-255. Make sure to tick the employed box to use the adjustment factor for the heatmap generation.

Be aware that entering an adjustment factor will have an influence on hotspot generation if a manual threshold is used!

Apply adj.

Applies the adjustment factor to the heatmap preview.

Employ

If ticked, the adjustment factor will be employed during heatmap generation.

Preview

When pressing the Preview button DDD calculates a heatmap of the regions of interest chosen before (Choose preview area). The slider on the right side of the display allows the user to navigate in the z-axis where possible (determined by the software).

Heatmap quantification

If checked quantification of the heatmap will be carried out. DDD will segment hotspots, export the hotspot threshold (0-255 in 8 bit format), the number of hotspots and the volume of individual hotspots.

Manual threshold

If a specific threshold (from 0 to 255 in 8 bit images) is desired (e.g. from an analysis of a non-treatment condition), it can be entered here. Be aware to adjust the heatmap calculation accordingly.

Automated thresholding

Fission/Fusion point
This is the standard automated hotspot segmentation. DDD will create hotspots at every value of the heatmap (1:256) and count the number of individual hotspots generated. The value at which the number of hotspots is highest and the furthest towards the high values (in conflicting situations) will be the hotspot threshold. After this value no more new hotspots will emerge. The number of hotspots is getting less and the density per individual hotspot increases.

Change point
Here the hotspot threshold is the value at which the number of hotspots decreases the most towards higher values i.e. the change point of the curve where number of hotspots is plotted against the hotspot threshold.

Background saturation
Here the hotspot threshold is the value at which the density of the non-hotspot regions i.e. the background is not growing "strong" anymore. It is the change point of the logistic function.

Hotspot size threshold
For all quantifications hotspots smaller than the specified volume in cubic micrometer are not analyzed.

Advanced options

Directionality analysis

If enabled, DDD will lay a 3D raster, as specified by Probe spacing XY and Probe spacing Z, of the matrix created by the centroids of the data. For the directionality analysis DDD need the input of a distance channel, as can be produced by the Distance Transformation Xtension in Imaris, saved as a series of .tiff images. To only include raster points within the tissue, the user can upload a tissue mask (voxels within tissue = 255, voxels outside tissue = 0). If no tissue mask is available, all raster points will be considered.

Then, DDD will go through all the raster points searching for the highest value of the heatmap within a radius, as specified by the user. Then, the directionally of the vector pointing from the raster point towards this highest concentration is calculated and compared to the vector pointing towards the lowest value in the distance channel within the radius. The angular difference between both vectors is recorded and saved as a) per raster point and b) as an average per 1um in the distance channel provided.

Migration analysis

As for the directionality analysis DDD will lay a 3D raster, as specified by Probe spacing XY and Probe spacing Z, of the matrix created by the centroids of the data. To only include raster points within the tissue, the user can upload a tissue mask (voxels within tissue = 255, voxels outside tissue = 0). If no tissue mask is available, all raster points will be considered.

Then, DDD will go through all the raster points searching for the highest value of the heatmap within a radius, as specified by the user. An unscaled vector is created pointing from the raster point towards the highest value on the heatmap, and distance as well as directionality are recorded. The endpoint of the vector becomes the starting point for the next iteration, where DDD will perform the same analysis again. The "cell" is allowed to "migrate" for as long as not one of the stop criteria is reached. The stop criteria are: "Backwards travel" over 180 degrees and too small of a distance gain upon migration (1 micrometer). The endpoint coordinates are saved as an Excel sheet as well as in .txt format for easy plotting in Imaris using the XTCreateSpotsFromFile XTension. Also, the distances travelled are saved for every raster point enabling an easy observation of how long the bulk of cell would travel until reaching a local maximum.

Sensing radius

This is the radius around a raster point that is considered when looking for the highest values of the heatmap. In other words it is the area has access to, which can be determined by size alone or also additional movement or the ability to probe more distant areas by protrusions.

Probe spacing XY

This value determines how many raster points you will have in the xy direction. The smaller the number the more raster points will be created and the longer the runtime of the code will be (Voxelsize XY = Probe spacing XY: every pixel will be a raster point; Probe spacing XY cannot be smaller than Voxelsize XY). A good value depends on imaging settings and tissue constitution. We would recommend starting with 50.

Probe spacing Z

This value determines how many raster points you will have in the z direction i.e. how many z stacks will be considered in the analysis. The smaller the number the more z stacks will be considered (Voxelsize Z = Probe spacing Z: every z stack will be considered; Probe spacing Z cannot be smaller than Voxelsize Z). The runtime of the code will be longer the smaller the number. A good value depends on imaging settings and tissue constitution. We would recommend starting with 2 or 3.

GO

Clicking the GO button start the tool with all the settings set prior. The user is prompted to name the heatmap tiff file around which a new folder is created. Depending on the options chosen in Advanced options, more input might be required. If the cursor on top of the user interface turns from a "waiting cursor" back to a normal "selection cursor" the operation is done.
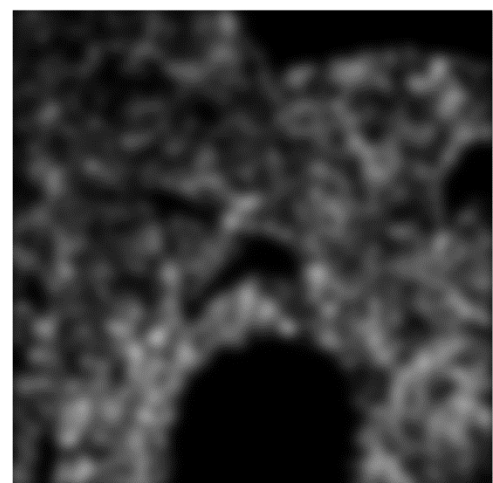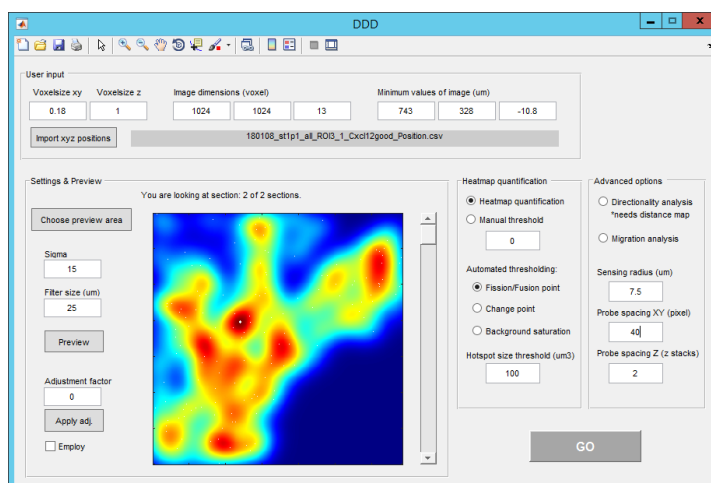
Demo data

Using the demo data provided, a 3D heatmap (as seen below in gray scale), and these first 10 path lengths from the migration analysis should result. Settings for the migration analysis should be as depicted in the screenshot.

Time until heatmap is produced: ca. 10 seconds

Time needed for hotspots calculation: 2-3 min

Time needed for migration analysis: 6-7 min

Length of migration paths (um): 5.864857, 9.087818, 2.368881, 8.014192, 17.11372, 12.0214, 4.521692, 4.34554, 7.927672