Below are the main steps of the analyses described in "Heterogeneity in VEGF Receptor-2 mobility and organization on the endothelial cell surface leads to diverse activation models by VEGF" by da Rocha-Azevedo et al. 2020.

Software installation

The software has been tested on **Matlab R2018b** on **Linux 64-bit OS**. It includes several mex-files that might need recompilation for other Operating Systems.

The software requires the following **Matlab toolboxes**:

- Statistics and Machine Learning Toolbox
- Control System Toolbox
- Robust Control Toolbox
- Optimization Toolbox
- Signal Processing Toolbox
- Symbolic Math Toolbox
- Image Processing Toolbox
- Datafeed Toolbox
- Bioinformatics Toolbox
- Computer Vision System Toolbox
- Curve Fitting Toolbox
- Parallel Computing Toolbox
- Matlab Distributed Computing Server

The download package contains all functions (but not the Matlab toolboxes) necessary to run the described analysis steps, except for those in the u-track package (for single particle tracking), the diffModesF2F package (for diffusion mode analysis from frame-to-frame displacements), and the YALMIP package (for estimating the particle oligomerization state from its intensity and merging and splitting history). These packages are necessary to run the below pipeline, and can be downloaded from here:

- u-track: https://github.com/DanuserLab/u-track.
- diffModesF2F: https://github.com/kjaqaman/diffModesF2F
- YALMIP: https://yalmip.github.io/.

The descriptions below explain briefly the function(s) for each analysis step. For detailed information on the input and output arguments of these functions, please refer to the help section of each function.

For individual time courses (i.e. individual experiments):

1. Detect, track and analyze receptor motion:

1A. Create movie list of single-molecule movies in a time course
Function call: UICreateMovieList;
Follow prompts. In the step of entering the emission wavelength, enter one wavelength per channel per prompt. Once all wavelengths have been entered, press cancel.
When asked to find a place to save the move list, navigate to directory and also type file name (e.g. ML1.mat).
Select all movies at once. They should be all in the same directory.

1B. Particle tracking
Function call: uTrackPackageGUI;
Follow u-track instructions. Can open whole movie list at once.
Run all 3 steps of u-track, including the track analysis step.

1C. Diffusion mode analysis from frame-to-frame displacements
Function calls:
- Open movie list in workspace: ML = MovieList.load;
- Load the contents of e.g. diffModeDividerStructExample1.mat (located inside diffModesF2F/ExampleData directory). NOTE: This diffusion mode divider structure is for VEGFR2. Other molecules will have other diffusion properties and will need their own diffusion mode divider structure. Please see documentation of diffModesF2F package for more details.
- Call analysis code:
analyzeDiffusionModesMLMD(ML,[],[],0,[],[],[],[],[],diffModeDividerStruct,0);

2. In parallel, generate cell masks:

The below steps allow one to manually generate a cell mask based on e.g. a brightfield image of the cell. If the cell mask can be generated in an alternative manner, based on different images or automatically instead of manually, that is also compatible with the full analysis pipeline, as long as step C is executed to link each single-molecule movie with its mask.

2A. Create movie list for bright field images
Function call: UICreateMovieList;
This goes through the same set up prompts as for single-molecule movies. Most of them are irrelevant, but go through them to make the movie list. Call the movie list BFML1.mat, for example.

2B. Make the masks
This is done through the GUI of uTrackPakageGUI
Function call: uTrackPackageGUI
Load the brightfield movie list. This will list the individual images in the first interface. Then, for each image, select “Tools” and “Add region of interest”. In the new interface, select “Draw new

region of interest". Draw mask manually, and select save. After all masks have been created, exit uTrackPackageGUI.

2C. Associate the masks with their single-molecule movies
Function calls:
- Open SINGLE_MOLECULE movie list in workspace: ML = MovieList.load;
- Associate masks with movies: importCellMaskMLMD(ML); This function allows you to manually select the mask for each single-molecule movie.

For multiple time courses:

These steps are performed after running the above steps for all individual time courses that are to be grouped together.

3. Combine multiple movie lists into one combined movie list

Function call: UICreateCombinedMovieList
Follow prompts. When asked to find a place to save the combined move list, navigate to directory and also type file name (e.g. CML1.mat). When asked to select movie lists to combine, select them one by one, pressing "open" after each selection. When done selecting, press "cancel."

4. Basic time course analysis

Function call: UITimeCourseAnalysis
When asked to select combined movie lists for analysis, select them one by one, pressing "open" after each selection. When done selecting, press "cancel."
Default parameters work well for a "complete" dataset of about 8-10 individual time courses, with one movie every ~3 min per dataset. Parameters can be adjusted to accommodate different situations. For example, time interval for averaging can be set to a value different from 3 min. If dataset is small, then uncheck "detect outliers" and "ignore isolated points."
UITimeCourseAnalysis makes plots in multiple directories. The only relevant plots are those in the "figuresAve" directory. The plots in the other two directories are no longer used or maintained in our analysis pipeline.

5. Merge/split/motion analysis:

This analysis requires the tracks and their times as sorted by UITimeCourseAnalysis. They can be obtained by opening the file "analysisData.mat," saved in the directory where the time course analysis results are saved. In this file, there is a cell array called "summary" (one entry per combined movie list analyzed), to be used as input for the next two analysis steps.

5A. Prepare tracks for analysis

Function calls:
- Load combined movie list. For example: CML = CombinedMovieList.load('CML1.mat');
- Load the contents of diffModeDividerStruct2.mat (same as in Step 1C above).
- Load the contents of "analysisData.mat," inside the directory where the time course analysis results are stored. The variable of interest is "summary." This is a cell array with number of entries equal to number of combined movie lists analyzed together. Take the entry that matches the loaded combined movie list.
- Navigate in matlab command line to directory where results are to be saved.
- Run the function prepareTracksForMSAnalysis. See function help for input and output details. The output will be saved in the current directory.

5B. Perform analysis
Function call: analyzeMergeSplitVsMotionStats. See function help for input and output details.

5C. Summarize analysis
Function call: summarizeMergeSplitVsMotionStats. See function help for input and output details.

6. Primary-secondary channel analysis:

In the code the primary channel is often called "master" and the secondary channel is often called "follower."

6A. Prepare tracks for analysis
Same steps as in 5A above. The only difference is that the input followerFlag = 1 here (it is set to 0 in 5A).

6B. Perform analysis
Function call: analyzeMergeSplitMotionVsFollowerStats. See function help for input and output details.

6C. Summarize analysis
Function call: summarizeMergeSplitMotionvsFollowerStats. See function help for input and output details.