This is an instruction manual for RLNEK users. The objective of this manual is to catalyze the user-experience by providing an example of the interactive session while running RLNEK Module 2. This should demonstrate how to: 1) locate the directory with relevant Trackmate files, 2) input experimental system parameters, and 3) enter multiple trials/replicates for any given condition. RLNEK Module 0 and Module 1 are analogous.

Running directory of ".py" scripts

RLNEK will automatically run in the directory where the python script is saved for all Modules. If you would like to change the running directory either save the python script in the desired directory or change the directory in the script (**Figure 1**).

```
import pandas as pd
import numpy as np
from scipy import stats, optimize, special, interpolate
from matplotlib import pyplot as plt, cm as cm
import operator
import math
import os
import sys
import warnings
import csv
os.chdir('directory/of/your/choice')
```

Figure 1. Necessary inbuilt modules for RLNEK and selecting the directory for RLNEK to operate in.

N_T Optimization

In order to use Module 2, a curve-fitted value of N_T is required to calculate the capture efficiency N_b/N_T. Open the file "NT_Optimization.py" on a console to begin. The optimized N_T value is calculated for each flow rate by measuring the number of captured cells, N_b, at one applied tensile force (or flow rate) and several combinatorial site densities, $m_r m_l$.

Run the file, and enter system parameters as well as CCD FPS, stopping criteria distance, receptor site density (m_r), and non-specific binding time (t_{min}) (**Figure 2**). To use the default values for D_min and t_min (0.5 μm and 0.2 seconds, respectively), press the Enter key when prompted to input those values.

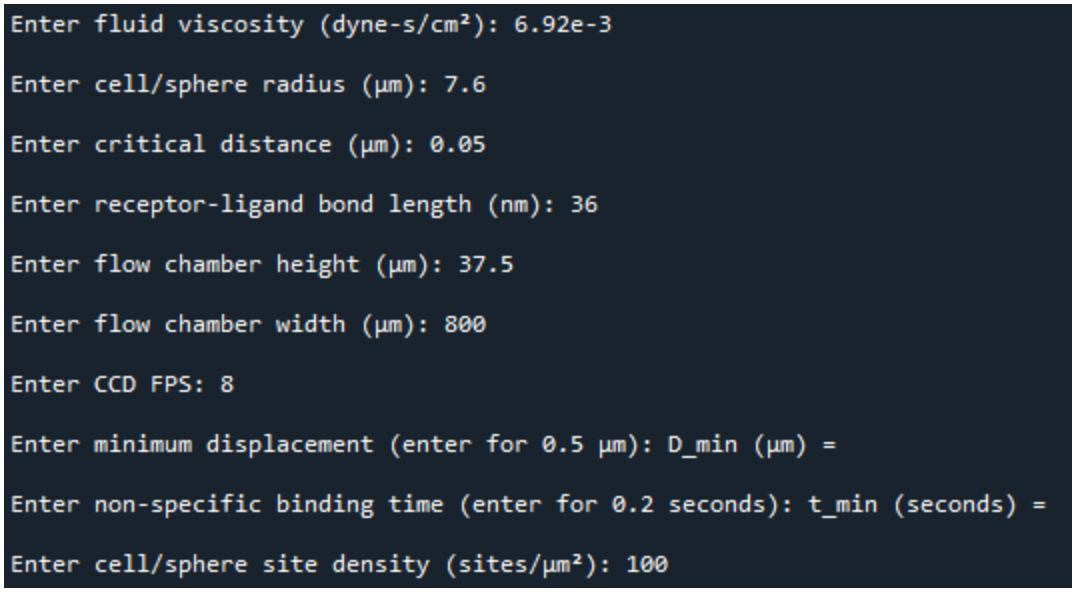

Figure 2. Inputting system parameters

Next, the user can input all Trackmate files to be analyzed (**Figure 3**). RLNEK will ask the user for a flow rate and then the ligand site densities (m_l) analyzed under that flow rate. Then, RLNEK will prompt the user to input the “track” and “spots” files from Trackmate.

Ensure that the order of files inputted will correspond. For example, **Figure 3** shows that when prompted for “tracks” files, the “10sites_receptor_ligand_5ulhr_8FPS_Trial_1_tracks” file is first inputted. Then, when prompted for “spots” files, the corresponding “spots” file, “10sites_receptor_ligand_5ulhr_8FPS_Trial_1_spots”, is inputted. After entering all flow rate and site densities, enter “n” when prompted for “y” or “n” to stop inputting data.

```
Enter "y" to input data or "n" to quit: y

Enter flow rate (μL/hr): 5

For Q = 5.00 (μL/hr), enter site densities (sites/μm²): 10, 50, 100

For flow rate = 5.00 (μL/hr) and site density = 10.000000 (sites/μm²), enter name of "tracks"
file(s) from Trackmate: 10sites_receptor_ligand_5uLhr_8FPS_Trial_1_tracks

For flow rate = 5.00 (μL/hr) and site density = 10.000000 (sites/μm²), enter name of "spots"
file(s) from Trackmate: 10sites_receptor_ligand_5uLhr_8FPS_Trial_1_spots

For flow rate = 5.00 (μL/hr) and site density = 50.000000 (sites/μm²), enter name of "tracks"
file(s) from Trackmate: 50sites_receptor_ligand_5uLhr_8FPS_Trial_1_tracks

For flow rate = 5.00 (μL/hr) and site density = 50.000000 (sites/μm²), enter name of "spots"
file(s) from Trackmate: 50sites_receptor_ligand_5uLhr_8FPS_Trial_1_spots

For flow rate = 5.00 (μL/hr) and site density = 100.000000 (sites/μm²), enter name of "tracks"
file(s) from Trackmate: 100sites_receptor_ligand_5uLhr_8FPS_Trial_1_tracks

For flow rate = 5.00 (μL/hr) and site density = 100.000000 (sites/μm²), enter name of "spots"
file(s) from Trackmate: 100sites_receptor_ligand_5uLhr_8FPS_Trial_1_spots

Enter "y" to input data or "n" to quit: n
```

Figure 3. Inputting experimental conditions and Trackmate files for one trial.

This module can also be used for multiple trials of data (**Figure 4**). Make sure to ***separate*** the file names and N_T values for ***different trials with a comma***. Make sure to input the same number of N_T values as “tracks” (or “spots”) files.

```
Enter "y" to input data or "n" to quit: y

Enter flow rate (μL/hr): 5

For Q = 5.00 (μL/hr), enter site densities (sites/μm²): 10, 50, 100

For flow rate = 5.00 (μL/hr) and site density = 10.000000 (sites/μm²), enter name of "tracks"
file(s) from Trackmate: 10sites_receptor_ligand_5uLhr_8FPS_Trial_1_tracks,
10sites_receptor_ligand_5uLhr_8FPS_Trial_2_tracks,
10sites_receptor_ligand_5uLhr_8FPS_Trial_3_tracks

For flow rate = 5.00 (μL/hr) and site density = 10.000000 (sites/μm²), enter name of "spots"
file(s) from Trackmate: 10sites_receptor_ligand_5uLhr_8FPS_Trial_1_spots,
10sites_receptor_ligand_5uLhr_8FPS_Trial_2_spots,
10sites_receptor_ligand_5uLhr_8FPS_Trial_3_spots
```

Figure 4. Inputting Trackmate files for multiple trials.

This is all that is required for RLNEK user input. RLNEK will output the N_T values for each force, calculated for each flow rate. This output will be organized in order of what flow rates the user inputted. The files shown in Figure 2 were used to obtain the result in **Figure 5**. The N_T value shown in **Figure 5** can then be used in Module 2 for the corresponding conditions (i.e., all data with a flow rate of 5 μL/hr). More details are provided in the Module 2 section.

```
For flow rate = 5.0000 µL/hr & force = 33.3969 pN: N_T = 21.3136.
```

Figure 5. N_T values for each flow rate inputted

Module 2

Open the file “Module 2.py” on a console to begin. Run the code, and enter system parameters as well as CCD FPS, stopping criteria distance, cell receptor site density m_r, and non-specific binding time, just like with the “NT_optimization.py” file.

Then, like with N_T optimization, the user can input all “tracks” and “spots” files from Trackmate to be analyzed. **Figure 6** shows an example of how to input files and corresponding data, if the user only has ***one trial*** for one condition (i.e., one flow rate and one m_l). Be sure to input the corresponding “tracks” and “spots” files, just like with “NT_Optimization”. N_T values are determined from the N_T optimization module.

Notice that **Figure 6** only included Trackmate files for one site density. To input data for multiple site densities, simply type “y” instead of “n” in the last line shown in **Figure 6**.

```
Enter "y" to input data or "n" to execute: y

Enter flow chamber site density (sites/µm²): 10

For flow chamber site density = 10.000000 (sites/µm²), enter flow rates (µL/hr), separated by commas and without new lines: 5,10,20

For flow rate = 5.00 (µL/hr) and flow chamber site density = 10.000000 (sites/µm²), enter name of "tracks" file(s) from Trackmate:
10sites_receptor_ligand_5uLhr_8FPS_Trial_1_tracks

For flow rate = 5.00 (µL/hr) and flow chamber site density = 10.000000 (sites/µm²), enter name of "spots" file(s) from Trackmate:
10sites_receptor_ligand_5uLhr_8FPS_Trial_1_spots

For each trial of flow rate = 5.00 (µL/hr) and flow chamber site density = 10.000000 (sites/µm²), enter N_T values(s): 18.333

For flow rate = 10.00 (µL/hr) and flow chamber site density = 10.000000 (sites/µm²), enter name of "tracks" file(s) from Trackmate:
10sites_receptor_ligand_10uLhr_8FPS_Trial_1_tracks

For flow rate = 10.00 (µL/hr) and flow chamber site density = 10.000000 (sites/µm²), enter name of "spots" file(s) from Trackmate:
10sites_receptor_ligand_10uLhr_8FPS_Trial_1_spots

For each trial of flow rate = 10.00 (µL/hr) and flow chamber site density = 10.000000 (sites/µm²), enter N_T values(s): 50

For flow rate = 20.00 (µL/hr) and flow chamber site density = 10.000000 (sites/µm²), enter name of "tracks" file(s) from Trackmate:
10sites_receptor_ligand_20uLhr_8FPS_Trial_1_tracks

For flow rate = 20.00 (µL/hr) and flow chamber site density = 10.000000 (sites/µm²), enter name of "spots" file(s) from Trackmate:
10sites_receptor_ligand_20uLhr_8FPS_Trial_1_spots

For each trial of flow rate = 20.00 (µL/hr) and flow chamber site density = 10.000000 (sites/µm²), enter N_T values(s): 75

Enter "y" to input data or "n" to execute: n
```

Figure 6. Inputting Trackmate files and N_T for one trial per condition. The N_T value for 10 sites/µm^2 was obtained from “NT_Optimization” and shown in Figure 3. The other N_T values were arbitrarily chosen just to demonstrate RLNEK’s functionality.

RLNEK will also accept ***multiple trials*** under one flow rate and one m_l (**Figure 7**). Make sure to ***separate*** the file names and N_T values for ***different trials with a comma***. Enter the same number of N_T as “track” (or “spots”) files.

```
Enter "y" to input data or "n" to execute: y

Enter flow chamber site density (sites/μm²): 10

For flow chamber site density = 10.000000 (sites/μm²), enter flow rates (μL/hr), separated by
commas and without new lines: 5,10,20

For flow rate = 5.00 (μL/hr) and flow chamber site density = 10.000000 (sites/μm²), enter name of
"tracks" file(s) from Trackmate: 10sites_receptor_ligand_5uLhr_8FPS_Trial_1_tracks,
10sites_receptor_ligand_5uLhr_8FPS_Trial_2_tracks,
10sites_receptor_ligand_5uLhr_8FPS_Trial_3_tracks

For flow rate = 5.00 (μL/hr) and flow chamber site density = 10.000000 (sites/μm²), enter name of
"spots" file(s) from Trackmate: 10sites_receptor_ligand_5uLhr_8FPS_Trial_1_spots,
10sites_receptor_ligand_5uLhr_8FPS_Trial_2_spots,
10sites_receptor_ligand_5uLhr_8FPS_Trial_3_spots

For each trial of flow rate = 5.00 (μL/hr) and flow chamber site density = 10.000000 (sites/μm²),
enter N_T values(s): 18.333, 18.333, 18.333
```

Figure 7. Inputting data for multiple trials under one condition.

After inputting all data, input “n” to quit and start data analysis. RLNEK will execute and compute stopping events for all conditions and trials (WARNING: this may be time intensive). Afterwards, the user will be prompted to enter the diffusivity, D, and reactive radius, α (**Figure 8**).

```
Enter receptor-ligand diffusivity (μm²/s): 0.15

Enter ligand reactive radius (nm): 2
```

Figure 8. Inputting diffusivity and reactive radius

After entering this data, RLNEK will compute the remaining parameters to analyze the receptor-ligand kinetics and output k_{off} vs. force, N_b/N_T vs. force, and k_+ vs. force graphs to the user’s local directory. A csv file will be saved “RLNEK.csv” that summarizes these calculations for all input files. Additionally, all stopping events and k_{off} values are output to “RLNEK_koff.csv” with their corresponding TRACK_ID. The intrinsic reaction rate k_{in} and other parameters of interest can be obtained by input into the console (e.g., kin_avg). Please refer to the “appendix.pdf” file, “README.txt” file, and the corresponding manuscript for more information on the parameters of interest.