**General Descriptions:**

**1. Check and input experiment-related information from the following three Excel files:**

- `additional\_info.xlsx`: for each RNA construct, including:

*• `ratio\_mu`: The fraction of pre-mRNA in the intron signal, measured by qPCR.*

*• `decay\_rate`: Pre-mRNA decay rates obtained from decay experiments, used as fixed input parameters for the kinetic model.*

- `global\_folder.xlsx`: *Specifies the directory paths for raw data, processed outputs, and result storage.*

- `well\_info.xlsx`: *Stores well-level metadata, including well IDs, doxycycline (Dox) induction times, and construct identities.*

*• Column 1: indicates the position of the well on the 96-well plate (e.g., A1, A2, etc.)*

*• Column 2: Construct and induction information — specifies the RNA construct used in the well and the corresponding doxycycline (Dox) induction time*

*• Column 3: Probe target(1 or 2) — indicates whether the intronic probe targets intron 1 or intron 2*

**2. Run `pre\_cellpose\_ad.m` in MATLAB to process the raw `.nd2` files generated from the experiment.**

This script converts the images into `.tif` format compatible with Cellpose, and performs intensity normalization to make the images suitable for segmentation.

**3. Use Cellpose to run two pretrained models:**

- `20230618-Li20x-splicing`: Segments and identifies transfected cells.

- `20230707-decay-dapi1`: Segments all cells in the image. This is used in downstream analysis

to estimate background signal generated by the camera.

**4. Run `splicing\_main.m` in MATLAB to begin the main splicing analysis pipeline.**

- splicing\_main.m depends on

**Matlab scripts**：

LoadCellBoundaries : *extract single-cell information from cellpose-generated masks*

AutoNucleusOtsu : *Nuclear segmentation by Otsu’s methods*

EstimateBgFromAllCells : *Background estimated by non-cell regions*

SelectTransfected : *Transfected Cell Selection*

SubtractBackground : *Background Correction*

QualityCheck : *This script is used to visualize the cells that were classified as transfected cells. Specifically, it displays the original images with overlays or masks to highlight selected transfected cells, enabling manual inspection of selection accuracy.*

ProbesCalibration : *This script estimates the relative fluorescence efficiency between intronic and exonic probes by comparing their signal intensities in a calibration dataset. The goal is to determine the correction factor needed to normalize their signals for downstream kinetic modeling.In the imaging setup used, probe concentrations and imaging parameters were pre-adjusted so that the efficiency ratio between intronic and exonic probes is approximately 1. Therefore, the fitted calibration result is expected to yield a ratio close to 1.*

SummaryAllwells\_splicing : *summarizes the splicing data across all wells by extracting the relative abundance of pre-mRNA ([P]) and spliced RNA ([S]) at each time point.*

SummaryAllcons\_splicing : *performs kinetic fitting of splicing data for each individual construct.*

**Matlab functions:**

autocontrast.m : *performs automatic contrast adjustment on images.*

Splicing\_model\_fixeddecay.m : *implements a splicing kinetics model in which the pre-mRNA splicing rate is fixed based on values obtained from previous experimental measurements.*

**Example workflow for splicing analysis pipeline:**

1) Initialize environment variables:

$ source ~/.bashrc

2) Navigate to the analysis code directory:

$ cd Desktop/Li\_96well/splicing-codes

3) Preprocess raw .nd2 images and generate Cellpose-compatible input:

$ matlab -batch pre\_cellpose\_ad

4) Return to the base directory:

$ cd ..

5) Activate the Cellpose conda environment:

$ conda activate cellpose

6) Run Cellpose segmentation to detect transfected cells:

$ cellpose --dir 20250130-splicing-esel28/ad-images \

--pretrained\_model model/20230618-Li20x-splicing \

--chan 2 --chan2 3 --save\_png --no\_npy --verbose --diameter 41.536

7) Create a parallel folder `ad-images-dapi` if needed, to store

additional segmentation masks (e.g., used for background estimation).

8) Run model that segments all cells in the image for background estimation:

$ cellpose --dir 20250130-splicing-esel28/ad-images-dapi \

--pretrained\_model model/20230707-decay-dapi1 \

--chan 3 --chan2 0 --save\_png --no\_npy --verbose --diameter 36.355

9) Deactivate the conda environment:

$ conda deactivate

10) Navigate back to the MATLAB code directory:

$ cd splicing-codes

11) Run the main splicing analysis pipeline:

$ matlab -batch splicing\_main