**General Descriptions:**

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

- `current\_constructs.xlsx`: ` *Lists the construct names selected for current analysis.*

*Names must* ***match exactly*** *with those in ` summary\_cellpose\_all.xlsx `.*

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

- `summary\_cellpose\_all.xlsx`: *contains experiment-level metadata and defines all relevant parameters for image analysis.*

**Description of columns in summary\_cellpose\_all.xlsx:**

**all\_consname**:  
A unique identifier for each experimental condition or construct.

**all\_image\_type**:  
Specifies the mode of image acquisition. Can be either 'manual' or 'auto'.

**all\_folder**:  
Primary folder where image files are stored.

**all\_subfolder**:  
Subdirectory within the primary folder where images are located.

**all\_image\_name**:  
The filename of the .nd2 image to be analyzed.

**all\_R** *(1, 2, 3, or 4)*:  
Channel assigned for RNA FISH signal.

**all\_G** *(1, 2, 3, or 4)*:  
Channel assigned for the second marker (e.g., protein marker).

**all\_B** *(1, 2, 3, or 4)*:  
Channel assigned for the nuclear speckle marker (e.g., SRRM2 or SON).

**all\_DAPI** *(1, 2, 3, or 4)*:  
Channel assigned for DAPI nuclear staining.

**all\_boundary\_removal\_cyto** *(0 or 1)*:  
If set to 1, any cell whose cytoplasm touches the image boundary will be excluded.

**all\_boundary\_removal\_nu** *(0 or 1)*:  
If set to 1, any cell whose nucleus touches the image boundary will be excluded.

**all\_nucleus\_minV**:  
Minimum pixel area threshold for a nucleus to be considered valid.

**all\_nucleolus\_minV**:  
Minimum pixel area threshold for a detected nucleolus to be retained.

**all\_nucleolar\_removal** *(0 or 1)*:  
If set to 1, detected nucleoli will be excluded from downstream analysis.

**all\_sat\_num\_threshold\_R:**Maximum allowed number of saturated pixels (i.e., max-intensity pixels) in a cell for the RNA FISH channel.  
Cells exceeding this threshold will be excluded to avoid bias from overly bright signals.

**all\_sat\_num\_threshold\_G**:  
Same as above, but for the protein marker channel.

**all\_trans\_threshold**:  
Intensity threshold used to define transfected cells. Typically applied to cytoplasmic fluorescence in the RNA FISH channel.

**all\_num\_of\_std\_B**:  
Thresholding parameter for nuclear speckle detection. The threshold is computed as:  
mean intensity (after DoG filter) + [this value] × standard deviation.  
Higher values make the speckle detection more stringent.

**all\_sigma1\_B**:  
First sigma (standard deviation) used in the double Gaussian filter for speckle detection.

**all\_sigma2\_B**:  
Second sigma used in the double Gaussian filter.

**all\_minvol\_B**:  
Minimum area (in pixels) for nuclear speckles. Speckles smaller than this will be excluded.

**all\_minnum\_B**:  
Minimum number of speckles required within a cell. Cells with fewer speckles will be excluded from analysis.

**all\_remove\_bg\_R**, **all\_remove\_bg\_G**, **all\_remove\_bg\_B** *(value = 0, 1, or 2)*:  
Background subtraction setting for each channel:

* 0: No background removal.
* 1: Subtract background estimated from untransfected cells.
* 2: Subtract background estimated from regions with no cells (background-only image areas).

**2. Run `Pre\_cellpose.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 pretrained model.**

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

- **Post\_cellpose.m** depends on

**Matlab scripts**：

LoadCellBoundaries: *Extract single-cell information from cellpose-generated masks.*

Auto\_nucleus\_Otsu: *Nuclear segmentation by Otsu’s algorithm.*

SortTransfectedCells: *Transfected Cell Selection.*

SegSpec\_untrans: *Nuclear speckle segmentation on untranfected cells.*

SegSpec\_trans: *Nuclear speckle segmentation on tranfected cells.*

Spec\_P: *Performs intensity-based quantification for downstream analysis, including the calculation of partition coefficients between nuclear speckles and the surrounding nucleoplasm.*

**Matlab functions:**

speckle\_thre.m: *Main function for nuclear speckle segmentation.*

DoG.m: *Function that applies a Difference of Gaussians (DoG) filter.*

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