SUPPLEMENTAL METHODS:

SEGMENTATION:

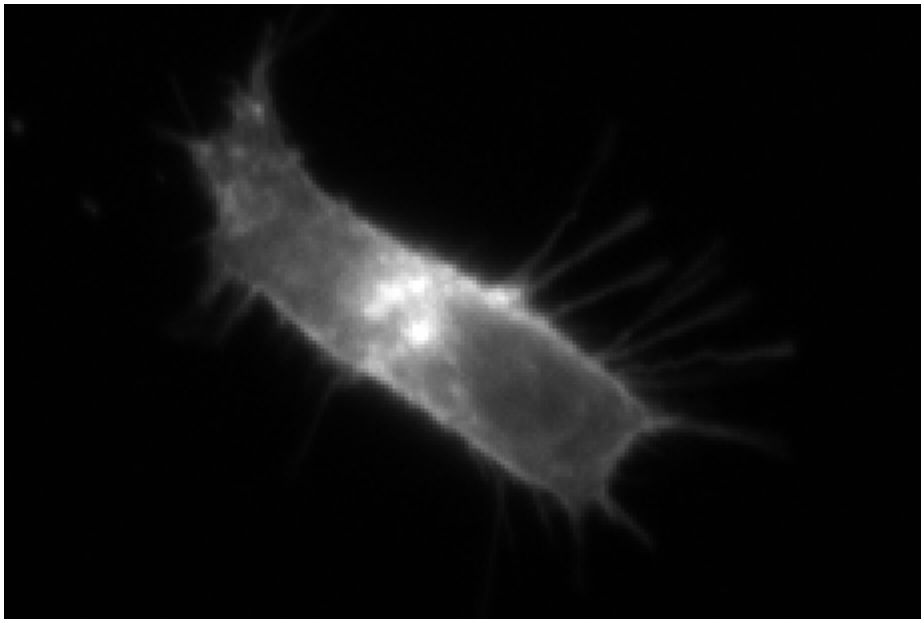
Receptor:
- Granular segmentation
  - Kernel = 3
  - Sensitivity = 50

Arrestin:
- Granular segmentation
  - Kernel = 3
  - Sensitivity = 50

Nucleus segmentation algorithm:
- Nuclear segmentation algorithm
  - 100% sensitivity

Colocalized Receptor and Arrestin:
- Step 1, Preprocessing:
  - Run Macro (Combine Min)
    - Input channels 6 & 7
    - Output channel 8
- Step 2, Intensity-based Segmentation
  - Minimum threshold = 1
  - Maximum threshold = 4095
  - This assured that only pixels that represented both receptor and arrestin were colocalized with one another.

ComboCellBody segmentation algorithm:
Input: YFP-Receptor, channel 1

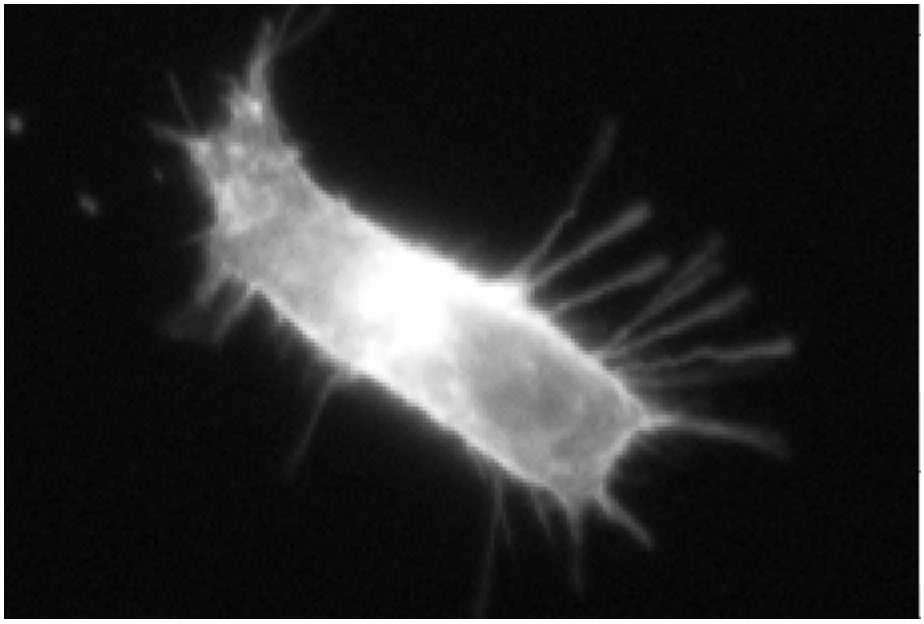


Step 1: Preprocess Macro

Information Equalization

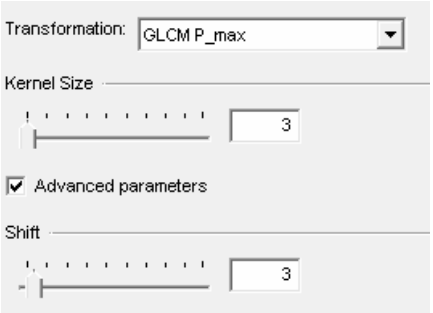
Output to Ch 9 for temporary storage

(to ensure normalized intensity values from one transfection to another)



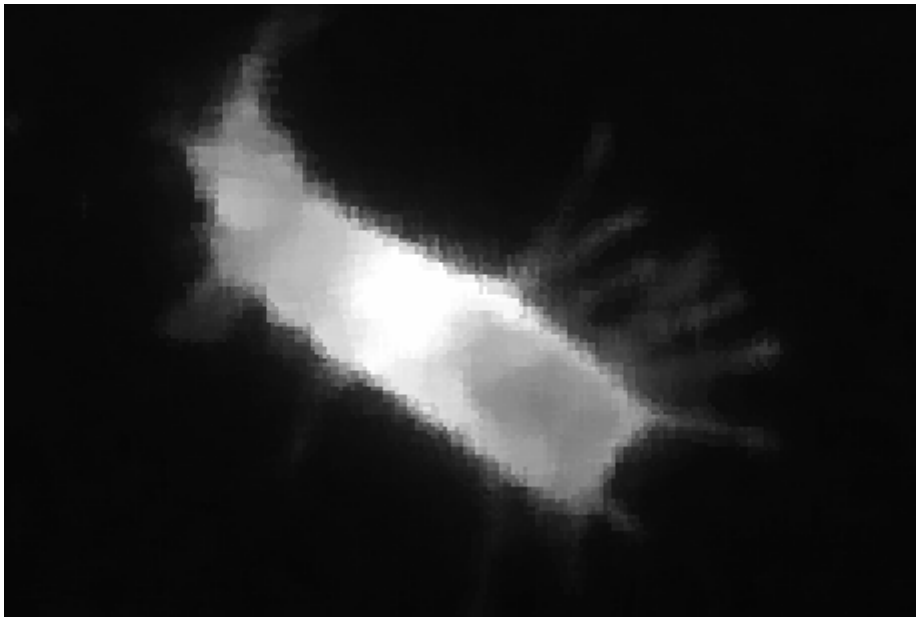
Step 2:

Texture Transformation



This was done to achieve homogeneity of cytoplasmic areas that was significantly disrupted upon granularization of the YFP-tagged receptor as it internalized over time. Gray level co-occurrence matrices (GLCM) are a calculation of occurrences of a particular gray value within a designated space. That is, by calculating the number of pixels with similar gray levels within a certain radius (kernel), we are able to generate another image (x by y matrix) of the gray level occurrences for every individual pixel. This results in an image with a smoothened intracellular texture, easing contiguous cytoplasmic segmentation.

These pixels that represent the cytoplasmic intensity can be emphasized by the fact they are more abundant than the bright puncta that form upon receptor internalization, and the bright puncta are de-emphasized via a relatively smaller abundance within the 3 pixel window used in this step.



Step 3:

Segmentation

Intensity

Minimum threshold at 40 gray level (determined empirically, based on output of GLCM P_max smoothing)

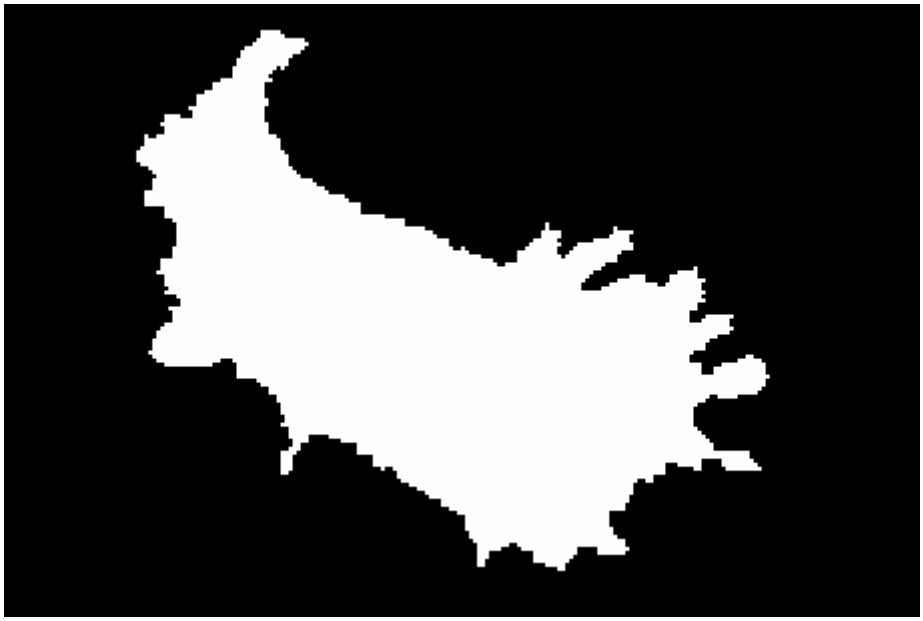
Maximum threshold at 4095

Filled

Use acceptance criteria

[Area]>100 μm^2

Update Output Image (channel 5)



Step 4:

Postprocessing

Sieve (Binary)

Keep >= 100 μm^2

Border Object Removal

All borders

Sieve (Binary)

Keep > 100 μm^2

Clump Breaking

Second segmentation: nucleus

Don't include secondary targets

Perinuclear segmentation:

Identical settings as the nuclear area.

(this was programmed separately to allow for modification of the perinuclear mask [e.g. dilation to increase the size of the perinuclear mask] without affecting nuclear area, a useful measure of apoptosis).

TARGET LINKAGES: Targets were linked to each other in order that receptor and arrestin puncta could be associated to individual cells and to subcellular regions (perinuclear). This was achieved via multiple target linkages, each ultimately assigning every individual target of interest to the primary target set of ComboCellBody, which allows for every measure to be analyzed on a cell by cell basis.

Primary Total cell Linkages:

Primary	Secondary	Title
ComboCellBody	Receptors	Receptors in Cells
	Arrestin	Arrestin In Cells
	Colocalizations	Coloc In Cells

Primary Subcellular Linkages:

Primary	Secondary	Title
Perinuclear	Receptors	Receptors in Peri
	Arrestin	Arrestin in Peri
	Colocalizations	Coloc in Peri

Primary Subcellular Region Linkages:

Primary	Secondary	Title
ComboCellBody	Perinuclear	CellBody-Peri

(created in the order that objects were assigned to a subcellular region and were assigned to the correct cell)

Second-Level Linkages (Composed One to One):

Title	Primary	Secondary	Matching Path
Linked Receptors in Peri	CellBody-Peri	Receptors in Peri	(ComboCellBody\Perinuclear)
Linked Arrestin in Peri	CellBody-Peri	Arrestin in Peri	(ComboCellBody\Perinuclear)
Linked Coloc in Peri	CellBody-Peri	Coloc in Peri	(ComboCellBody\Perinuclear)

This linkage results in the effective organization of targets in the hierarchy outlined in Table S1, with every measure referred up the hierarchy to report within ComboCellBody.

MEASURES: For each linkage corresponding to a particular object (in this case representing an individual cell), two measures were generated, Area and Count (totals per cell or subcellular area). Other useful measures were calculated on every cell, including:

Position
- X location
- Y location
- Distance moved from prior location
- Polar angle of movement

Morphometric:
- Form Factor
- Area
- Length
- Major Axis length
- Minor Axis Length

Intensity:
- YFP intensity (raw)
- YFP intensity (post-norrmalization/information equalization)
- dsRed intensity

Identification:
- Label
- Linked Track ID
- Tracking Events

CELL TRACKING: ComboCellBody objects were tracked using Developer's built-in Cell Tracking proximity algorithm with the following parameters:

Relative threshold	1
Outlier detection	2

Only Pos X and Pos Y were used, each with an equal weight of 1.0.

EXCEL TEXT-BASED EXTRACTION: Well, field, and time information were encoded in a singular text string “Section” located in the first column “A” and were extracted using the LEFT(), VALUE(), SUBSTITUTE(), and MID() formulas below:

Example [Section]: B - 1 (fld 1 - 510000)

1. [Well] = LEFT(A2,5)
2. [Field] = VALUE(MID(A2,12,1))
3. [Time (min)] = VALUE(SUBSTITUTE((MID(A2,16,10)),")",""))/60000
    a. Time is reported in Section as milliseconds
    b. SUBSTITUTE is used to replace the end parenthesis “)” returned in MID formula applied to shorter timepoints with a blank space to avoid errors in the VALUE function, which converts text to numerical format. E.g. “0)”→”0”