

E-cadherin tension sensor FRET image acquisition and analysis in *Drosophila* border cells

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Materials

Reagents

- Schneider's *Drosophila* medium (Life technologies cat. no. 21720-001)
- Insulin, 10mg/ml (Sigma cat. no. I1882-100MG)
- Fetal Bovine Serum (Life technologies cat. no. 10437-028)
- Halocarbon oil 27 (Sigma cat. no. H8773)
- Immersion medium Immersol W 2010 (Carl Zeiss cat. no. 444969-0000-000)

Equipment

- Dissecting microscope
- 2-well concavity slide (VWR cat. no. 100491-032)
- Lumox dish 50mm (Sarstedt cat. no. 94.6077.410)
- Zeiss LSM 710 microscope
- Fine Tip Transfer Pipets (Thermo Scientific cat. no. 1371125)
- Dumont #5 forceps (Fine Science Tools cat. no. 11251-30)
- Fisher brand no. 1 coverslips, size: 22x22mm (Fisher, cat. no. 12-542-B)
- Fisher brand no. 1 coverslips, size: 40x22mm (Fisher, cat. no. 12-545C)

Procedure

Preparations before live imaging

1) Select young female *Ecad*^{TS} and *Ecad*^{TS-C} flies that are 3-4 days old. Keep them on plain fly food before fattening.

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2) Right before imaging, fatten flies with fresh yeast for 16-18 hours at 25 °C. Yeast granules after absorbing moisture cover about 1/3 of the surface of fly food.

3) On the day of imaging, prepare a new or unscratched Lumox dish.

4) Take out an aliquot of insulin solution (10µl for 500µl imaging medium) from -80°C fridge to let it thaw at room temperature.

5) Mix thawed insulin with Schneider's Drosophila medium supplemented with 20% Fetal Bovine Serum to make complete live imaging medium.

CRITICAL STEP: For dissection and FRET imaging, always use fresh live imaging medium (less than 20 days old).

CRITICAL STEP: We found that the correct range of pH (6.85-6.95) is important for border cell to be healthy and migrate well (average speed is about or more than 0.6 μ m/min).

3. Dissect fly ovary and isolate stage 9 egg chambers

6) Immediately before imaging, dissect around five fattened female flies to isolate fly ovaries and subsequently stage 9 egg chambers according to protocols established before (1, 2).

CRITICAL STEP: Well-fattened ovaries should show a nice distribution of early and late stage egg chambers in the ovariole. Don't use your flies for imaging if the ovaries look sick or under-fattened (Figure 1).

? [Troubleshooting](#)

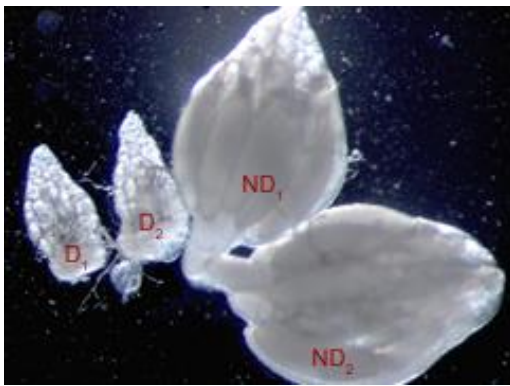


Figure 1. Images of under-fattened (left) and well-fattened (right) ovaries (From Eanes Lab).

7) After dissection, remove as many mature egg chambers as possible since they easily deplete nutrients in the medium during imaging.

8) Use a fine tip transfer pipette to transfer egg chambers for imaging to a small Eppendorf tube.

9) Wash once with 300 μ l complete live imaging medium.

Prepare imaging cassette for confocal imaging:

10) Carefully break one 22x22mm coverslip in the middle to two equal parts (11x22mm) using the shaft region of forceps. Wipe off fragments on the coverslip using Kim wipe.

11) Apply 5 μ l live imaging medium onto two separate spots on the dish (5 μ l x 2)

12) Use forceps to carefully transfer two 11x22mm coverslips to the Lumox dish membrane, onto the pre-applied medium droplets, with the smooth side of the coverslips facing inward

13) Use a plastic pipette tip to carefully arrange the coverslips so that they are about 18mm apart, and parallel to each other

14) Transfer sample in 90µl live imaging medium to the center of two coverslips

15) Gently cover the sample using a 22x40mm coverslip. Bubbles should be avoided in medium.

CRITICAL STEP: when you slightly tilt the Lumox dish the egg chambers should be just barely able to move. If they move too quickly then there's too much medium: egg chambers would shift during imaging. If they are unable to move then there's insufficient medium: egg chambers may deform due to squeezing during imaging.

16) Apply small droplets of halocarbon oil 27 to the rims of coverslip and let them spread and cover all the sides to prevent evaporation. 15µl of halocarbon oil in total is sufficient.

Image acquisition

17) On a Zeiss LSM 710 upright microscope, mount the Lumox dish with sample onto the motorized stage.

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18) Apply a drop of water immersion medium to the coverslip, and lower the 40x/1.1W LD C-Apo water objective to touch the immersion medium.

? [Troubleshooting](#)

19) Under "Locate" tab in Zen software, turn the system online.

20) Switch to GFP filter. Use microscope eyepiece to adjust focal plane and locate the egg chambers for imaging.

CRITICAL STEP: In order to reduce photobleaching, do not expose samples in epifluorescence for too long. And do not let the objective touch the side of the Lumox dish. This will shift focus and possibly damage the sample.

21) Switch to "acquisition" tab in Zen software. Set up three imaging tracks. Track #1 uses a 458nm Argon laser for excitation, and acquires mTFP and FRET images simultaneously using channel 1 (463-501nm) and channel 2 (519-566nm). We use 100% laser power, and adjust pinhole size to 3 Airy Units. Use MBS 458/514 for main beam splitter. Track #2 utilizes a 514nm Argon laser for excitation, and acquires Venus image using channel 2 (519-566nm). Track #3 uses DPSS 561nm laser for excitation of Lifeact-mRFP channel, and uses channel 3 (567-700nm) to acquire image. Channel gains are no more than 800 to reduce background noise. Switch tracks every frame.

22) Adjust frame size to 512x512 pixels. Set averaging to 8 times for maximum signal to noise ratio. Use maximum speed.

23) Every time when setting changes (laser power change etc.), calibrate the signal crosstalk and bleed-through using Cad-mTFP and Cad-Venus only sample (steps 24-25).

24) Image Cad-mTFP sample using Track #1 to calculate mTFP to Venus bleed-through parameter. Then make a selection box on the border cells to measure the intensities of the selections in channel 1 and channel 2. Calculate bleed-through parameter by dividing intensity in channel 2 by that in channel 1 (usually around 25%-30%).

25) Image Cad-Venus sample using Track #1 and Track #2 to calculate the excitation of Venus by mTFP laser. Then calculate ratio of channel 2 signals in #2 to #1. This usually is 5% in our case and is ignored.

Notes: Because mTFP cannot be excited at Venus excitation wavelength and the Venus bleed-through into mTFP channel is weak, these two calibrations are not carried out. All the acquisition parameters should be the same with Cad-mTFP and Cad-Venus only controls.

26) Select all stage 9 border cells as early as those just detached from the anterior end of the egg chamber, and as late as those that have reached midpoint of migration path.

CRITICAL STEP: Only select fully detached border cell cluster for image acquisition.

We consider cluster as detached if it only has a thin strip connecting with anterior end as shown in the RFP channel (Fig. 2C) or totally unconnected (not shown).

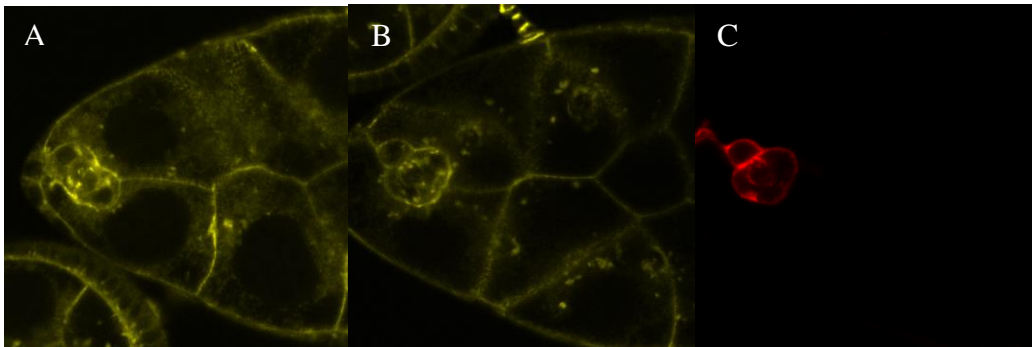


Figure 2. Examples of undetached (A) and detached (B-C) border cell clusters. (A-B) Venus channel in FRET acquisition. (C) Lifeact-mRFP channel in same cluster as (B).

27) E-cadherin tension sensor should express well and localize nicely to cell boundaries (Figure 3A). Do not use your sample for imaging if membrane localization is not clear (Figure 3B). Lifeact-mRFP should also express well in every border cell. Avoid egg chambers that look damaged with broken follicle cells.

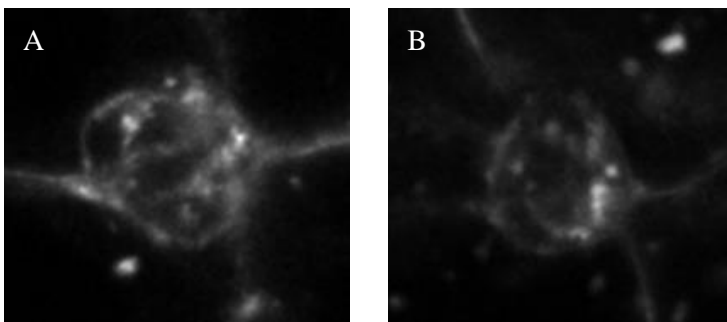


Figure 3. Examples of clear (A) and unclear (B) membrane localization
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28) Under “Live”, adjust focus so that border cell mid-plane is in focus.

29) Under 2x magnification, rotate crop box so that it is parallel to the migrating border cell cluster, with the front protrusion facing right side of the box.

30) Acquire the image using Tracks #1, 2, & 3.

Image analysis

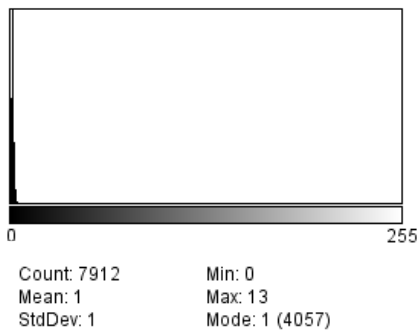
Remove image background

31) Open the mTFP, Venus, and FRET images in ImageJ software.

32) Measure background using a rectangular selection box around 20 μ m in height and width. Avoid selecting sample region.

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33) Use “Analyze->Histogram” to get the background mean and standard deviation.



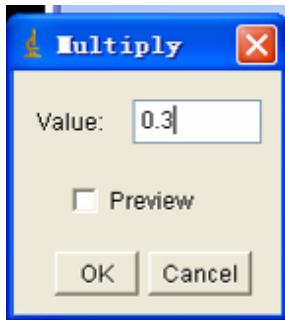
34) Calculate background value as “mean+3x StdDev”.

Select the entire image. Subtract background value using “Process->Math->Subtract” function.

(CRITICAL STEP: do not use the “Process->Subtract Background” function as it uses a rolling ball algorithm. This function subtracts a highly non-uniform background and can cause potential problems.)

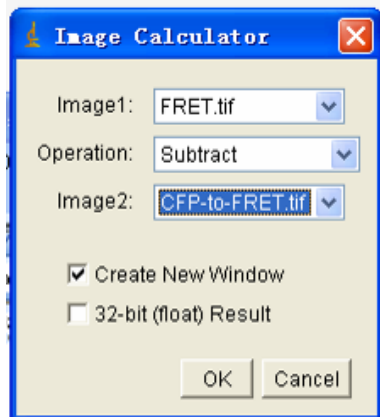
Remove channel crosstalk and bleed through

Select the mTFP image, multiply it by the previously calculated mTFP to Venus channel bleed-through factor (~25%-30%) using “Process->Math->Multiply” function to get mTFP-to-FRET image.



Use the Venus image, multiply it with the factor of Venus excited by mTFP excitation light using the same function to get Venus-to-FRET image. This is negligible so we don't include it in our calculations

Subtract the mTFP-to-FRET image from the FRET image using "Process->Image Calculator" function and generate the pure FRET image FRET-pure. Select the "Create new" window option otherwise the original image will be lost.



Apply "Process->Smooth" on mTFP and FRET-pure images.

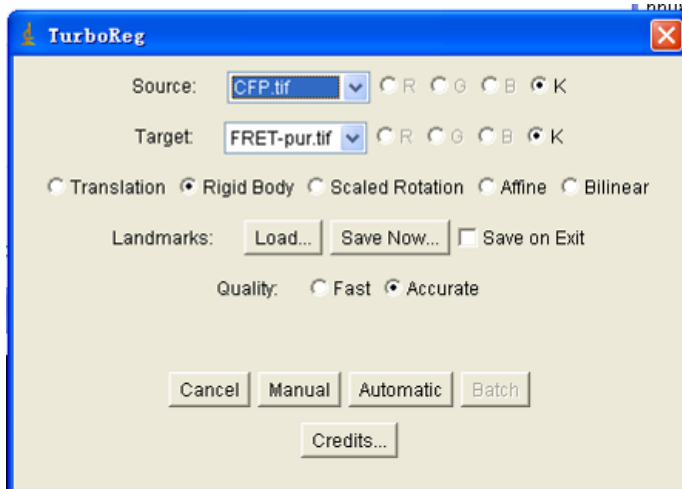
Register FRET and mTFP images (optional)

Shifts between different channels can be significant in epi-fluorescence microscope because different filter may not align perfectly in the light path. For confocal imaging, this problem is usually less severe. However, using image registration can still reduce artifacts, especially near the edge of the structures.

Here one can use a third party plug-in called TurboReg.

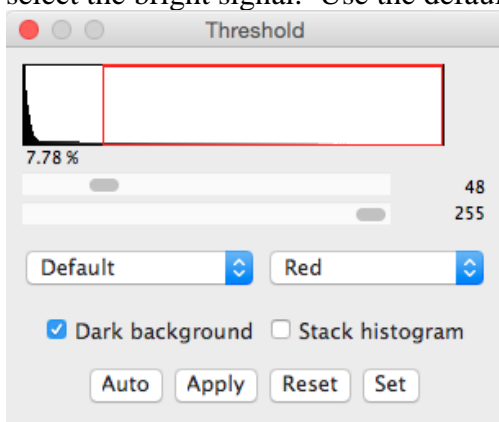
Usually, because the channel shifts will not deform the image, we choose "rigid body".

Usually we register the mTFP channel to the FRET-pure channel to generate a registered mTFP, mTFP-reg.



Generation of Mask

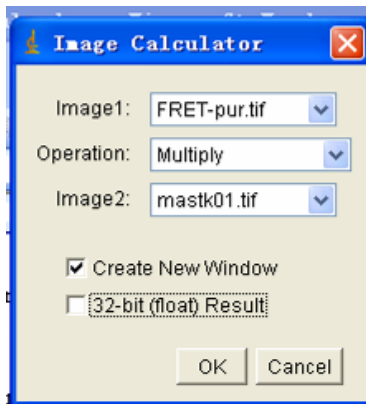
Duplicate the FRET-pure channel using Image->Duplicate function to create FRET-pur2. Use Image->adjust->threshold to create binary mask. Image. Choose Dark background to select the bright signal. Use the default algorithm.



For convenience, the generated mask should be divided by 255 to create a 0/1 binary image, mask01. (Use “Process->Math->Divide” function)

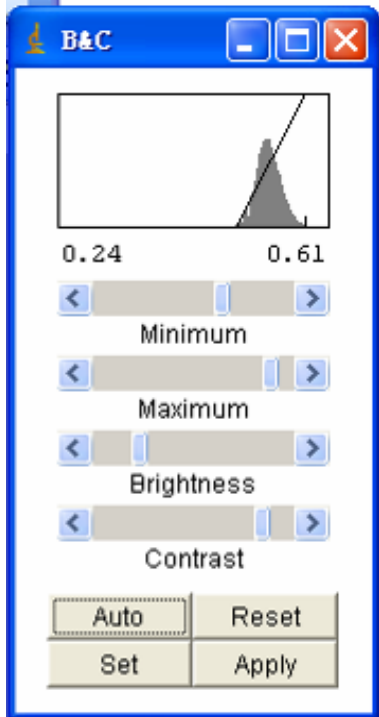
Generate FRET image and display

First, multiply the mask01 with FRET-pur channel using “Process->Image Calculator” to get a new image “FRET-masked”.



Then divide the “FRET-masked” with “mTFP-reg” to get the final FRET image using same Image Calculator. Choose 32-bit float option this time.

FRET index distribution can be seen from Image->Adjust->Brightness/Contrast or from Analysis->Histogram.



By changing the lookup-table using Image->Lookup Tables (or Plugins->LUT), one can visualize the dynamic range using different color coding, like rainbow RGB, Red-hot etc. Using the same Image->Adjust->Brightness/Contrast tool, one can choose the optimal range of display to best capture the dynamic range of the probe (this will not change the actual value of each pixel).

Color ramp can be added by Plugins->LUT->Add Ramp function.

Save the 32-bit image in tiff format.

Convert FRET image to RGB image for display purposes.

Selection of front and back of the cluster for FRET comparison

Make a 7 μ m x 7 μ m ROI selection box (can be saved at Analyze -> tools -> ROI manager). If there is a front protrusion, make a selection so that the tip of the protrusion just touches the right boundary of the box; if there is a back protrusion, tip of the protrusion should touch the left boundary of the box

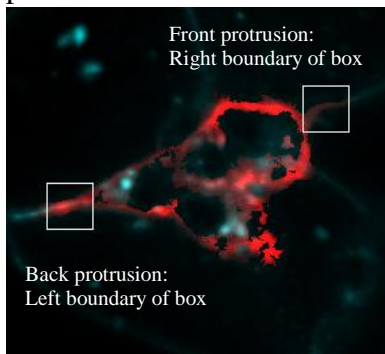


Figure 4. Selection of front and back protrusions

If the protrusion doesn't have clear RFP signal, could only be visualized after manually increase intensity, and is discontinuous with the main cluster, the protrusion is probably not in focus when you acquire the image. FRET index will only indicate nurse cell-nurse cell boundary tension. Discard such images or focus better when acquiring image.

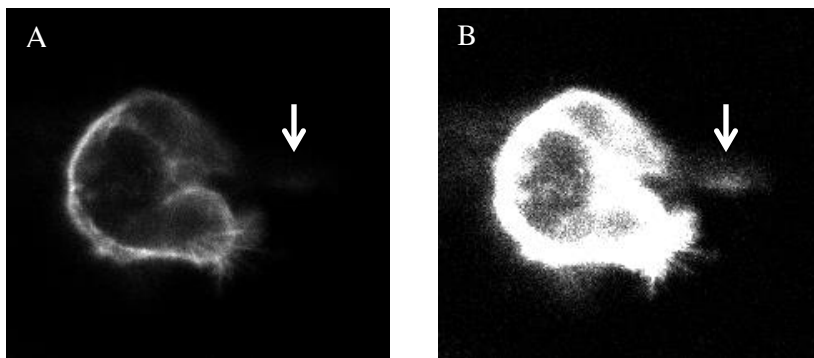


Figure 5. RFP channel showing image before (A) and after (B) manual increase of intensity. Arrow: position of front protrusion.

In some cases, the front protrusion has strong Lifeact-mRFP signal, but weak to non-existent tension sensor or control signal (Figure 6). This is probably due to the fast formation of front protrusions. We exclude such samples.

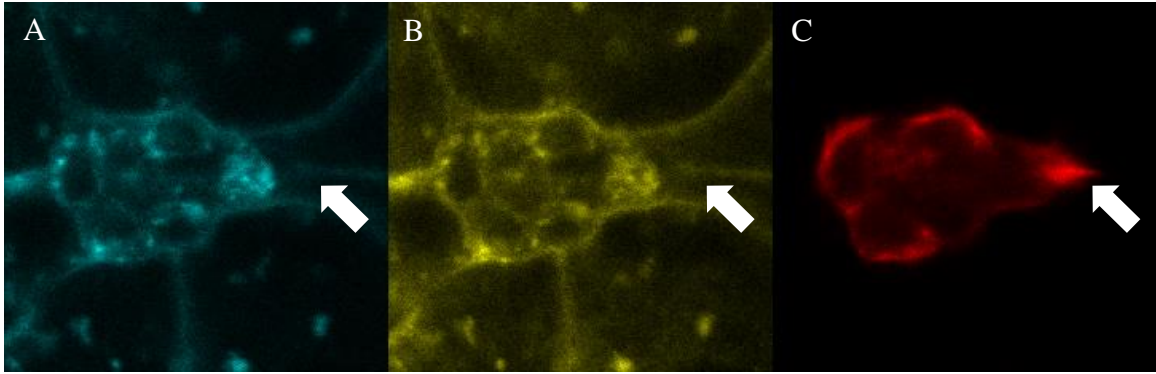


Figure 6. Absence of tension sensor signal in the front protrusion in mTFP (A) and Venus (B) channels while Lifeact-RFP signal (C) is present. Arrows: tip of front protrusion.

If the front protrusion in RFP channel doesn't co-localize with any tension sensor signal (Fig. 7C small arrow), select the region on border cell cluster most adjacent to the protrusion (Fig. 7A. big arrow).

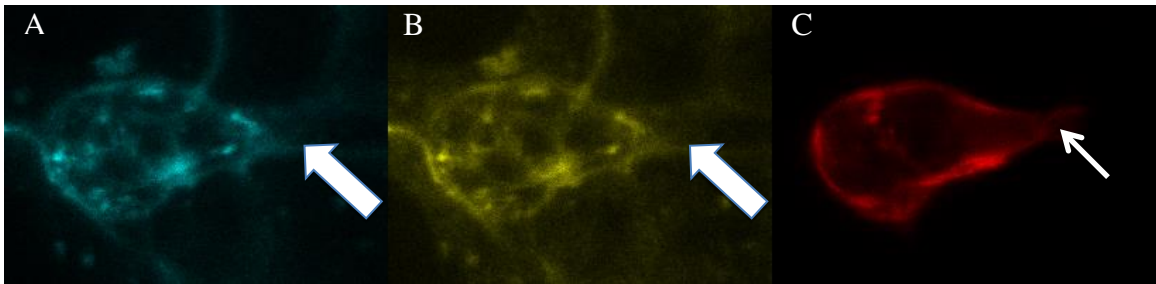


Figure 7. Selection of region closest to unclear protrusion.

If there are multiple front protrusions, choose one whose tip extends most posteriorly. Similarly if there are multiple back protrusions, choose one whose tip extends most anteriorly. The protrusion chosen should be in focus.

If there is no protrusion (Figure 6A, box 1), or protrusion is short (Figure 6A, box 2), adjust the *width* of the box so that you only select the border cell-nurse cell boundary membrane, the outer-most point of the membrane touches boundary of the box (Figure 6A, box 1) and avoid aggregates (Figure 6A, box 2).

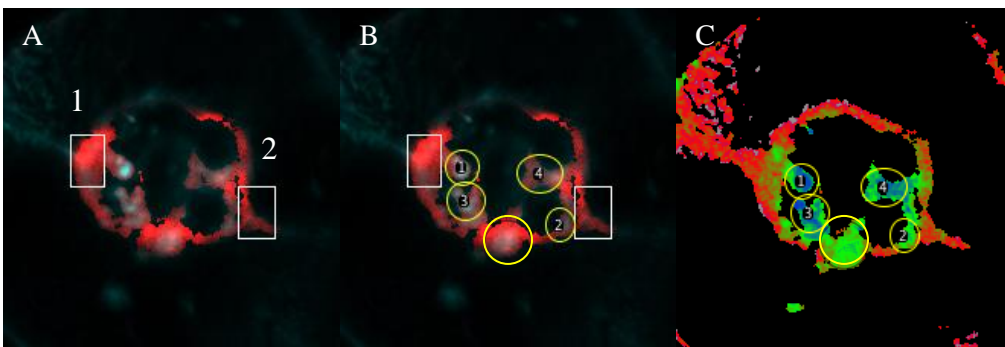
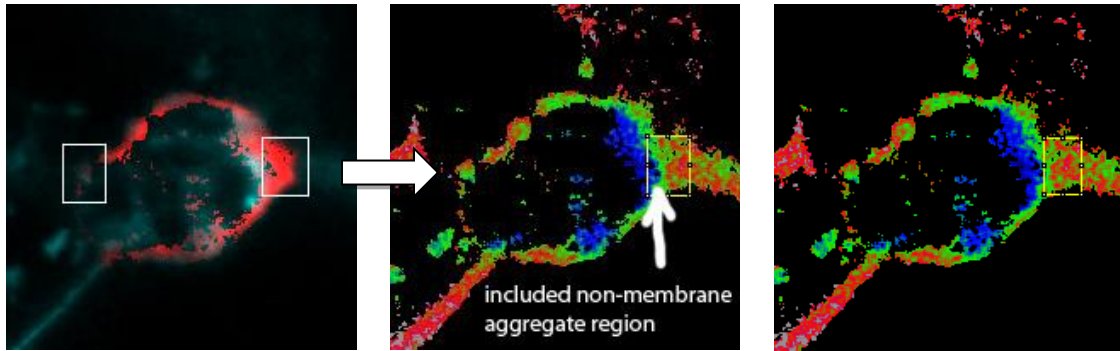


Figure 6. Narrower selection boxes that restrict selection to border cell membrane and avoid internal aggregates (circled regions).

Due to the properties of mTFP, it tends to form aggregates inside the cluster. Aggregates are usually inside the border cell, below the membrane. They should be round and bright in mTFP channel (circled in Figure 6B). We will avoid those aggregates (i.e. Figure 6B oval selection 2) when measuring membrane FRET. Sometimes it is uncertain what are aggregates and what are real membrane signal. Go to the final FRET image (Figure 6C). The circled aggregate regions show clear and abrupt drop in FRET index compared to adjacent membrane and should be avoided.

Only select regions that are located on the membrane

Solution: do selection on the final FRET image and use RFP image as reference.



*(YA's selection on overlay of mTFP and RFP image:
hard to avoid internal "aggregate" region)*

*(DC's selection on FRET
image: membrane-localized)*

Figure 7. Selection is better done on the final FRET image.

References:

1. Prasad M, Jang AC, Starz-Gaiano M, Melani M, & Montell DJ (2007) A protocol for culturing *Drosophila melanogaster* stage 9 egg chambers for live imaging. *Nature protocols* 2(10):2467-2473.
2. Prasad M, Wang X, He L, & Montell DJ (2011) Border cell migration: a model system for live imaging and genetic analysis of collective cell movement. *Methods in molecular biology* 769:277-286.