Biosensor Image Processing

Ratiometric Biosensors

LFD Workshop Oct 2009
Control of cell behavior through localized activation of key signaling proteins

Visualizing protein activation rather than localization

Rac localization

Rac activation

Kraynov et al. 2000 Science
GTPase Activation Visualized by Biosensors in Live Cells
Sensitized-emission

Quantitation in living cells: Ratio imaging

\[ \text{FRET Em} / \text{Donor Em} = \text{Activity}^* \]

Emission Ratio is unaffected by uneven illumination, cell thickness and biosensor distribution, etc.
Design

All custom mirrors
Tight bandpass designs
Main turret dichroic eliminated
Optimized for live-cell imaging
Biosensor types

**Single-chain biosensor:**
- Raw data stacks (FRET and CFP or EGFP and mero87)
- Matching shade images; camera noise control

**Bimolecular biosensor:**
- Raw data stacks (FRET, CFP and YFP)
- Matching shade images
- Calibration image of FRET and CFP from CFP - cell
- Calibration image of FRET and YFP from YFP - cell
Biosensor Image Processing (single-chain)

- Shade correction
- Background subtraction
- Morphing*
- Masking
- X-Y registration
- Ratio
- Photobleach correction
Biosensor Image Processing (single-chain)

Shade correction
Background subtraction
Morphing*
Masking
X-Y registration
Ratio
Photobleach correction
Shading correction

Wide-field epi: uneven illumination, out of focus light
Shading correction

Wide-field epi: uneven illumination, out of focus light

1) Take cell-free FOV at same exposure condition
   1: focus should be at the right plane
   2: immersion oil should be centered
   3: same media/buffer conditions (volume also)

2) Take camera noise correction images
   1: shutter closed, exposure times should be the same as cell images
   2: Scaling factor for division *
Shading correction

Wide-field epi: uneven illumination, out of focus light

\[ I_{sc} = \frac{(I_{raw} - noise) \times (S.F.)}{(shade - noise)} \]

\[ \text{ave}(shade - noise) \quad \text{lim} \ shade \to noise? \]

\[ S.F. = \frac{\text{ave}(I_{raw} - noise)}{\text{constant} \ (e.g., 1000)} \]

Scaling factor: needs to keep Isc in 16-bit depth (65535)

Median filter to smooth out noise
Shading correction
Biosensor Image Processing (single-chain)

- Badpixel correction
- Shade correction
- **Background subtraction**
- Morphing*
- Masking
- X-Y registration
- Ratio
- Photobleach correction
Background subtraction

Subtract from the entire FOV an average value from a small ROI drawn away from the cell

1: Nothing protrudes into ROI
2: measure ROI at corresponding planes

Demo...
Background subtraction
Biosensor Image Processing (single-chain)

- Badpixel correction
- Shade correction
- Background subtraction
- Morphing*
- Masking
- X-Y registration
- Ratio
- Photobleach correction
Coordinate transformation: morphing

Critical for multi-camera applications

Pixel-pixel match for ratio is critical

For single camera, chromatic effects can be addressed

A pre/post acquisition calibration needed

multi-speck beads

Consistent use of the reference channel (CFP)
Coordinate transformation: morphing

Critical for multi-camera applications

Pixel-pixel match for ratio is critical

Using multispectral beads on FRET / CFP channels (two cameras)
Track each point source light, produce morphing parameters for polynomial-fit based transformation

This should correct for: lateral chromatic effects, camera mounting, shear, magnification, x-y translation etc.
Coordinate transformation: morphing

- Image rotation
- Image shear/curvetures
- Different magnification at different ports

- Fit using 3\textsuperscript{rd}-order polynomial for coordinate transformation
Coordinate transformation: morphing

- Image rotation
- Image shear/curvetures
- Different magnification at different ports

Original

Aligned
Coordinate transformation: morphing
Biosensor Image Processing (single-chain)

- Badpixel correction
- Shade correction
- Background subtraction
- Morphing*
- **Masking**
- X-Y registration
- Ratio
- Photobleach correction
Image masking

Noise in BKGD makes ratio noisy

Produce binary mask to include cell area only
Ratio: not masked, not morphed, not registered
Image masking

Noise in BKGD makes ratio noisy
Produce binary mask to include cell area only
Equalize histogram to make thresholding simpler
Image masking

Noise in BKGD makes ratio noisy
Produce binary mask to include cell area only
Equalize histogram to make thresholding simpler

Cell features and multiple masks:
filopods versus lamellipodia

Multiply binary masks into cell images
Image masking

- Noise in BKGD makes ratio noisy
- Produce binary mask to include cell area only
- Equalize histogram to make thresholding simpler

Cell features and multiple masks:
- filopods versus lamellipodia

Automated, unsupervised approaches are available:
- Cytometry 2006: k-means clusters based thresholding

Multiply binary masks into cell images
Biosensor Image Processing (single-chain)

- Badpixel correction
- Shade correction
- Background subtraction
- Morphing
- Masking
- \textbf{X-Y registration}
- Ratio
- Photobleach correction
X-Y subpixel registration

Ratio: pixel-pixel match is critical

Using masked images, normalized cross-correlation is used to determine best match

Use of masked images to improve accuracy

For images taken with single camera, X-Y registration may be sufficient. Check also using morph to determine how much shifts are in your FOV
Discrete-normalized cross correlation for X-Y registration

\[ c(x, y) = \frac{\sum_{i}^{M} \sum_{j}^{N} g_{x+i, y+j} \cdot h_{i,j}}{\sqrt{\sum_{i}^{M} \sum_{j}^{N} g_{x+i, y+j}^2 \cdot \sum_{i}^{M} \sum_{j}^{N} h_{i,j}^2}} \]

Original grayscale

Masked binary


X-Y translational registration

Correct | Incorrect
--- | ---
A

Correct | Incorrect
--- | ---
B

Cytometry 2006
X-Y subpixel registration

Ratio: pixel-pixel match is critical

Using masked images, normalized cross-correlation is used to determine best match

Use of masked images to improve accuracy

Apply one X-Y value to the whole stack

Consistent use of the reference channel
Biosensor Image Processing (single-chain)

- Badpixel correction
- Shade correction
- Background subtraction
- Morphing*
- Masking
- X-Y registration
- Ratio
- Photobleach correction
Ratio

Divide masked, registered FRET by masked CFP

1: Floating point consideration

2: Effect of scaling factor (we use 1000)

note the total dynamic range of biosensor
Ratio
Scaling factor = 100
Scaling factor = 1000
**Ratio**

Divide masked, registered FRET by masked CFP

1: Floating point consideration

2: Effect of scaling factor

Not yet photobleach corrected...
Biosensor Image Processing (single-chain)

- Badpixel correction
- Shade correction
- Background subtraction
- Morphing
- Masking
- X-Y registration
- Ratio
- Photobleach correction
Photobleach correction

Exponential decay in fluorescence in timelapse

Minimizing photobleach (later)

Measure whole-cell average intensity per time

Produce function fit, then take the inverse

Assumptions:

#1: Majority of biosensor not responding

#2: 2nd order kinetics applies (CFP / YFP)

\[ y = a \cdot e^{-b} + c \cdot e^{-d} \]
Photobleach correction

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Command window
To get started, select HELP or Menu from the Help menu.

Figure 1
[Graph showing fitted curve]
Review

Cell-free FOV at the SAME condition for shading
BKGD measured at every time point/plane
Calibrate your FOV and morph your data
Mask your data for what you want to look at
X-Y registration, masked images make better match
Ratio and scaling factor
Photobleach correction, assumptions important
Minimizing photobleach and maximizing cell health

De-gas your media (37C water bath, 1~2hrs)

Argon gas to remove oxygen (30s~1min)

HEPES buffer (10~20mM) for open chamber situations

Oxyfluor (1:100) and DL-lactate 1mM, let it work at 37C for 30min~1hr

Exposure conditions:

Use ND filters, DIC optics, maximize light throughput, minimize light loss

Longer exposure at low intensity better than short exposure at high intensity

Don’t keep looking at the cell! Open shutter only as absolutely necessary
Sensitized emission: Bimolecular sensors

Donor and acceptor portions are NOT linked

CFP and YFP are NOT equimolar

Moderately challenging to engineer

Complicated quantitation

Bleedthrough removal required

Rac1 sensor: Dynamic range: ~400%
Bleedthrough: how to correct

1) Take CFP ex / CFP em of CFP-only cell
2) Take CFP ex / YFP em of CFP-only cell
3) Take CFP ex / YFP em of YFP-only cell
4) Take YFP ex / YFP em of YFP-only cell

Plot intensity of FRET (C-Y) against intensities of CFP (C-C) and YFP (Y-Y)

#1: Same exposure conditions as actual experiments
#2: shade correction, BKGD subtraction, masking
FRET_{B-CFP} = 0.3258 \times [CFP] \\
FRET_{B-YFP} = 0.2032 \times [YFP] \\
FRET_{Corrected} = FRET_{raw} - 0.3258 \times [CFP] - 0.2032 \times [YFP] \\
Ratio_{Corrected} = FRET_{Corrected} / CFP
When implementing in your own lab:

SimFCS Ratio module (next)

For full scale processing, start simple:
   First, do one time point only
   Think about how to extend to multi-planes

Any questions: lhodgson@aecom.yu.edu