Biosensor Image Processing

Ratiometric Biosensors

LFD Workshop Oct 2008
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

Rac1

Cdc42

RhoA

YFP

CFP

Cdc42

GTP

EGFP

YFP

CFP

RhoA

GDP
Why multi-channel epi?

Quantitation in living cells. Ratio imaging

Example: MeroCBD

EGFP → CBD → DYE

Ratio = 2 regardless of cell thickness
Biosensor Images

**Single-chain biosensor:**
- Raw data stacks (FRET and CFP or EGFP and I-SO)
- Matching shade images

**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)

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

CCD chip inherent defects - always present
Bad pixel correction

CCD chip inherent defects – always present
As CCD chip ages, more may show up!
Subtract BKGD value to produce hot pixel image
Multiply by large number to make it max out
If 2 cameras, just add them

Demo...
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
   Caveat #1: focus should be at the right plane
   Caveat #2: immersion oil should be centered

2) Divide Cell image by shading image for a flat-field
   Caveat #1: floating point problem
   Caveat #2: Scaling factor for division

Demo...
Background subtraction

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

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

Demo...
Coordinate transformation: morphing

Critical for multi-camera applications

Pixel-pixel match for ratio is critical
Coordinate transformation: morphing

- Image rotation

- Curvature

- Different magnification at different ports

- A priori calibration using stage micrometer

- Fit using 3rd-order polynomial for coordinate transformation
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

- Grid-type micrometer
- multi-speck beads

Consistent use of the reference channel (CFP)

Demo...
Image masking

Stochastic noise in BKGD areas makes ratio noisy

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

Stochastic noise in BKGD areas makes ratio noisy

Produce binary mask to include cell area only

- Cell features and multiple masks (intensity thresholding)
- Photobleaching and intensity matching
- Separate masks for CFP versus FRET
- Multiply binary masks into cell images

Demo...
X-Y subpixel registration

Ratio: pixel-pixel match is critical

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

Cannot use raw image, must be masked
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

Cross-correlation based X-Y registration
Good for single camera data sets...

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

Cannot use raw image, must be masked

Apply one X-Y value to the whole stack

Consistent use of the reference channel (CFP)

Demo...


Ratio

Divide masked, registered FRET by masked CFP

Caveat #1: Floating point consideration
Caveat #2: Effect of scaling factor
Scaling factor = 100
Scaling factor = 1000
Ratio

Divide masked, registered FRET by masked CFP

Caveat #1: Floating point consideration
Caveat #2: Effect of scaling factor

Not yet photobleach corrected...

Demo...
Photobleach correction

Exponential decay in fluorescence in timelapse

Minimizing photobleach (off topic)

Assumptions:

#1: Majority of biosensor not responding

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

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

Produce function fit, then take the inverse

Demo...
Review

Keep track of bad pixels

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 only

Ratio and scaling factor

Photobleach correction, assumptions important
Minimizing photobleach and maximizing cell health

- De-gas your media (37°C water bath, 1~2hrs)
- Argon gas to remove oxygen (30s~1min)
- HEPES buffer (10~20mM) for open chamber stuff
- Oxyfluor (1:100) and DL-lactate 1mM, let it work at 37°C 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 staring at the cell! Open shutter only as absolutely necessary
Sensitized emission: Bimolecular probes

Bleedthroughs:

1) CFP only cells: FRET channel and CFP channel
2) YFP only cells: FRET channel and YFP channel

Relate CFP (or YFP) intensity to FRET channel intensity

#1: Same exposure conditions as actual experiments
#2: Shade correction, BKGD subtraction, masking

Corrected Ratio:

\[ R_c = \frac{\text{FRET}_c/\text{CFP}}{} = \frac{\text{FRET} - \alpha \text{CFP} - \beta \text{YFP}}{\text{CFP}}\]

Example...