Motivations

Processes

An example
- counting molecules
- analyzing errors

Motivations

Analyzing a specimen
- how many? how much? how fast? etc.

Analyzing & comparing microscopes
- which one should I use? which one should I buy?

Standardization & troubleshooting
- “But it worked last time…”

Experimental design
- would I be able to see X?

Quantification is used for many different purposes, but typically begins with the same simple steps.

1) Fix common image defects
   (to permit comparisons between different regions of one image)

2) Normalize to constant exposure
   (to permit comparisons between different images)

3) Adjust display parameters to optimize visibility for further analysis

The subsequent steps usually need to be customized for each project.

4) Select targets

5) Measure

6) Statistical analysis to estimate precision and confidence limits
1) Fix or compensate common image defects

- Remove “hot” pixels
- “Flat-fielding”
  - additive corrections
    (e.g., dark current in fluorescence microscopy, non-uniform illumination in transmitted light microscopy)
  - multiplicative corrections
    (e.g., non-uniform illumination in fluorescence microscopy)

2) Normalize exposure
   (e.g., photosensor correction, or include internal reference)
1) Fix or compensate common image defects

   Remove "hot" pixels
   "Flat-fielding"
   additive corrections
   (e.g., dark current in fluorescence microscopy, non-uniform illumination in transmitted light microscopy)
   multiplicative corrections
   (e.g., non-uniform illumination in fluorescence microscopy)

2) Normalize exposure
   (e.g., photosensor correction, or include internal reference)

3) Adjust display parameters
   (e.g., choose LUT, set display min, max, and gamma)

Understand the difference between modifying the display, and modifying the image.

In preparing figures for presentation for which the primary goal is to demonstrate relative location rather than relative intensity, it is often necessary to use non-linear contrast enhancement tools to modify the image. This manipulation is done on a copy of the images, and described in the figure legend or Methods section. The original raw images must be archived in unmodified form.
**CCD camera:**
> > 500 distinguishable levels of intensity

**Display monitors, print media:**
~30 distinguishable levels of intensity

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**A case study**

- **the motivation**
- counting molecules in live cells
- analyzing the limits to precision
**Immuno-gold labeling of APR1**

Fangliang Zhang & Eiji Nagayasu

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**Mom's Christmas Cookies**

Cream some sugar and butter together

Spice flour with some salt, ground cinnamon, ground cloves, and allspice

Use the flour mixture to dust some chopped candied pineapple, candied cherries, and pecan halves.

Beat 1 egg at a time into the flour and the creamed butter-sugar, then add some white wine.

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**APR1-mCherryFPspots**

integrated intensity, background corrected

mean = 55257    sem = 1098

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**EGFP-tubulin**

**APR1-mCherryFP**

**APR1-mCherryFPspots**

integrated intensity, background corrected

mean = 55257    sem = 1098
mCherryFP-Sindbis virus
240 copies of mCherry per particle

mCherryFP-Sindbis spots
integrated intensity, background corrected
mean = 1007    sem = 3.5

APR1-mCherry
1956 ± 41 molecules

5 μm
Yu-chen Hwang
Kulika Chomvong

26 ± 1 DCX
484 ± 18 tubulin dimers
88 ± 2 APR1
22 cortical microtubules
A case study

the motivation

counting molecules in live cells

analyzing the limits to precision

Poisson distribution

\[ P(r) = \frac{(np)^r e^{-np}}{r!} \]

Variance = mean = np

Average photons/pixel (np)
The distribution of photon yields is much broader than expected. Why?

Evaluate noise sources using a very simple specimen — repeated images of one single, very small, very bright, photostable, fluorescent bead, on the optic axis, with no background.

image 4096 pixels (64 x 64)

"background" = outside the box (4015 pixels)

"bead" = 81 pixels inside the box (9 x 9)

measure the background corrected integrated bead intensity in 100 sequential images of the same bead
Basic Image Quantification

Measured brightness
100 images of one fluorescent bead

Expected errors: Poisson

Illumination fluctuation?
Possible sources of extra noise:

- light source fluctuations not captured by photosensor
- random error in camera/shutter, giving variable exposure times
- miniscule vertical bead excursions, within an evanescent field
- funky photochemical/photophysical events in the bead fluorophore population
2 non-fluorescent light sources show the expected level of variation.

3 solutions of a fluorophore (Alexa 594) show the expected level of variation.
fluorescent beads exhibit time/history-dependent fluorescence using a water immersion lens (no evanescent field)

Sources of extra noise: Conclusions

light source fluctuations not captured by photosensor?
   if this contributes to noise, the effect is small

random error in camera/shutter, giving variable exposure times?
   camera/shutter timing variability is below detection level

miniscule vertical bead excursions, within an evanescent field?
   Excess noise remains the same when evanescent field is eliminated.

funky photochemical/photophysical events in the bead fluorophore population
   This seems to account for most of the excess noise.
   This funkiness is not a general property of fluorescence imaging.

The distribution of photon yields is much broader than expected. Why?