Fluorescence Imaging Analysis: The Case of Calcium Transients.

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Mathématiques Appliquées à Paris 5 (MAP5)

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ENP Course: June 20 2017

Outline

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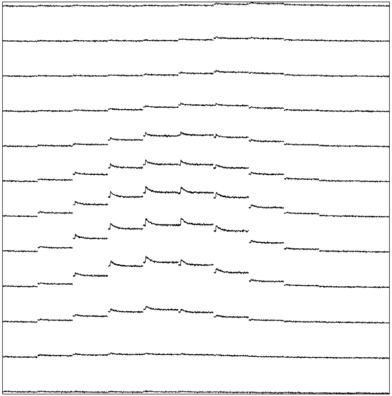
Introduction

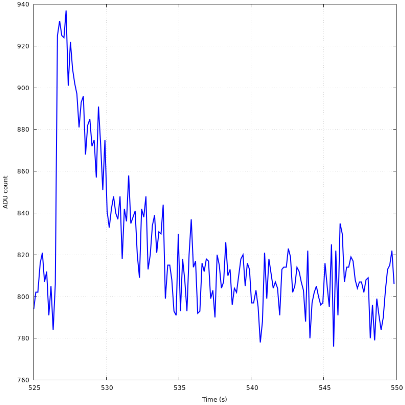
CCD camera noise

The variability inherent to fluorescence imaging data (1)



ADU counts (raw data) from Fura-2 excited at 340 nm. Each square corresponds to a pixel. 25.05 s of data are shown. Same scale on each sub-plot. Data recorded by Andreas Pippow (Kloppenburg Lab. Cologne University).

The variability inherent to fluorescence imaging data (2)



One of the central pixels of the previous figure.

What do we want? (1)

Given the data set illustrated on the last two slides we might want to estimate parameters like:

- the peak amplitude
- the decay time constant(s)
- the baseline level
- the whole time course (strictly speaking, a function).

What do we want? (2)

If we have a model linking the calcium dynamics—the time course of the free calcium concentration in the cell—to the fluorescence intensity like:

$$\frac{\mathrm{d}Ca_t}{\mathrm{dt}} \left(1 + \kappa_F(Ca_t) + \kappa_E(Ca_t)\right) + \frac{j(Ca_t)}{v} = 0\,,$$

where Ca_t stands for $[Ca^{2+}]_{free}$ at time t, v is the volume of the neurite—within which diffusion effects can be neglected—and

$$j(Ca_t) \equiv \gamma(Ca_t - Ca_{steady})\,,$$

is the model of calcium extrusion—Ca_{steady} is the steady state \$[Ca^{2+}]$_{\text{free}}$\$—

$$\kappa_F(Ca_t) \equiv \frac{F_{total}\,K_F}{(K_F + Ca_t)^2} \quad \text{and} \quad \kappa_E(Ca_t) \equiv \frac{E_{total}\,K_E}{(K_E + Ca_t)^2}\,,$$

where F stands for the fluorophore en E for the *endogenous* buffer.

What do we want? (3)

In the previous slide, assuming that the fluorophore (Fura) parameters: F_{total} and K_F have been calibrated, we might want to estimate:

- the extrusion parameter: γ
- the endogenous buffer parameters: E_{total} and K_E

using an equation relating measured fluorescence to calcium:

$$Ca_t = K_F \, \frac{S_t - S_{min}}{S_{max} - S_t} \, ,$$

where S_t is the fluorescence (signal) measured at time t, S_{min} and S_{max} are *calibrated* parameters corresponding respectively to the fluorescence in the absence of calcium and with saturating $[Ca^{2+}]$ (for the fluorophore).

What do we want? (4)

- The variability of our signal—meaning that under replication of our measurements *under the exact same conditions* we wont get the exact same signal—implies that our estimated parameters will also fluctuate upon replication.
- Formally our parameters are modeled as *random variables* and it is not enough to summarize a random variable by a single number.
- If we cannot get the full distribution function for our parameters, we want to give at least ranges within which the true value of the parameter should be found with a given probability.
- In other words: an analysis without confidence intervals is not an analysis, it is strictly speaking useless since it can't be reproduced—if I say that my time constant is 25.76 ms the probability that upon replication I get again 25.76 is essentially 0; if I say that the actual time constant has a 0.95 probability to be in the interval [24,26.5], I can make a comparison with replications.

A proper handling of the "variability" matters (1)

Let us consider a simple data generation model:

$$Y_i \sim \mathcal{P}(f_i)\,, \quad i = 0, 1, \ldots, K\ ,$$

where $\mathcal{P}(f_i)$ stands for the *Poisson distribution* with parameter f_i :

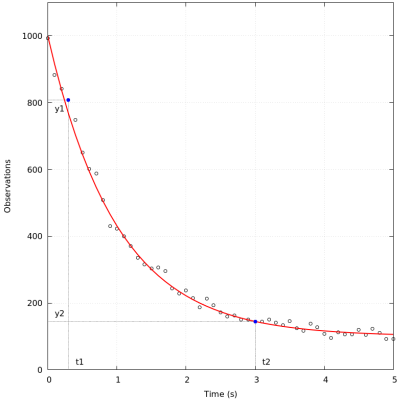
$$\mathrm{Pr}\{Y_i = n\} = \frac{(f_i)^n}{n!} \exp(-f_i)\,, \quad \text{for} \quad n = 0, 1, 2, \ldots$$

and

$$f_i = f(\delta i | f_\infty, \Delta, \beta) = f_\infty + \Delta\, \exp(-\beta\, \delta i)\ ,$$

δ is a time step and f_∞, Δ and β are model parameters.

A proper handling of the "variability" matters (2)



Data simulated according to the previous model. We are going to assume that f_∞ and Δ are known and that (t_1, y_1) and (t_2, y_2) are given. We want to estimate β.

Two estimators (1)

We are going to consider two estimators for β:

- The "classical" least square estimator:

$$\tilde{\beta} = \arg\min \tilde{L}(\beta) \ ,$$

where

$$\tilde{L}(\beta) = \sum_j \left(y_j - f(t_j \mid \beta)\right)^2 \ .$$

- The least square estimator applied to the *square root* of the data:

$$\hat{\beta} = \arg\min \hat{L}(\beta) \ ,$$

where

$$\hat{L}(\beta) = \sum_j \left(\sqrt{y_j} - \sqrt{f(t_j \mid \beta)}\right)^2 \ .$$

Two estimators (2)

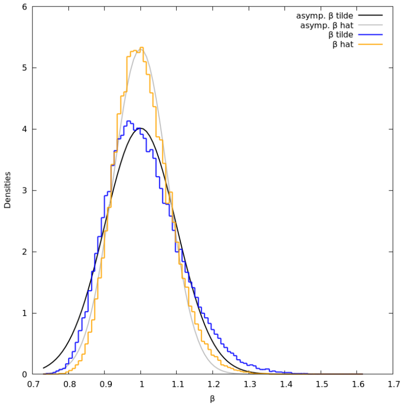
We perform an empirical study as follows:

- We simulate 100,000 experiments such that:

$$(Y_1, Y_2) \sim \big(\mathcal{P}(f(0.3|\beta_0), \mathcal{P}(f(3|\beta_0)\big) ,$$

  with $\beta_0 = 1$.
- For each simulated pair, $(y_1, y_2)^{[k]}$ $(k = 1, \ldots, 10^5)$, we minimize $\tilde{L}(\beta)$ and $\hat{L}(\beta)$ to obtain: $(\tilde{\beta}^{[k]}, \hat{\beta}^{[k]})$.
- We build histograms for $\tilde{\beta}^{[k]}$ and $\hat{\beta}^{[k]}$ as density estimators of our estimators.

Two estimators (3)



Both histograms are built with 100 bins. $\hat{\beta}$ is clearly better than $\tilde{\beta}$ since its variance is smaller. The derivation of the theoretical (large sample) densities is given in Joucla et al (2010).

Where are we ?

Introduction

CCD camera noise

CCD basics

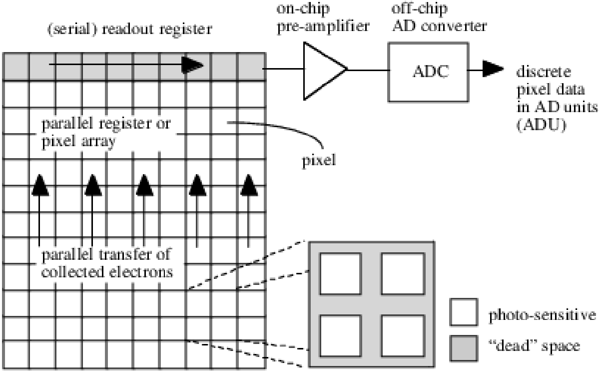


Figure 4: A full frame CCD as employed in scientific CCD cameras.

Source: L. van Vliet et col. (1998) Digital Fluorescence Imaging Using Cooled CCD Array Cameras (figure 3).

"Noise" sources in CCD (1)

- The "Photon noise" or "shot noise" arises from the fact the measuring a fluorescence intensity, λ, implies counting photons—unless one changes the laws of Physics there is nothing one can do to eliminate this source of variability (improperly called "noise")—:

$$\mathrm{Pr}\{N = n\} = \frac{\lambda^n}{n!} \exp -\lambda \, , \quad n \; = \; 0, 1, \ldots \, , \quad \lambda > 0 \; .$$

- The "thermal noise" arises from thermal agitation which "dumps" electrons in potential wells; this "noise" also follows a Poisson distribution but it can be made negligible by *cooling down* the camera.

"Noise" sources in CCD (2)

- The "read out noise" arises from the conversion of the number of photo-electrons into an equivalent tension; it follows a normal distribution whose variance is independent of the mean (as long as reading is not done at too high a frequency).
- The "digitization noise" arises from the mapping of a continuous value, the tension, onto a grid; it is negligible as soon as more than 8 bit are used.

A simple CCD model (1)

- We can easily obtain a simple CCD model taking into account the two main "noise" sources (photon and read-out).
- To get this model we are going the fact (a theorem) that when a large number of photon are detected, the Poisson distribution is well approximated by (converges in distribution to) a normal distribution with identical mean and variance:

$$\mathrm{Pr}\{N = n\} = \frac{\lambda^n}{n!} \exp -\lambda \approx \mathcal{N}(\lambda, \lambda) \; .$$

- In other words:

$$N \approx \lambda + \sqrt{\lambda}\,\epsilon \; ,$$

where $\epsilon \sim \mathcal{N}(0, 1)$ (follows a standard normal distribution).

A simple CCD model (2)

- A read-out noise is added next following a normal distribution with 0 mean and variance σ_R^2.
- We are therefore adding to the random variable N a new independent random variable $R \sim \mathcal{N}(0, \sigma_R^2)$ giving:

$$M \equiv N + R \approx \lambda + \sqrt{\lambda + \sigma_R^2}\,\epsilon \,,$$

where the fact that the sum of two independent normal random variables is a normal random variable whose mean is the sum of the mean and whose variance is the sum of the variances has been used.

A simple CCD model (3)

- Since the capacity of the photo-electron weels is finite (35000 for the camera used in the first slides) and since the number of photon-electrons will be digitized on 12 bit (4096 levels), a "gain" G smaller than one must be applied if we want to represent faithfully (without saturation) an almost full well.
- We therefore get:

$$Y \equiv G \cdot M \approx G\,\lambda + \sqrt{G^2\,(\lambda + \sigma_R^2)}\,\epsilon\ .$$

For completeness: Convergence in distribution of a Poisson toward a normal rv (1)

We use the moment-generating function and the following theorem (*e.g.* John Rice, 2007, *Mathematical Statistics and Data Analysis*, Chap. 5, Theorem A):

- If the moment-generating function of each element of the rv sequence X_n is $m_n(t)$,
- if the moment-generating function of the rv X is $m(t)$,
- if $m_n(t) \to m(t)$ when $n \to \infty$ for all $|t| \leq b$ where $b > 0$
- then $X_n \xrightarrow{D} X$.

For completeness: Convergence in distribution of a Poisson toward a normal rv (2)

Lets show that:

$$Y_n = \frac{X_n - n}{\sqrt{n}} \ ,$$

where X_n follows a Poisson distribution with parameter n, converges in distribution towards Z standard normal rv.
We have:

$$m_n(t) \equiv \mathrm{E}\left[\exp(Y_n t)\right] \ ,$$

therefore:

$$m_n(t) = \sum_{k=0}^{\infty} \exp\left(\frac{k-n}{\sqrt{n}} t\right) \frac{n^k}{k!} \exp(-n) \ ,$$

For completeness: Convergence in distribution of a Poisson toward a normal rv (3)

$$m_n(t) = \exp(-n)\exp(-\sqrt{n}t)\sum_{k=0}^{\infty}\frac{\left(n\exp\left(t/\sqrt{n}\right)\right)^k}{k!}$$

$$m_n(t) = \exp\left(-n - \sqrt{n}t + n\exp(t/\sqrt{n})\right)$$

$$m_n(t) = \exp\left(-n - \sqrt{n}t + n\sum_{k=0}^{\infty}\left(\frac{t}{\sqrt{n}}\right)^k\frac{1}{k!}\right)$$

$$m_n(t) = \exp\left(-n - \sqrt{n}t + n + \sqrt{n}t + \frac{t^2}{2} + n\sum_{k=3}^{\infty}\left(\frac{t}{\sqrt{n}}\right)^k\frac{1}{k!}\right)$$

$$m_n(t) = \exp\left(\frac{t^2}{2} + n\sum_{k=3}^{\infty}\left(\frac{t}{\sqrt{n}}\right)^k\frac{1}{k!}\right)$$

For completeness: Convergence in distribution of a Poisson toward a normal rv (4)

We must show:

$$n\sum_{k=3}^{\infty}\left(\frac{t}{\sqrt{n}}\right)^k\frac{1}{k!}\to_{n\to\infty}0\quad\forall\ |t|\leq b,\quad\text{where}\quad b>0\,,$$

since $\exp(-t^2/2)$ is the moment-generating function of a standard normal rv. But

$$\left|n\sum_{k=3}^{\infty}\left(\frac{t}{\sqrt{n}}\right)^k\frac{1}{k!}\right|\to_{n\to\infty}0\quad\forall\ |t|\leq b,\quad\text{where}\quad b>0$$

implies that since

$$-\left|n\sum_{k=3}^{\infty}\left(\frac{t}{\sqrt{n}}\right)^k\frac{1}{k!}\right|\leq n\sum_{k=3}^{\infty}\left(\frac{t}{\sqrt{n}}\right)^k\frac{1}{k!}\leq\left|n\sum_{k=3}^{\infty}\left(\frac{t}{\sqrt{n}}\right)^k\frac{1}{k!}\right|\,.$$

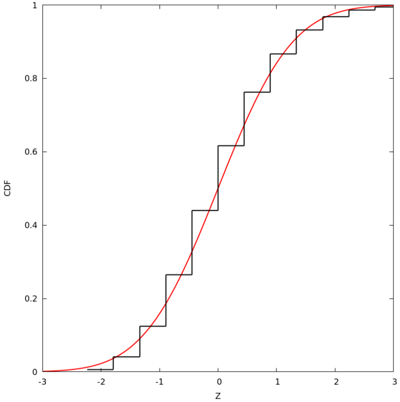
For completeness: Convergence in distribution of a Poisson toward a normal rv (5)

But for all $|t| \leq b$ where $b > 0$

$$\begin{aligned} 0 \leq \left| n \sum_{k=3}^{\infty} \left(\frac{t}{\sqrt{n}}\right)^k \frac{1}{k!} \right| &\leq n \sum_{k=3}^{\infty} \left(\frac{|t|}{\sqrt{n}}\right)^k \frac{1}{k!} \\ &\leq \frac{|t|^3}{\sqrt{n}} \sum_{k=0}^{\infty} \left(\frac{|t|}{\sqrt{n}}\right)^k \frac{1}{(k+3)!} \\ &\leq \frac{|t|^3}{\sqrt{n}} \sum_{k=0}^{\infty} \left(\frac{|t|}{\sqrt{n}}\right)^k \frac{1}{k!} \\ &\leq \frac{|t|^3}{\sqrt{n}} \exp\left(\frac{|t|}{\sqrt{n}}\right) \to_{n\to\infty} 0\,, \end{aligned}$$

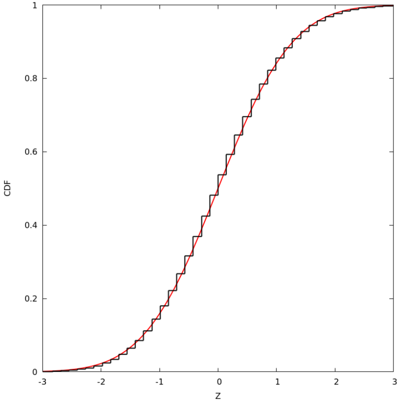
which completes the proof.

For completeness: Convergence in distribution of a Poisson toward a normal rv (6)



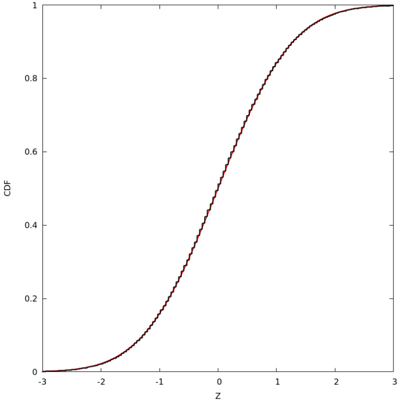
Cumulative distribution functions (CDF) of Y_5 (black) and Z a standard normal (red).

For completeness: Convergence in distribution of a Poisson toward a normal rv (7)



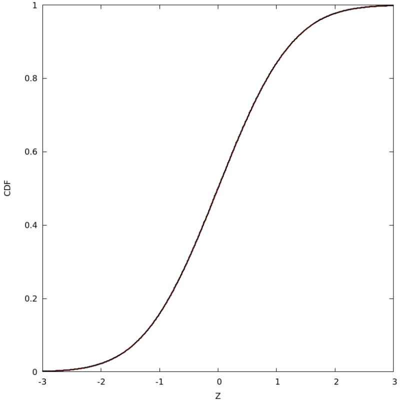
Cumulative distribution functions (CDF) of Y_{50} (black) and Z a standard normal (red).

For completeness: Convergence in distribution of a Poisson toward a normal rv (8)



Cumulative distribution functions (CDF) of Y_{500} (black) and Z a standard normal (red).

For completeness: Convergence in distribution of a Poisson toward a normal rv (9)



Cumulative distribution functions (CDF) of Y_{5000} (black) and Z a standard normal (red).