REMARKS ON THE STATISTICAL STUDY OF PROTEIN-PROTEIN INTERACTION IN LIVING CELLS

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Abstract. In this note, we focus on a selection model problem: a mono-exponential model versus a bi-exponential one. This is done in the biological context of living cells, where small data are available. Classical statistics are revisited to improve existing results. Some unavoidable limits are also pointed out.

1. Introduction

The measurement of molecular dynamic interactions and their respective proportions in living cells or tissues is a major question in biological and medicine research. The Förster resonance energy transfer (FRET) is one of the best known approaches to observe and quantitatively study protein-protein interactions at a subcellular level (1). The FRET measurement can be currently performed by fluorescence lifetimes imaging microscopy (FLIM for short) in living cells and tissues. It can be achieved via the time correlated single photon counting (TCSPC) method which provides a lifetime decay curve per site (2). To be interpreted, this curve is fitted by selecting the “best” (with respect to a given statistical criterion) multi-exponential model. Contrary to a mono-exponential model, a bi-exponential one witnesses interaction between two proteins. Our aim is to find, pixel per pixel, which of these models is accurate. But one difficulty is that the number of observed photons per pixel is small for any statistical treatment in order to preserve the living cell and therefore cannot be increased. An attempt to deal with the problem can be found in [7]. Our aim here is to go further in this direction pointing out some improvements and limits. Some account of statistical methods in this area can be found in [5] and [6].

1.1. Modelling fluorescence lifetimes. It is not necessary to describe here in details FLIM and TCSCP. We only need to understand that lifetimes are measured as differences between excitation times (pulses) and emission times of photons. Denote by $r$ the period between two consecutive pulses. Here $r$ is 12 nanoseconds, near values taken in practice. What is actually measured is a lifetime modulo $r$ since we cannot be sure from what pulse it goes.

It is assumed that lifetimes come from say $K$ species and are observed in the interval $[0, r)$ after infinitely many pulses. In these conditions, each lifetime species $k$ ($1 \leq k \leq K$) admits the following probability density:

\[
 f_k(t) = \alpha_k \exp(-\alpha_k t) \frac{\mathbb{I}_{[0,r)}(t)}{1 - \exp(-\alpha_k r)}
\]

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where $\alpha_k$ is the inverse mean lifetime of the $k$-th species. A uniform noise is added with density

$$f_0(t) = \frac{\mathbb{1}_{[0,r)}(t)}{r}.$$  

If $\pi_k$ denotes the proportion of the $k$-th species ($\pi_0$ refers to the noise’s one), we get the probability density of the fluorescence lifetime by writing

$$g(t) = \sum_{k=0}^{K} \pi_k f_k(t).$$

1.2. Modelling the photon emission. Let $I_k$ be the mean photon number of species $k$ detected between two pulses. Assume that photons occurrences are independent. Then the total number of detected photons is Poisson distributed with intensity $T \sum_{k=0}^{K} I_k$ if observations take place during $T$ pulses. For a later use, it is convenient to set

$$I = \sum_{k=0}^{K} I_k.$$  

Note that we have

$$\pi_k = \frac{I_k}{I}.$$  

Since the noise intensity $I_0$ will be supposed known, it is convenient to consider proportions $\pi_k'$ among all species with $k \geq 1$ except $k = 0$. Thus, we have for $k \geq 1,$

$$\pi_k' = \frac{I_k}{I - I_0} = \frac{I}{I - I_0} \pi_k.$$  

1.3. Maximum likelihood estimation (MLE) and likelihood ratio test. The aim is firstly the determination of the most probable parameter $\theta := (\alpha_1, \ldots, \alpha_K, I_1, \ldots, I_K)$ from observed lifetimes modulo $r$ denoted by $t_1, \ldots, t_n$. The noise intensity $I_0$ is supposed known. The related log-likelihood is then

$$\mathcal{L}(\theta) = \mathcal{L}(\theta; t_1, \ldots, t_n) = -IT + n \log(IT) - \log(n!) + \sum_{i=1}^{n} \log(g(t_i)).$$

For physical reasons, in particular since lifetimes are sure to be between 30pc and 30ns, we may and do assume that $\theta$ lies in a compact parameter set.

Numerical optimisation of the likelihood (5) is made easier by knowing derivatives:

$$\frac{\partial g(t)}{\partial I_k} = \sum_{i=0}^{K} \frac{I_i}{T^2} [f_k(t) - f_i(t)] = \frac{f_k(t) - g(t)}{T};$$

$$\frac{\partial g(t)}{\partial \alpha_k} = \mathbb{1}_{[0,r)}(t) \frac{I_k}{T} \frac{e^{-\alpha_k t}}{(1 - e^{-\alpha_k r})^2} (1 - \alpha_k t + (\alpha_k t - \alpha_k r - 1)e^{-\alpha_k r});$$

$$\frac{\partial \mathcal{L}(\theta)}{\partial I_k} = -T + \frac{n}{T} + \sum_{i=1}^{n} \frac{f_k(t_i) - g(t_i)}{g(t_i)} = -T + \frac{1}{T} \sum_{i=1}^{n} \frac{f_k(t_i)}{g(t_i)};$$

$$\frac{\partial \mathcal{L}(\theta)}{\partial \alpha_k} = \sum_{i=1}^{n} \frac{\partial g(t_i)}{\partial \alpha_k} = \frac{I_k}{T} \sum_{i=1}^{n} \frac{\partial f_k(t_i)}{\partial \alpha_k} g(t_i).$$

Denote by $\theta_1^*$ the most probable parameter if there is $K$ species.

To decide next which model from $K = 1$ or $K = 2$ is the most accurate, a classical statistic is the likelihood ratio

$$D := |\mathcal{L}(\theta_2^*) - \mathcal{L}(\theta_1^*)|. $$
From a theoretical point of view, since we are dealing with the number of components of a mixture model, even the asymptotics under the null hypothesis are not the usual $\chi^2$ statistics. It can be expressed as a supremum over a Gaussian process on a subset of a four-dimensional unit sphere (in our case) endowed with the “right” covariance function ([1] and references therein). However this process depends also on the “true” point $\theta$. Since all calculations are complicated, it is easier to simply simulate if we want to know the level of a test associated with a given threshold.

Notice on the other hand that simulations hint that the likelihood ratio test is quite efficient for knowing the number of components in a mixture with compact parameter set (see for example [4] or [3]).

2. Selection of the number of exponential species $K$

2.1. Comparisons. We restricted ourselves to test $K = 1$ versus $K = 2$. It can be already a difficult and interesting question, if few observed photons are available. With the help of simulated observations, we first optimised $\theta$ by MLE for each $K$ and next tested $K = 1$ versus $K = 2$ via the likelihood ratio statistics $D$.

Compared to the one given in [7], the preceding statistical test is as efficient but with about 100 times less observations. For the reader’s convenience and for comparison, consider the table obtained in [7]:

| Nbr of photons / $\Delta \chi^2$ | 10.0 | 20.0 | 30.0 | 40.0 | 50.0 | 90.0 | Error(%) |
|----------------------------------|------|------|------|------|------|------|----------|
| 1000                             | 35.7 | 34.8 | 34.3 | 34.6 | 34.9 | 45   | > 20     |
| 10000                            | 13.7 | 12.0 | 11.9 | 12.1 | 12.9 | 27.3 | < 20     |
| 100000                           | 4.2  | 1.7  | 2.3  | 2.7  | 4.7  | 26.3 | < 5      |
| 1000000                          | 1.7  | 0.0  | 0.0  | 0.0  | 3.3  | 32.7 | < 2      |

Table 1. Frequency of selection of the wrong model. It depends on the observations number and a $\Delta \chi^2$ criterion which consists in comparing the $\chi^2$ statistics for $K = 1$ and $K = 2$. Simulations were performed on a mix of $1/\alpha_1 = 0.6$ ns and $1/\alpha_2 = 2.4$ ns with different proportions $\pi'_1 = 0.077, .2, .43, 1$ with 100 noise photons. 30 simulations per condition.

In similar simulation conditions, we have obtained the following:

| Nbr of photons | Mean error rate (%) | Best threshold | Mean error rate at threshold 4 |
|----------------|---------------------|----------------|-----------------------------|
| 1000           | 12.8                | .85            | 20                          |
| 10000          | 0.3                 | 4              | 0.3                         |
| 100000         | 0                   | 4              | 0                           |

Table 2. Frequency of selection of the wrong model. It depends on the observations number and a likelihood ratio criterion. Simulations were performed on a mix of $1/\alpha_1 = 0.6$ ns and $1/\alpha_2 = 2.4$ ns with different proportions $\pi'_1 = 0.077, .2, .43, 1$ with 100 noise photons. 500 simulations for each proportion and number of photons.

Here, mean error rate is the average over the simulation number of the percentage to select the wrong model. Best threshold means threshold that gives the smallest mean error rate; using a very crude optimisation. Notice that the strange values $0.077, .2, .43, 1$ of $\pi'_1$’s proportion correspond to values $.25, .5, .75, 1$ of proportion.
\[ \eta_1 = \frac{\pi_1 \alpha_2}{\pi_1 \alpha_2 + \pi_2 \alpha_1} \] considered in [7]. A consequence is that we never test more short-life photons than long-life. Moreover the case \( \pi_1' = .077 \) is not very far away from the mono-exponential case. In particular, with 1000 photons, among which 100 noise photons, the expected number of photons with 0.6 nanoseconds lifetime is less than the number of noise photons. If we compute the error rate for 1000 photons without that case, we obtain for instance a 2.6% error rate for the likelihood ratio test at threshold 3.

2.2. Simulation scheme. The data set generation algorithm is as follows:
1. Sample \( n_k \) the number of photons for each species \( k \), including noise \((k = 0)\), from a Poisson distribution of parameter \( TI_k \).
2. Draw \( n_k \) lifetimes with distribution density \( f_k \) for each species \( k \).
3. Return the set of all the sampled lifetimes, regardless of \( k \).

Some differences between simulation methods should be noted:
- We use random Poissonian number of photons rather than fixed number of photons: we take into account the “offset noise”.
- Instrumental response: we neglect the .03 nanoseconds long instrumental response function.
- Exact times vs channels: we did not use bins and worked as if we knew the exact detection times.

Nevertheless these differences should have little effect and comparisons still make sense.

3. Further comments
3.1. With closer lifetimes. If we choose \( 1/\alpha_1 = 1 \) ns and \( 1/\alpha_2 = 2 \) ns as mean lifetimes, it is harder to select the right number of species:
- With 10000 photons and \( \frac{\pi_0}{1 - \pi_0} = .01 \) as noise ratio:
  - If \( \pi_1' = \pi_2' = .5 \) or \( \pi_1' = .75, \pi_2' = .25 \), no wrong selection should occur,
  - If \( \pi_1' = .25, \pi_2' = .75 \), the error rate is about 1.1% when the threshold is calibrated so as to balance errors “mono towards bi” and “bi towards mono”.
- With 1000 photons and \( \frac{\pi_0}{1 - \pi_0} = .01 \) as noise ratio: if \( \pi_1' = \pi_2' = .5 \), the error rate is about 15% when the threshold is calibrated so as to balance errors “mono towards bi” and “bi towards mono”.

If we choose close mean lifetimes such as \( 1/\alpha_1 = 1.4 \) ns and \( 1/\alpha_2 = 1.6 \) ns, we are too close to the “border” of the model, and about 1 million photons is required to distinguish the two components. By border, we mean a proportion close to 0 or 1/\( \alpha_1 \) close to 1/\( \alpha_2 \) so that identifiability problems occur with small samples. Asymptotically, when we get \( n \) times closer to the border of a mixture model, we need \( n^4 \) times as many photons to get the same statistical efficiency, for any procedure [2].

3.2. Absolute limits. The former sentence about rates when we get nearer the border is a first expression of limits that cannot be broken, no matter how smart the statistical procedure. To give a small taste of what to expect, here are the best error rate when having to choose specifically between two possible sets of lifetime parameters and corresponding distribution probabilities \( f^1 \) and \( f^2 \), with equal \textit{a priori} probabilities. In that situation, which is easier than the one studied in the article, the optimal choice is the one with greater observed likelihood, and the error rate is \( \frac{1}{2} - \frac{1}{4} \| f^1 - f^2 \|_1 \).
• With 32 observed photons and a signal to noise ratio of 1/10, choose between a mono-exponential with lifetime 2.4 ns, and a bi-exponential with proportions 0.077 and 0.923 and lifetimes 0.6 and 2.4 ns: optimal error rate $> 25\%$.

• With 32 observed photons and no noise, choose between a mono-exponential with lifetime 2.6 ns, and a bi-exponential with proportions one half and lifetimes 2.5 and 2.7 ns: optimal error rate $> 49.75\%$.

The second case is almost as bad as a coin toss, ignoring the data.

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