The analysis of fluorescence microscopy images for FRET detection

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Abstract: This paper describes a new method of analysis of fluorescence images containing a mixture of fluorophores. Parametric maps showing the relative quantity and distribution of each individual fluorophore are generated using a predictive model of image formation. Error analysis applied to the model supplies objective criteria for choosing the optimal filters for image acquisition in the regions in the spectra where error is lowest. The method has been successfully applied to the detection of Fluorescence Resonance Energy Transfer (FRET) which provides evidence of interaction between proteins in a cell. The study of protein-protein interactions is of crucial importance in basic biomedical sciences.

1 Introduction

Individual cells from living organisms can be specifically stimulated by their extracellular environment to perform a defined task, for example a hormone from the blood supply may stimulate cell growth. This requires the transduction of the extracellular stimulant into an intracellular signal. Intracellularly, the transduction of such a signal can be mediated through consecutive modular interaction between two or more proteins. It is probable that the pathology of many diseases results from complete loss or aberrant interactions between such proteins. Although the homogeneous purification of specific proteins facilitates the in vitro characterisation of an interaction, these studies provide no information with regards to the spatial and temporal regulation of interactions within a living cell; information that is likely to be essential to enable future therapeutic interventions. With the aid of standard optical microscopy it is possible to see whether two proteins co-localise (i.e. they are close one to another in the cell), but not whether they interact. To achieve this, the proteins of interest are each tagged with a different coloured fluorescent molecule (fluorophore), for example red and green. Each of the fluorophores is individually stimulated and images of the resulting fluorescence are taken through the matched filters (e.g. red and green). In the registered images yellow hues indicate the locations where both the red and the green fluorophores co-localise. As well as not giving any indication as to whether or not these proteins interact, the reliability of this analysis is sometimes questioned because registration errors or accidental overlap in depth can generate false positives. In Fluorescence Resonance Energy Transfer imaging (FRET) the proteins of interest are tagged with specially matched fluorophores. One of them will fluoresce only if it is at a Förster distance (1-10 nm) from the other, which is likely to occur only if the two tagged proteins interact. The occurrence of FRET is taken to provide much firmer evidence of interaction, but the FRET induced fluorescence is much weaker than the primary fluorescence and hence difficult to detect reliably.

This paper presents a novel semi-quantitative method of the analysis of fluorescence microscopy images developed to provide a principled method of FRET detection. The next section describes FRET in more detail. Section 3 outlines the modelling of the fluorescence emissions and of the imaging pathway, and sketches out the error analysis. The selection of the optimal filters and the implementation of the inversion process which computes the relative levels of the emission by a given fluorophore are presented in section 4. The remaining sections describe the experiments, their results and the conclusions.

2 Fluorescence Resonance Energy Transfer (FRET)

Fluorescence is a phenomenon in which light at an appropriate wavelength excites electrons in a molecule; the electrons returning to a resting state emit light at a longer wavelength. FRET involves two molecules (fluorophores). The donor molecule receives the initial energy from a photon whose wavelength is within a specific spectral excitation range. During the subsequent decay stage a photon with the longer wavelength is emitted. The acceptor molecule must have its absorption spectrum tuned to the emission spectrum of the donor molecule. When it receives a photon from the donor it fluoresces (weakly) at a longer wavelength. The energy loss in a donor makes its own fluorescence level decrease. As FRET can only occur within 1-10 nm, the detection of the emission from the acceptor means that the two molecules are very close. If they are attached as markers to the proteins of interest, this is strongly indicative of the protein interaction. This evidence can be further corroborated by the drop in the fluorescence level by the donor molecule. FRET suffers from several problems of which the main ones are the low signal levels, the overlap of the donor and the acceptor spectra, the excitation of the acceptor by the donor excitation wavelengths, and the background fluorescence.

3 Predictive modelling

The analysis employed in this work draws on the understanding of the image formation process which can explain the relationship between the quantities we try to measure and image values to which they correspond. Given the spectral definition of all the individual fluorophores that can be present in a sample it is possible to model the spectra resulting from their mixtures in different proportions (Sect. 3.1). The next modelling step computes the effects of transmission through spectral filters and the capture of photons by camera electronics (Sect. 3.2). In this way it is possible to predict image values originating from various mixtures of fluorophores.

3.1 Fluorescence spectra

The pair of fluorophores used in this work is Cyan Fluorescent Protein (CFP) and Yellow Fluorescent Protein (YFP). Their respective excitation and emission spectra are shown in Fig.1(a) and (b). CFP acts as a donor in FRET and YFP as an acceptor. Fig.1(c) shows the CFP emission spectrum together with YFP excitation spectrum demonstrating the spectral overlap which is necessary for FRET (30% or more).

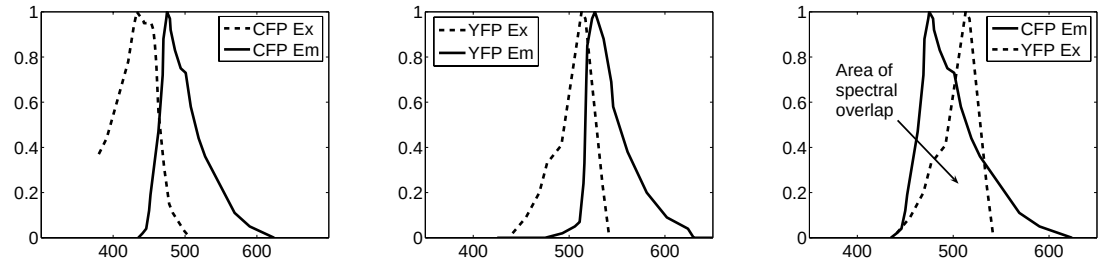

Figure 1: Excitation (Ex) and emission (Em) spectra for (a) CFP and (b) YFP; (c) spectral overlap between the CFP (donor) emission range and the YFP (acceptor) excitation range necessary for FRET.

The spectra in Figure 1 are normalized so that the peak emission has value 1. In practical imaging the magnitude of the emitted fluorescence depends on the magnitude of the excitation light (which "donates" photons), its spectral overlap with the excitation spectrum of the acceptor, and the local concentrations of the fluorophores. If a sample contains a mixture of CFP and YFP, its fluorescence spectrum should be a linear combination of their emission spectra, $P_C(\lambda)$ and $P_Y(\lambda)$. A well known analysis method, known as spectral unmixing, uses this assumption of linearity to identify the contributions of individual fluorphores from spectra containing their mixtures [1]. However, the purity of these spectral responses is compromised by additional emissions which may originate from background fluorescence, cell autofluorescence and other sources. These unwanted background contributions (not noise!) are difficult to model theoretically, but they need to be incorporated into the model. In this case their theoretical modeling was replaced by the experimental collection of data from the slides containing cells not tagged with fluorophores. The background spectra $P_B(\lambda)$ were incorporated into the model and a full set of spectral emissions was generated by the equation

$$P(c,y,b,\lambda) = c \cdot P_C(\lambda) + y \cdot P_Y(\lambda) + b \cdot P_B(\lambda), \text{ where } c \in [0,1],\ y \in [0,1],\ b \in [0,1] \quad (1)$$

Taken together this family of spectra constitutes a spectral emission model. The model has three variable parameters: the level of CFP emissions *c*, the level of YFP emissions *y*, background fluorescence *b*. Each combination of the quantities of *c*, *y* and *b* forms a parameter vector **p**, which is associated through the model with the appropriate spectrum. Image values corresponding to the spectra are modeled by convolving each spectrum with the response functions of the appropriate colour primaries, for example red, green and blue. A vector of such primaries is called an image vector, **i**.

3.2 Imaging system

In quantitative image interpretation it is necessary to take into account the optical properties of the imaging system. Their effect on the image can then be discounted so that pixel values can be directly related to properties of interest. The optical properties are also needed for error analysis (see 4.1). In this work we have modelled the effects of camera and of spectral filters. Camera quantum efficiency $Q(\lambda)$ specifies the percentage of photons registered at each wavelength λ. Filter transmission, $F_n(\lambda)$, specifies the percentage of photons transmitted through a given filter *n* at each wavelength. Normally the incident light spectrum has to be known, however in

fluorescence imaging the light which stimulates the fluorophores is filtered out. The formation of an image vector $\mathbf{i} = [i_1, ..., i_N]$ can be represented as $i_n = \int P(\lambda) \cdot F\,n(\lambda) \cdot Q(\lambda)\, d\lambda$, n=1,…,N. (2)

4 Optimal filter selection and parameter recovery

The filter sets used in fluorescence imaging normally coincide with the emission peaks of the dyes used. Our previous experience [2] has shown that in quantitative analysis of spectral mixtures the best set of filters can be chosen through optimisation. The method for optimal filter selection is outlined in section 4.2. Prior to that section 4.1 looks at error analysis because the error with which the contributions of the individual fluorophores can be recovered is used as an optimisation criterion in the filter selection process.

4.1 Error analysis

We have considered two sources of error. Spectral error is a statistical error arising from uncertainty in the measurement of spectra. Such measurements incorporate photon counting, which is known to be characterised by Poisson noise. In this noise model the error is related to the photon count, P(λ), i.e. $\sigma_{spec}(\lambda)=\sqrt{P(\lambda)}$. (3)

The error for the image value i_n is thus $\sigma_{spec}(i_n) = \sqrt{\sum_{\lambda} (\sigma_{spec}(\lambda)\cdot F_n(\lambda)\cdot Q(\lambda))^2}$ [3]. (4)

The camera error is the denominator in the expression for the camera signal to noise ratio (SNR) [4]

$$SNR_n(\lambda) = \frac{F_n(\lambda)\cdot P(\lambda)\cdot Q(\lambda)\cdot t}{\sqrt{F_n(\lambda)\cdot P(\lambda)\cdot Q(\lambda)\cdot t + D\cdot t + Nr^2}}, \quad \text{thus } \sigma_{cam}(i_n) = \sum_{\lambda} \sqrt{F_n(\lambda)\cdot P(\lambda)\cdot Q(\lambda)\cdot t + D\cdot t + Nr^2} \qquad (5)\ (6)$$

where P(λ) is a photon count, $F_n(\lambda)$ is filter transmittance for filter n, Q(λ) is quantum efficiency, D is camera dark current, Nr is the maximum readout noise and t is exposure time.

4.2 Optimal filter selection

The error analysis above supplies objective criteria for choosing the regions in the spectra where error is lowest. However, the aim of this work is to recover the magnitudes of the fluorescent emissions, i.e. parameters *c*, *y* and *b* forming vector **p**. The optimisation criteria should be expressed in relation to these magnitudes. Using standard error propagation [3], the spectral and camera errors expressed as a function of parameter p_j are:

$$\sigma_{spec}(p_j) = \left(\sum_n \left(\sigma_{spec}(i_j) \frac{\partial p_j}{\partial i_n}\right)^2 \right)^{1/2} \quad \text{and} \quad \sigma_{cam}(p_j) = \left(\sum_n \left(\sigma_{cam}(i_j) \frac{\partial p_j}{\partial i_n}\right)^2 \right)^{1/2} \qquad (7)\ (8)$$

The total error is the sum of the total spectral error $\sum_j \sigma_{spec}(p_j)$ and the total camera error $\sum_j \sigma_{cam}(p_j)$. (9) (10)

In the filter optimisation process filters are assumed to have Gaussian shape, normalised so that the area under the filter is 1. Each filter is defined by two values: the central wavelength (CW) and FWHM. As for the recovery of three parameters (*c*, *y* and *b*) three filters are required, optimisation operates on a six-dimensional vector $[F_{CW1}\ F_{FWHM1}\ F_{CW2}\ F_{FWHM2}\ F_{CW3}\ F_{FWHM3}]$. A standard Matlab genetic algorithm was used to find the optimal filter values. Full details of the method for optimal filter selection can be found in [2].

4.3 Parameter recovery

The spectral emission model developed in 3.1 comprises spectra which correspond to all plausible combinations of parameters *c*, *y* and *b*. Now that the optimal filters are found, each spectrum can be substituted by a three-dimensional image vector **i** where $i_{n,cyb}$ is a spectrum $P(c,y,b,\lambda)$ convolved with filter $F_n(\lambda)$. The optimization procedure described in 4.2. has ensured that the mapping between the image vectors and the parameter vectors is one-to-one [2]. It is therefore possible to find a unique parameter vector **p** = [*c y b*] corresponding to a given image vector **i**. If *f* denotes a function which maps the parameter vectors onto the corresponding image vectors, f^{-1} performs the inversion. In practice f^{-1} is non-linear and discrete and its implementation has to involve interpolation to compute vectors **p** for an arbitrary vectors **i**. We have used an inversion algorithm based on the Taylor expansion [2]. The parametric image *j* is created simply by placing a value of a parameter p_j at a location (x,y) corresponding to $f^{-1}(\mathbf{i}(x,y))$.

5 Experiments

The purpose of the experiments was to determine whether the new method correctly identifies fluorescence emissions from individual fluorophores (CFP and YFP), and correctly identifies the occurrence of FRET.

Phospholipase D1b (PLD1b) and ADP-ribosylation factor 6 (ARF6) are two proteins that have been shown to interact within cells by other methods and were therefore labelled with CFP and YFP respectively to facilitate the identification of FRET by this method (Powner et al., 2002). Four sets of slides were prepared: (a) a set containing untagged cells; (b) cells with CFP-labelled ARF6 protein only; (c) cells with YFP-labelled PLD1b protein only; and (d) a set where both proteins were labelled and thus likely to generate FRET (see Fig. 3). The slides were placed at a stage of the inverted fluorescence microscope (TE300, NIKON). White light generated by a mercury lamp was passed though a filter set BV-2A to produce illumination in the excitation range. A computer-controllable set of interference filters (VariFilters, CRI) was mounted between the microscope's camera port and a high quality B/W digital camera (Retiga Exi, QImaging). Images were taken at the spectral wavelengths corresponding to the optimal filter bands $[F_{CWi} \quad F_{FWHMi}] = [[497, 7]; [528, 8]; [558\ 21]]$. The exposure time at each band was set individually to ensure as large as possible dynamic range and to avoid over- and under-exposure. Digital images were then numerically normalised to correspond to the same exposure time. The inversion process was applied at every pixel and produced three parametric maps corresponding to the levels of CFP, YFP and the background.

6 Results

Figure 3 shows examples of the parametric maps for the four sets of experimental data. It can be seen that all the maps are qualitatively correct. Most excitingly, the last sample, in which FRET occurs, shows both the presence of YFP fluorescence and the drop in the level of CFP fluorescence.

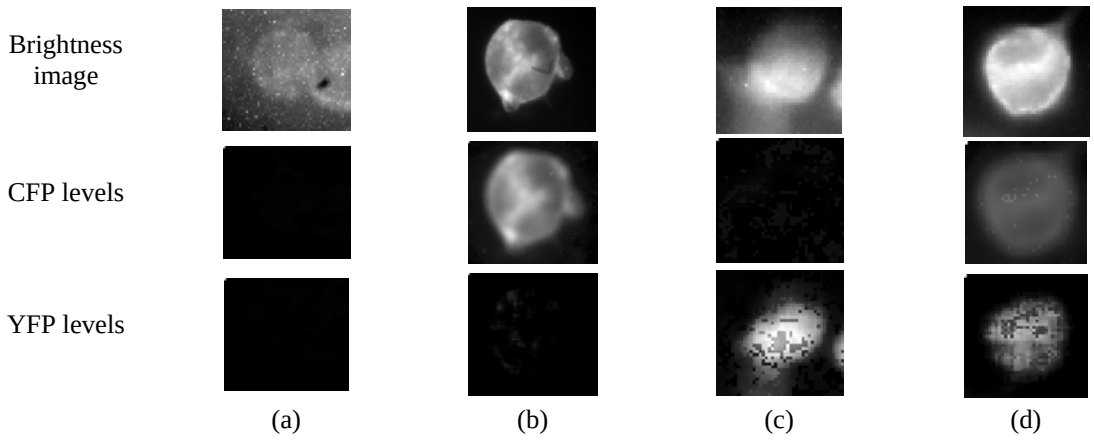

Figure 3: (a) a set containing untagged cells; (b) cells with CFP-labelled protein ARF6 only; (c) cells with YFP-labelled PLD1b protein only; and (d) a set showing the evidence of FRET. (Colour images in Appendix).

7 Discussion and conclusions

This paper has presented a new method of analysis of fluorescence images containing a mixture of fluorophores. It generates parametric maps, each showing a relative quantity of one individual fluorophore at a given location. Unlike spectral unmixing methods, this method does not require the acquisition of the complete spectral data. Instead, only several bandpass filtered images are needed, their number being equal to the number of fluorophores in the mixture plus one (to account for background fluorescence). The quantities in the parametric maps are related to the magnitudes of the fluorescence in the sample through a predictive spectral model. The method has been successfully applied to the detection of FRET, which provided evidence of interaction between proteins ARF6 and PLD1b in a cell. The work described in this paper constitutes "proof of concept" for the new method and space constraints have prevented inclusion of further detail. Additional systematic studies are planned to enable full statistical verification and explore other applications in fluorescence microscopy imaging.

References

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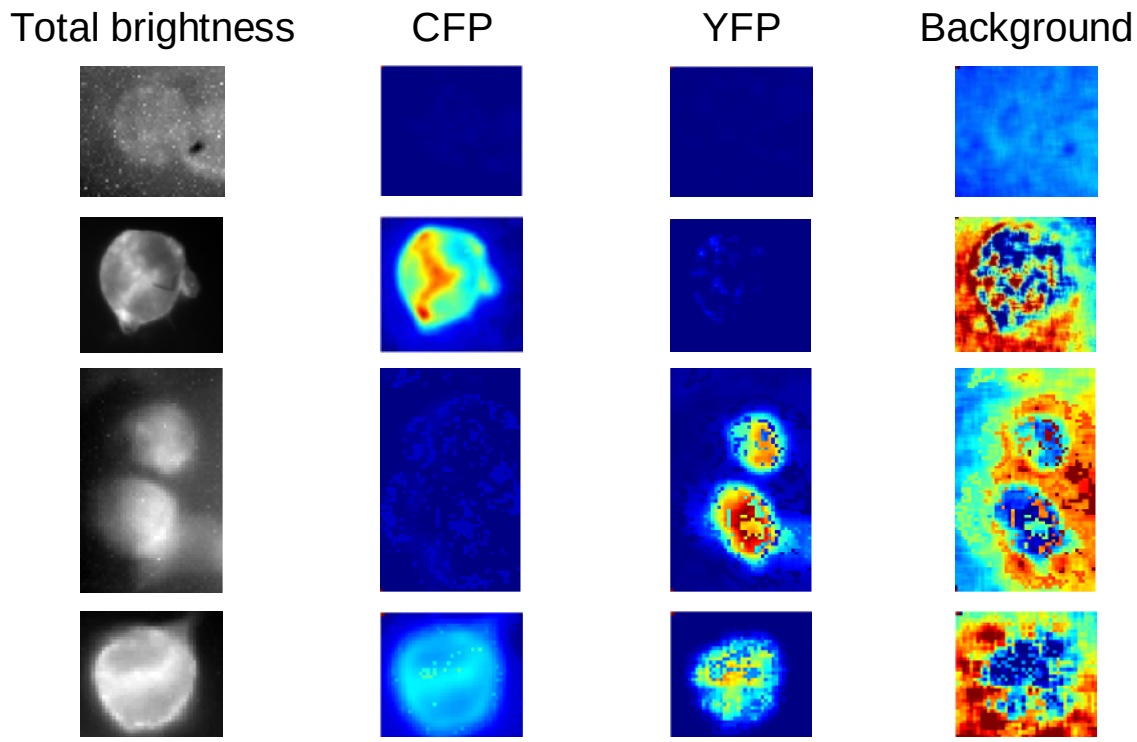
Appendix

Figure 3 (in colour):

(a) a set containing untagged cells; (b) cells with CFP-labelled protein ARF6 only; (c) cells with YFP-labelled PLD1b protein only; and (d) a set showing the evidence of FRET.



(a) (b) (c) (d)