Co-localization of cell surface receptors at high spatial resolution by single-particle fluorescence imaging

I. Karakikes, R.E. Barber, I.E.G. Morrison, N. Fernández and R.J. Cherry

Department of Biological Sciences, University of Essex, Wivenhoe Park, Colchester, Essex CO4 3SQ, U.K.

Abstract
Dual-wavelength single-particle fluorescence imaging has been used to quantify the co-localization of receptors and/or ligands on cells by widefield microscopy. Methods for correction of chromatic aberration and identification of submicroscopic artefacts are presented, with data for the lipopolysaccharide/CD14 and MHC class II/CD74 systems.

Introduction
SPFI (single-particle fluorescence imaging) utilizes the sensitivity of cooled slow-scan CCD (charged-coupled device) cameras to image submicroscopic fluorescent particles, such as phycobiliproteins acting as probes for selected receptors [1–3]. At low surface density (~1 particle µm⁻²) the probes appear as spots of diffraction-limited width that can be modelled as two-dimensional Gaussian envelopes; their position and intensity parameters can be obtained by image processing and used to quantify receptor dimerization [2] and mobility [3].

We are now exploiting the ability to image fluorophores of different wavelength characteristics to investigate interactions between pairs of distinct surface protein species. Antibodies against the two species are derivatized by either PhyE (R-phycoerythrin) or APhyC (allophycocyanin) at 1:1 ratios, and cells are labelled and imaged sequentially at the appropriate excitation wavelength using suitable emission filters. In the present paper, the image analysis methods are described briefly, and results are presented for two systems.

Interactions between cell surface proteins are implicated in many signalling processes. LPS (lipopolysaccharide) is a constituent of bacterial cell walls that is known to trigger immune responses via the CD14 receptor [4]; LPS bound to cells is thus probably co-localized with CD14. The MHC class II molecules are assembled in the endoplasmic reticulum to cells is thus probably co-localized with CD14. The MHC class II molecules are assembled in the endoplasmic reticulum with the aid of cytoplasmic Ii (invariant chain) [5], but associations with CD74 receptors (the plasma membrane isoform of Ii) in living cells have not been determined.

Materials and Methods
The preparation and characterization of (1:1) antibody–phycobiliprotein conjugates has been described previously[6]; all fluorophores were from Molecular Probes. Anti-CD14 (26ic) and anti-HLA-DR (DA6.231) were labelled by PhyE, and anti-CD74 (BU45) was conjugated with APhyC. LPS was labelled by Alexa-488 hydrazide on oxidized carbohydrate moieties [7]. These probes were applied to either M1DR1/DM/Ii cells [8] or CHO-CD14 cells [9], and were fixed to prevent movement between images.

Imaging was performed by a Wright Instruments back-illuminated CCD camera attached to an Olympus IX70 microscope, using an NA0.85 40x objective, mercury arc illumination and Omega Optical Inc. filter sets. Image analysis to extract position, width and intensity parameters for each spot has been described [1]; correlations between spots at different wavelengths were established by a method similar to that used for single particle tracking [3].

Chromatic aberration in images at different wavelengths can be seen as a radial shift in the positions of correlated spots with respect to an image centre \( \{x_o, y_o\} \). In addition, non-parallel coverslip and slide surfaces can generate offset between images. These effects can be corrected for a spot centred at \( \{x_p, y_p\} \) as:

\[
\{x_p, y_p\} = \{x_o + k(x_p - x_o), y_o + k(y_p - y_o)\}
\]

The values of the radial correction factor \( k \), \( x_o \) and \( y_o \) are determined for each set of images by first identifying correlated spots, and then fitting the shifts from the PhyE spot positions using this equation.

In many cells, submicroscopic autofluorescent spots exist and can be detected at emission wavelengths from 500 to 700 nm. These act as guides for correlating the chromatic aberration, but also would be identified as co-localized particles. Thus a third image was obtained at a wavelength where neither probe fluoresced (‘fluorescein’ filters when using PhyE and APhyC, or APhyC filters when using Alexa-488 and PhyE). Spots found in all three images were termed artefacts; those found in two images are correlated, while the remaining spots are uncorrelated receptors/ligands.

The extent of co-localization can be quantified by calculating the overlap integral between two spot envelopes,
Results and discussion

Figures 1(a) and 1(b) show fluorescent images of a small area of a CHO cell that has been incubated with Alexa-488–LPS and PhyE-labelled anti-CD14 antibody; a third image using APhyC filters (Figure 1c) detected artefacts. Correlated spot positions were analysed to determine the chromatic aberration and shifts using eqn (1); Figure 1(d) shows the green–blue shift in \( \{x, y\} \) plotted against distance from the best-fit optical centre position, with the straight line that provides the correction factor \( k \). A map of the corrected positions is shown in Figure 1(e). The intensities of the Alexa-488–LPS spots indicate that they correspond to small aggregates; control images of the probe on PLCS (polylysine-coated slides) (results not shown) suggested that single Alexa-488–LPS molecules would not be reliably detected against the autofluorescence background, so the uncorrelated PhyE–26ic may in fact be associated with undetected LPS.

Similar images for PhyE–DA6.231 and APhyC–BU45, using a ‘fluorescein’ filter set to identify artefacts, were similarly analysed, correlated and corrected to determine the extent of co-localization of their receptors. Control data from images of the probes adhering to PLCS are shown as a histogram of overlap integrals in Figure 2(a); it is apparent that the probes do not interact in the absence of their receptors, and the overlap integrals show few values above 0.8. The \( \Phi \) values for the on-cell correlated spots are shown in Figure 2(b); many values above 0.8 are seen, suggesting co-localization. Histograms of the intensities of the PhyE spots on PLCS and on the cell are shown in Figures 2(c) and 2(d), confirming that the receptors can be monomeric or dimeric, as found previously [2]. The APhyC spots on the cell also appear to derive from single and double particles that may correspond to monomeric and dimeric receptors (compare Figures 2e and 2f).

These results show that images showing chromatic aberration can be corrected by using the artefact spots to find
The solid lines are the best-fit normal distributions with a two-particle convolution [2].

parameters for a correction algorithm. When two different molecules are labelled for SPFI, the extent and molecular state of any associations can be assessed by comparing the intensities of the co-localized spots with those on control slides.

This work was supported by the Biotechnology and Biological Sciences Research Council.

References
3 Smith, P.R., Morrison, I.E.G., Wilson, K.M., Fernández, N. and Cherry, R.J. (1999) Biophys J. 76, 3331–3344

Received 21 July 2003