Measurements of associations of cell-surface receptors by single-particle fluorescence imaging

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Abstract
SPFI (single-particle fluorescence imaging) uses the high sensitivity of fluorescence to visualize individual molecules that have been selectively labelled with small fluorescent particles. The images of particles are diffraction-limited spots that are analysed by fitting with a two-dimensional Gaussian function. The spot intensities depend on whether they arise from one or more particles; this provides the basis for determining self-association of cell-surface receptors. We have used this approach to determine dimerization of MHC class II molecules and its disruption by interface peptides. We have also exploited the positional information obtained from SPFI to detect co-localization of cell-surface molecules. This involves labelling two different molecules with different coloured fluorophores and determining their positions separately by dual wavelength imaging. The images are analysed to quantify the overlap of the particle images and hence determine the extent of co-localization of the labelled molecules. The technique provides quantification of the extent of co-localization and can detect whether co-localized molecules occur singly or in clusters. We have obtained preliminary data for co-localization of lipopolysaccharide and CD14 on intact cells. We also show that HLA-DR (human leukocyte antigen-DR) and CD74 are partially co-localized and that interaction between these molecules involves the peptide-binding groove of HLA-DR.

Introduction
Signalling and other functions of cell-surface receptors often involve transient or long-lived associations with other membrane-bound molecules. Self-association, particularly dimerization, of receptors is also often essential to function. The existence of molecular associations may be deduced from applying biochemical and molecular biological techniques while more direct evidence of associations in intact cells is often obtained from FRET (fluorescence resonance energy transfer) measurements.

There has of late been much interest in techniques for detecting events at the level of single molecules. Such an approach can provide much more detailed information than that obtained from more traditional methods. In our laboratory, we have developed applications of SPFI (single-particle fluorescence imaging) to study molecular associations. The basic concept of SPFI is to use the high sensitivity of fluorescence to visualize individual receptors in the plasma membranes of living cells. Receptors are selectively labelled with a small fluorescent particle and the particles imaged using a cooled CCD camera attached to a fluorescence microscope [1–3]. Suitable probes are made by attaching a monoclonal antibody (or its Fab fragment) to either a phycobiliprotein or a fluorescent latex microsphere [4]. Phycobiliproteins are particularly advantageous because of their small size [PhyE (R-phycocerythrin) is 11 nm × 8 nm] and minimal non-specific binding. The images of the small particles used for SPFI are diffraction patterns that approximate to a two-dimensional Gaussian function. By fitting the intensity distribution in an individual fluorescent spot to a two-dimensional Gaussian function, the intensity of the spot and its position can be determined.

Determination of receptor self-association by SPFI
Analysis of spot intensities obtained in SPFI experiments provides a method for detecting self-association of receptors in living cells. The principle of the method is that monomers, dimers, trimers . . . of a receptor will bind 1, 2, 3 . . . particles provided the probe is added at saturating concentrations. The spot intensities thus have intensities that are multiples of the intensity of a single particle. A histogram of spot intensities will in principle exhibit peaks corresponding to different oligomeric states of the receptor (see Figure 1). In practice the peaks are considerably broadened even if, as is the case with phycobiliproteins, all particles have the same number of fluorophores. This is because the low-intensity images are inherently noisy, also exposure times that optimize signal-to-noise cause some random photobleaching of the probe. Consequently, histograms of spot intensities have to be deconvolved to determine the contributions from 1, 2, 3 . . . particles. To aid this analysis, the intensities of single particles are normally determined by imaging the probe bound to poly-L-lysine-coated slides.

Spots that have intensities corresponding to two or more particles occur when the particles are too close to be resolved
Figure 1 | Principle of method for detecting receptor self-association
Particles are bound at saturation to the receptors and the intensities of their images are determined. The intensity histogram will be a convolution of the intensity distributions of one or more particles depending on the oligomeric state of the receptors.

Dimerization of HLA-DR (HLA direct repeat)
We applied the SPFI approach to investigate dimerization of the MHC class II molecule, HLA-DR, in living cells [2]. MHC class II molecules play a central role in the capture and presentation of immunogenic peptides to CD4+ T-cells. HLA-DR had previously been found to crystallize as dimers [5] and SDS-stable dimers had also been detected in immunoprecipitation experiments [6]. We labelled HLA-DR on transfected fibroblasts with PhyE–Fab, imaged the cells and analysed the spot intensities. We found that at 22°C about a quarter of the spots arose from two particles. The proportion of two-particle spots decreased at 37°C. The results suggest that monomers and dimers of HLA-DR are in equilibrium in the plasma membrane of cells. The dimeric state would thus be favoured when the concentration of HLA-DR is increased, as occurs when antigen-presenting cells are infected and also locally at the immunological synapse. The issue of whether dimers are required for T-cell activation is not fully resolved, although site-directed mutations that are predicted to inhibit dimerization have been shown to inhibit activity of HLA-DR [7].

We have recently examined the effects of ‘interface peptides’ on dimerization of HLA-DR. Interface peptides are designed to bind to the sequence of amino acids occurring at a contact region between two monomers that form the dimer in the crystal structure. They can thus potentially inhibit dimerization by competing for essential binding sites. There are three contact regions between monomers in the HLA-DR crystal structure that are designated as interface regions I, II and III [5]. We have used SPFI to test interface peptides in regions I and III for their effect on HLA-DR dimerization. We find that dimerization is inhibited in both cases. An example is shown in Figure 2. These experiments confirm that at least the majority of dimers detected by SPFI are true molecular entities and not a consequence of chance proximity between monomers.

Determination of receptor co-localization by SPFI
In a further application of SPFI, we have recently developed methodology for detecting and quantifying co-localization of single molecules in intact cells. Previously, Enderle et al. [8] used near-field scanning optical microscopy to investigate co-localization of malarial parasite proteins with host skeletal proteins in infected erythrocytes. A pixel-by-pixel comparison of the images of fluorescent antibodies permitted detection of co-localization with a resolution of about 100 nm. Schutz et al. [9] imaged single fluorophores to detect ligand–receptor recognition between biotin and streptavidin in a model system. Fluorophores bound to biotin and streptavidin were co-localized within about 40 nm when biotin was bound to streptavidin immobilized on supported phospholipid membranes. More recently, Michalet et al. [10] described methodology for high-resolution distance measurements using confocal microscopy. The sample consisted of 40-nm TransFluoSpheres or 10-nm nanocrystals that were deposited on to glass coverslips in suspension and then dried.

The method of detecting co-localization by SPFI involves labelling two different receptors with different coloured (e.g. green and red) fluorescent particles. The positions of the green and red particles are determined separately by dual wavelength imaging and the images analysed for co-localization of the receptors. Where green and red particles are in close proximity, the overlap of their Gaussian profiles (termed the overlap integral) is determined (Figure 3). The overlap integral has a value of 1 when images of the red and green exactly coincide and zero when there is no overlap.

Co-localization of immunological receptors
We have used this method to investigate association of HLA-DR with CD74 on the cell surface. CD74 is the surface isoform of the invariant chain (Ii) [11]. It is well established that cytoplasmic Ii is required for the correct assembly of HLA-DR in the endoplasmic reticulum and its targeting to endocytic compartments [12,13] A segment of Ii known as CLIP (class II invariant chain peptide) occupies the peptide-binding groove of HLA-DR to prevent premature binding of endogenous peptides. In endosomes, the Ii is proteolytically degraded and CLIP is exchanged for exogenous peptides. The role of CD74 at the cell surface is, however, not well understood. We have recently examined the effects of ‘interface peptides’ on co-localization of HLA-DR and CD74 on the cell surface. In a model system, Fluorophores bound to biotin and streptavidin detected ligand–receptor recognition between biotin and streptavidin in a model system. Fluorophores bound to biotin and streptavidin were co-localized within about 40 nm when biotin was bound to streptavidin immobilized on supported phospholipid membranes. More recently, Michalet et al. [9] described methodology for high-resolution distance measurements using confocal microscopy. The sample consisted of 40-nm TransFluoSpheres or 10-nm nanocrystals.
understood. In humans, the tissue distribution of CD74 parallels the tissue distribution of MHC class II. Both receptors are expressed on antigen-presenting cells. It has been shown that CD74 is not simply residual Ii that has been carried to the cell surface bound to HLA-DR since it is translocated independently [14]. A plausible hypothesis, for which there is some evidence, is that CD74 clears MHC class II molecules with an empty peptide groove from the cell surface by inducing rapid endocytosis [14].

Evidence for an association of CD74 with the MHC class II molecule, HLA-DR, has previously been obtained from immunoprecipitation experiments [15]. To further investigate this association in intact cells, we labelled HLA-DR with PhyE–IgG and CD74 with APhyC–IgG (allophycocyanin–IgG) and performed dual-wavelength imaging to separately image the two particles. We then analysed the fluorescent spots to determine their positions and looked for coincidences of the two probes. After correcting for a displacement due to

*Figure 2* | **Inhibition of HLA-DR dimerization**
The percentage of two-particle spots (corresponding to dimers of HLA-DR) was determined in the presence and absence of peptides designed to bind to interface region III. A scrambled peptide was used as a control.

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*Figure 3* | (a) **Gaussian profiles of two overlapping spots in green and red images and (b) histogram of overlap integrals for APhyC bound to CD74 with PhyE bound to HLA-DR on transfected fibroblasts**
(a) The overlap integral ($\Phi$) is the shaded region and is defined by the separation of the two peaks ($\Delta r$) and the widths of the two profiles ($w_1$ and $w_2$ respectively). (b) Histogram of overlap integrals for APhyC bound to CD74 with PhyE bound to HLA-DR on transfected fibroblasts. High values of ($\Phi$) (> 0.8) occur rarely in random distributions and are thus indicative of co-localization of the two receptors.

\[ \Phi = \exp[-\frac{1}{2}\Delta r^2/(w_1^2 + w_2^2)] \]
chromatic aberration, we found a high level of coincidence between many APhyC and PhyE probes (Figure 3b). Because of the high spatial resolution of SPFI, it is likely that the co-localization of HLA-DR and CD74 molecules corresponds to a molecular association. An important feature of the method is that the number of co-localized spots can be quantified. The data indicate that about 25% of CD74 molecules are present in HLA-DR–CD74 complexes. In addition, quantification of the intensities of the co-localized spots as described above enabled us to deduce that both monomeric and dimeric HLA-DR molecules are associated with CD74.

As with studies of dimerization, it is necessary to consider the possibility of chance proximity of the two receptors. This was investigated both by a simulation and experimentally by imaging a mixture of APhyC and PhyE probes bound to a slide at similar densities to those found on the cells. Both approaches confirmed that high values of the overlap integral rarely occurred by chance.

We also tested the hypothesis that CD74 binds to HLA-DR molecules that have an empty peptide-binding groove. We found that incubating cells with either CLIP or a haemagglutinin peptide (HA307–319, which is known to bind tightly to the peptide-binding groove) inhibited the association of CD74 with HLA-DR. As well as supporting the hypothesis, these experiments also confirm that co-localization is due to a molecular association rather than random close proximity.

We have also used SPFI to observe co-localization of the receptor, CD14, with its ligand, LPS (lipopolysaccharide). LPS is the major component of the outer membrane of Gram-negative bacteria and triggers cells to synthesize and release inflammatory mediators including interleukins. LPS is the major component of the outer membrane of Gram-negative bacteria and triggers cells to synthesize and release inflammatory mediators including interleukins.

In vivo these mediators may provoke septic shock, involving multiple organ dysfunction, disseminated intravascular coagulation and respiratory failure. For these experiments, CD14 was labelled with an antibody coupled to PhyE while LPS was labelled on its carbohydrate moiety with Alexa 488. The antibody and the labelled LPS were bound to CHO cells expressing CD14 and Alexa and PhyE imaged separately. The antibody and the labelled LPS were bound to CHO cells expressing CD14 and Alexa and PhyE imaged separately. We have also used SPFI to observe co-localization of the receptor, CD14, with its ligand, LPS (lipopolysaccharide). LPS is the major component of the outer membrane of Gram-negative bacteria and triggers cells to synthesize and release a cascade of inflammatory mediators including interleukins 1, 6 and 12, interferon-γ and tumour necrosis factor [16,17]. In vivo these mediators may provoke septic shock, involving multiple organ dysfunction, disseminated intravascular coagulation and respiratory failure. For these experiments, CD14 was labelled with an antibody coupled to PhyE while LPS was labelled on its carbohydrate moiety with Alexa 488. The antibody and the labelled LPS were bound to CHO cells expressing CD14 and Alexa and PhyE imaged separately using dual-wavelength excitation (the antibody against CD14 does not inhibit LPS binding).

We found that the majority of the LPS–Alexa spots co-localized with CD14. We also observed many CD14 molecules that were not associated with LPS. Interestingly, the intensities of the LPS–Alexa spots indicated that they arose from small clusters of LPS molecules; the significance of this finding remains to be established. The intensities of the PhyE spots were similar to those measured when the probe was bound to a microscope slide and indicated that the CD14 molecules are largely monomeric.

These experiments illustrate how SPFI provides information that is not readily accessible by FRET or other techniques, namely (a) quantification of the proportion of molecules that are co-localized and (b) information on the number of molecules that are associated (e.g. the finding that a small cluster of LPS appear to be associated with a single CD14 molecule). In addition, SPFI may detect associations that are missed by FRET. FRET signals could be difficult to detect if only a small number of molecules are associated or because of unfavourable geometry of the donor–acceptor pairs. It should be noted, however, that whereas a FRET signal requires very close proximity between molecules (typically 0.5–5 nm), high values of the overlap integral in SPFI are detected for separations up to about 300 nm. A high overlap integral could thus result from either a molecular association or confinement of molecules within a small domain. SPFI could thus be advantageous for investigating domains in cell membranes, where FRET experiments have given conflicting results [18,19]. Probably the most informative approach would be to combine SPFI with FRET measurements. Together, they have the potential to elucidate in considerable detail the organization of molecules in cell membranes.

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