Probing the oligomeric status of G protein-coupled receptors by single-molecule fluorescence

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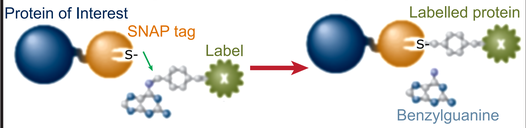
Approach

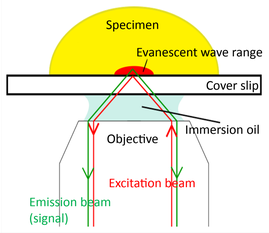
The oligomeric size of GPCRs determined using single-particle tracking is at variance with what is obtained using other techniques, including other fluorescence-based techniques such as FRET and BRET. We therefore generated constructs containing monomeric CD86, dimeric CD28 or the M_2 muscarinic receptor fused at the extracellular N-terminus with a SNAP tag, and expressed those constructs in Chinese hamster ovary (CHO) cells. The SNAP tags were labelled with the cell-impermeable dye SNAP-Surface 488 (**A**), and the cells were imaged on a total internal reflection (TIR) microscope (**B**). Time-lapse movies of fluorescent spots moving along the basal membrane of the cell were recorded and analyzed using GMimPro (Mashanov *et al.*, 2007) or MATLAB (Jaqaman *et al.*, 2008; Smith *et al.*, 2010) to obtain the locations and intensities of all single molecules in each frame (**C**). Single-molecule tracks were created by linking individual molecules in successive frames, using either the nearest-neighbour algorithm in GMimPro, or the global combinatorial optimisation algorithm in u-track. The pixel intensity histograms from raw images also were analysed using spatial intensity distribution analysis (SpIDA), which has been used previously to quantify the proportion of monomers and dimers present on the cell surface (Godin *et al.*, 2011).

A. Advantages of SNAP tags over eGFP



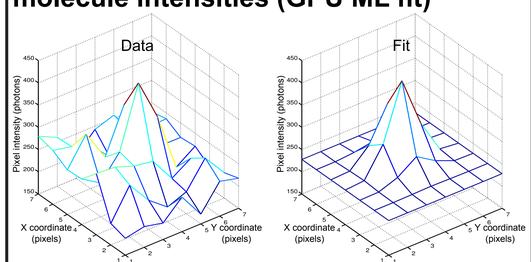
- Brighter synthetic fluorophores (*e.g.*, SNAP-Surface 488)
- Higher resistance to photobleaching
- Oxygen scavangers can be used to improve stability
- Only cell-surface labelling

B. Advantages of TIRF microscopy



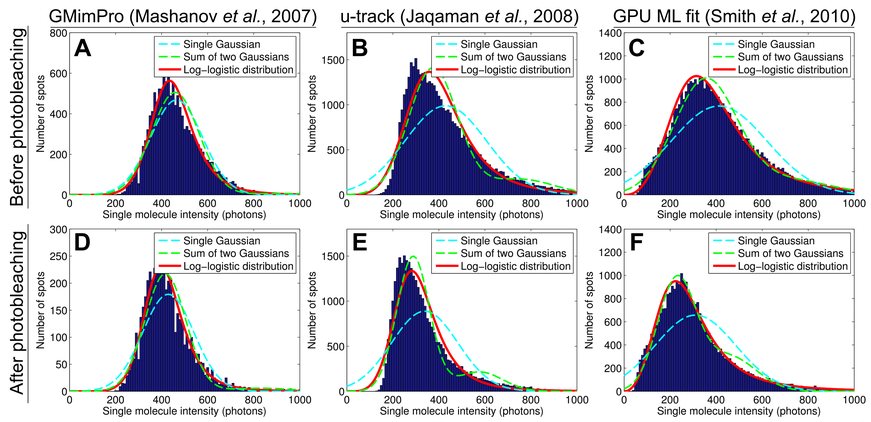
- Only ~100 nm above the coverslip is excited by the laser
- Decreased Raman and Rayleigh scattering improves signal-to-noise ratio of images
- Ultra-sensitive EM-CCD cameras allow imaging at a time resolution of 33 ms.

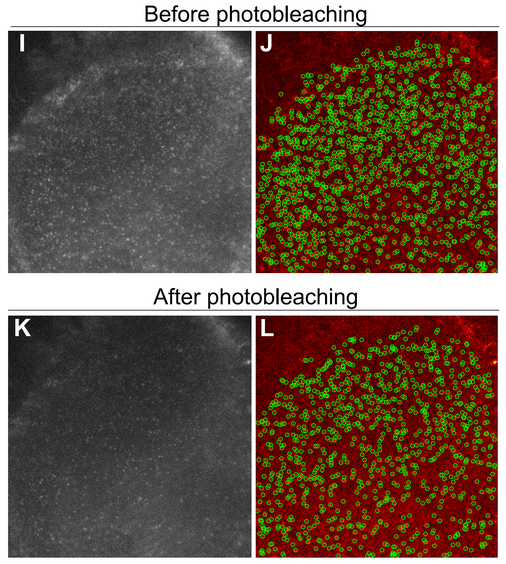
C. Maximum likelihood fitting of single molecule intensities (GPU ML fit)

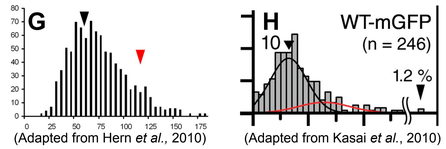


Images were scanned to locate potential single-molecule spots, defined as pixels with an amplitude higher than what would be expected to occur by chance (p = 0.05). The images were cropped around these candidate spots to produce a stack of 7 by 7 pixel squares. These squares were fit to achieve the maximum likelihood estimate of the position and intensity of single fluorophores (Smith *et al.*, 2010). The GPU implementation of the fitting algorithm could perform over 100 000 fits per second on a budget GTS 560 SE graphics card. A typical single-molecule spot and the corresponding fit are shown in the figure above.

Single-molecule intensity distributions for monomeric CD86 are not symmetric

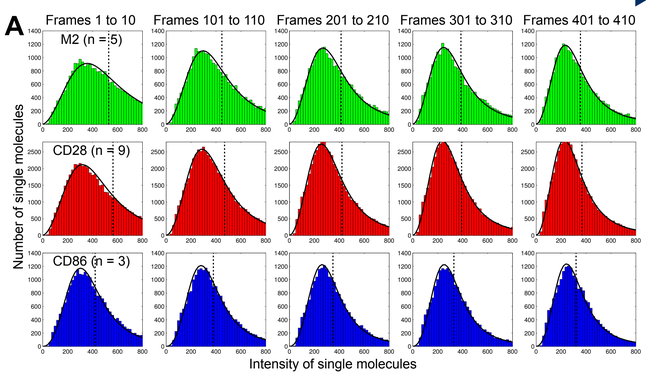




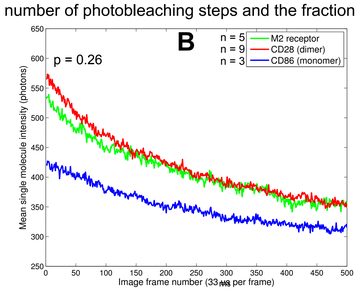


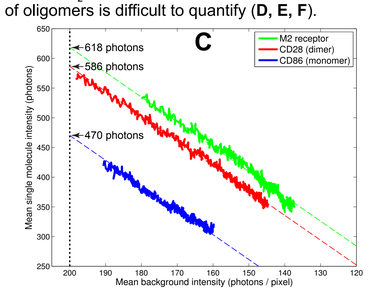
Histograms **A** to **F** show the distributions of single-molecule intensities that were collected over frames 1 to 30 (the first second of imaging, **A-C**), or over frames 1501 to 1530 (the last second of imaging, **D-F**) from a movie of a cell expressing monomeric CD86 tagged with a SNAP tag and labelled with SNAP-Surface 488. Images **I** and **K** show the 1st and 1501st frame of this movie, and images **J** and **L** show all single molecules that were found and measured in those frames using GPU ML fit (Smith *et al.*, 2010). Single-molecule intensities shown in histograms **A** and **D**, **B** and **E**, or **C** and **F** were measured by analysing the same images with GMimPro (Mashanov *et al.*, 2007), u-track (Jaqaman *et al.*, 2008), or GPU ML fit. GPU ML fit appears to be the best alogithm because it fits each potential single molecule by a 2D Gaussian rather than simply by sliding a Gaussian kernel over the image, and because it correctly assumes Poisson rather than Gaussian noise (**C in Methods**). All histograms are skewed to the right and are approximated well by a log-logistic distribution (red line), especially in the case of **C** and **F**. Similar skewed histograms have been reported previously for single molecules of the M_1 muscarinic receptor (Hern *et al.*, 2010; **G**) and the N-formyl peptide receptor (Kasai *et al.*, 2011; **H**), and they were interpreted to suggest the co-existance of monomers and dimers. These interpretations are questionable because single molecule intensities have a skewed distribution even in the case of a monomer, CD86.

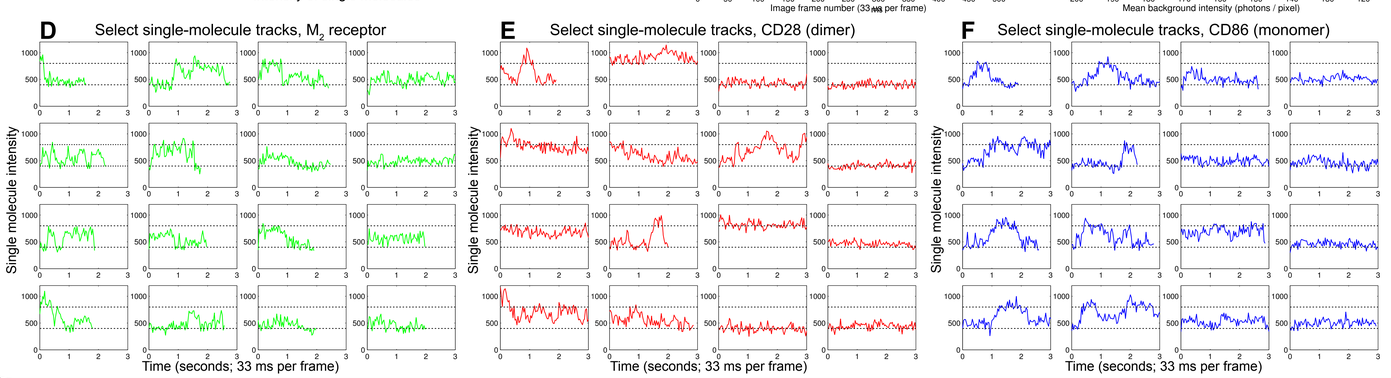
Photobleaching of single molecules



The M_2 muscarinic receptor, monomeric CD86 and dimeric CD28 produce similar single-molecule intensity distributions (**A**), and the mean of those distributions shifts left as the cells are photobleached (vertical dashed line, **A**). When plotted as a function of photobleaching time, dimeric CD28 and the M_2 muscarinic receptor have a higher mean single-molecule intensity than monomeric CD86, but this difference is not significant (p = 0.26, **B**). The lack of significance is mainly due to high cell-to-cell variation in cells expressing CD86, with one cell having a similar average single molecule intensity to what is observed for CD28. Mean single-molecule intensity (dashed line in **A**) is linearly related to the mean background intensity of the images, and both decrease as the cells are photobleached (**C**). Single-molecule intensity tracks for CD28, CD86 and the M_2 muscarinic receptor are similar, and the number of photobleaching steps and the fraction of oligomers is difficult to quantify (**D, E, F**).

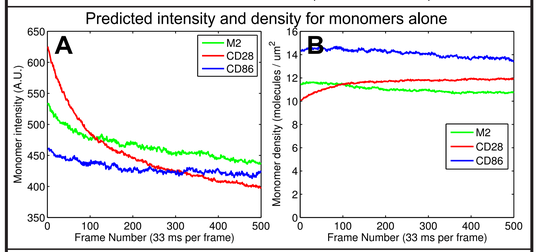


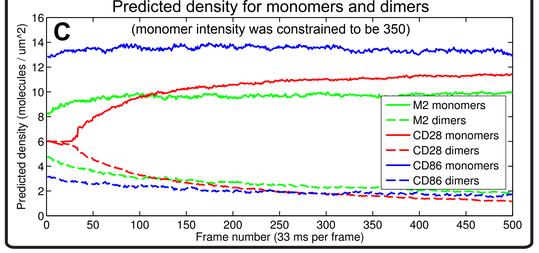




SpIDA

We also used spatial intensity distribution analysis (SpIDA; Godin *et al.*, 2011) to analyse single-pixel intensity histograms from the same images that we used for single-particle tracking. First we assumed that all cells expressing a given protein contained a single population of monomers, and we used SpIDA to estimate the monomer intensity and density that produced the best fit to the aquired histograms (**A, B**). CD86 was computed to have a lower initial single molecule intensity (**A**) and a higher density (**B**) than CD28, and the M_2 receptor was approximately half-way between these monomeric and dimeric controls (**A**, **B**). Next we assumed the co-existance of monomers and dimers, and constrained their intensities to be 350 and 700, respectively. The value of 350 is the monomer intensity calculated for photobleached cells expressing CD86. Over 50% of CD28 molecules, 40% of M_2 molecules, and 19% of CD86 molecules were found to exist as dimers, and in each case the fraction of dimers decreased and the fraction of monomers increased as the cells were photobleached (**C**). Surprisingly, even better fits to the histograms were obtained by assuming a mixture of monomers and tetramers or hexamers (data not shown).





Conclusions

- Single-molecule intensities always follow a skewed distribution that is described well by a log-logistic probability density function.
- The main factors determining the average intensity of single molecules are the average background intensity of the image and the expression level of the labelled protein.
- No significant difference was found between monomeric CD86, dimeric CD28 or the M_2 receptor with respect to the average single-molecule intensity, the number and pattern of photobleaching steps or the spatial intensity distribution (SpIDA).
- Analysing TIRF images through single-molecule tracking or through SpIDA can produce different results, pointing out the ambiguity of fluorescence intensity measurements.

References

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Acknowledgments

Heart and Stroke Foundation of Ontario

Canadian Institutes of Health Research

Ontario Graduate Scholarship