Single molecule studies of clathrin-coated vesicle disassembly

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Introduction

Using single molecule confocal ALEX microscopy[1] at the CLF's Octopus facility[2] we aim to monitor clathrin disassembly at the single molecule level using fluorescently labelled components of the clathrin disassembly apparatus: Hsc70, auxilin and clathrin[3]. In a previous experiment we carried out proof of principle single pair FRET analysis of clathrin cage complexes to detect intramolecular association events relevant to clathrin cage disassembly. As a result of the success of this pilot study we wished to extend our studies to address the larger questions surrounding the individual events which occur when individual clathrin triskelions dissociate, the mechanism by which Hsc70 is recruited to clathrin-auxilin cages and the events which preceed cage collapse.

Clathrin cage measurements

We attempted to monitor the disassembly of Clathrin cages via loss of FRET signal from separately donor and acceptor labelled triskelia that had been assembled into donor-acceptor cages. Figure 1 illustrates our ability to create donor-acceptor Clathrin cages that are FRET positive or negative. Figure 1a shows the typical signals acquired for separately donor and acceptor labelled triskelia which when polymerised into cages together exhibit FRET, Figure 1b shows the contrasting scenario where separate donor and acceptor labelled cages are mixed and may enter the confocal volume coincidentally but not exhibit any interaction giving rise to no FRET signal.
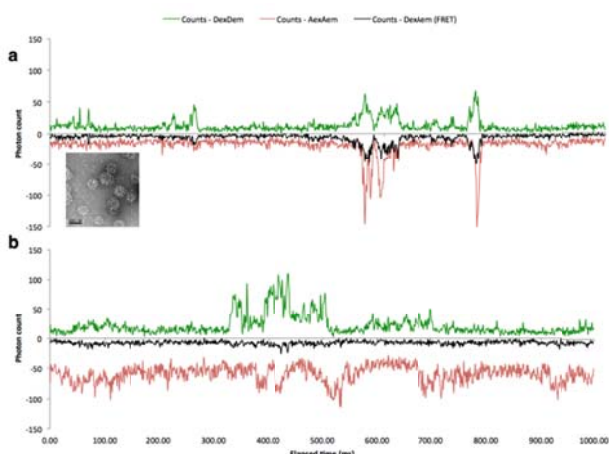

Figure 1: (a) Co-assembled donor (Alexafluor 546) and acceptor (Alexa-fluor 647) labelled triskelia giving rise to FRET positive cages, (b) independently assembled donor and acceptor labelled cages mixed exhibiting concurrent occupation of the confocal volume but not interaction as indicated by no FRET on the $D_{ex}A_{em}$ channel.

In running these control experiments we observed that signals from fluorescently labelled clathrin cages are significantly different to the single molecule bursts from site-specific labelled monomeric proteins. Targetting lysines on clathrin for labelling we reliably achieve ~6-9 fluorophores per clathrin triskelia (consisting of 3 clathrin heavy chain monomers), in the barrel conformation a Clathrin cage may have up to 324 fluorophores (assuming 36 triskelia). Figure 2 illustrates the dynamic range of bursts typically obtained for donor-acceptor labelled Clathrin cages, where signals are observed to vary over two orders of magnitude and also have variable burst profiles and lengths.
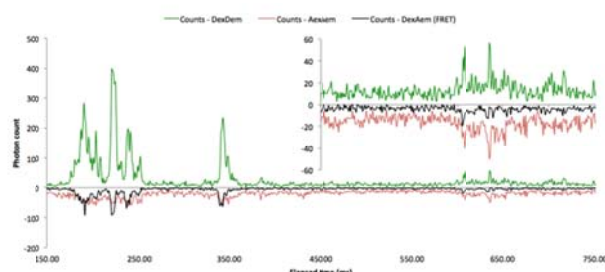

Figure 2: Typically observed $D_{ex}D_{em}$, $A_{exc}A_{em}$ and $D_{exc}A_{em}$ bursts observed for donor-acceptor labelled cages with $D_{exc}A_{em}$ signals indicating FRET. The insert illustrates the dynamic range over which fluorescent bursts are observed.

We have collected data on fluorescent events from donor-acceptor labelled cages over time to test their stability and also their disassembly when exchanged into buffers that promote disassembly or enzymatically by the addition of the adaptor proteins hsc70 and auxilin. Before analysing the data from these experiments we sought to assess the current analysis softwares ability to identify Clathrin cage bursts.

Figure 3 shows the identified bursts when using previously established burst searching parameters (Oxford – 7 nearest neighbours, 500 µs arrival threshold, RAL – 3 nearest neighbours, 1000 µs arrival threshold) and a range of parameters in between. Using the Oxford burst searching parameters a Clathrin burst is identified as multiple bursts and in contrast using the RAL parameters the whole Clathrin burst is identified, as well as noise. Our intention was to characterise the degree of disassembly by measuring the change in the number of fluorescent bursts from Clathrin cages. Upon inspection of the identified bursts it appears that burst searching parameters of 7 nearest neighbours and 1000 µs arrival threshold successfully identifies the start and finish of a Clathrin burst. However as shown in Figure 2 these same parameters identify multiple bursts in the instrument noise.
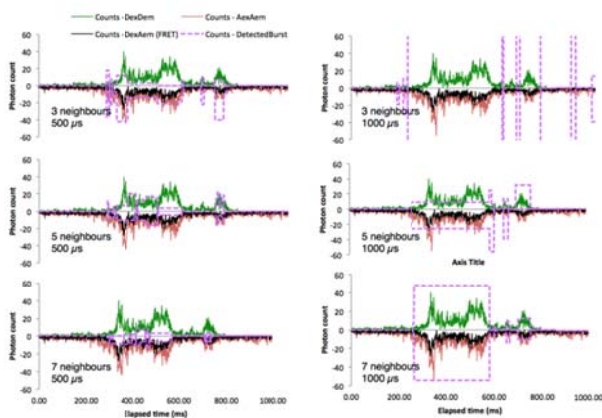
Figure 3: Burst searching parameters varied and resulting identification of burst size of FRET positive donor-acceptor Clathrin cages.
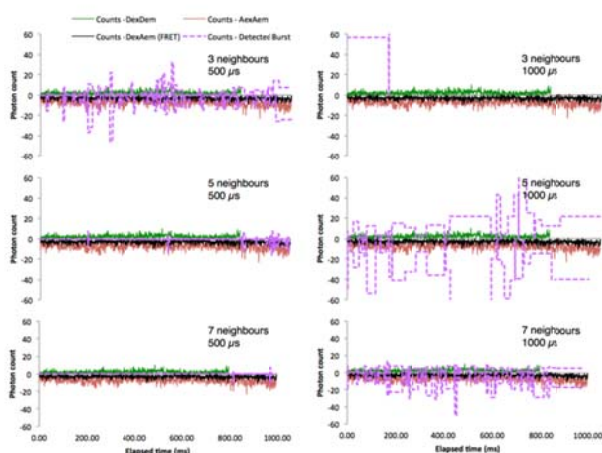

Figure 4: Burst searching parameters varied and resulting artefact burst identification.

After the burst matrix is produced using the nearest neighbours and arrival threshold values the burst matrix is thresholded to remove incorrectly identified bursts. The threshold values for Gexc and AA are 10 & 40 respectively for Figure 4. Figure 5 illustrates the effect on identified bursts when significantly larger thresholds (Gexc/AA = 200/200) are applied to the 7 nearest neighbours, 500 µs burst matrix. It is apparent that bursts are still identified in the instrument noise.
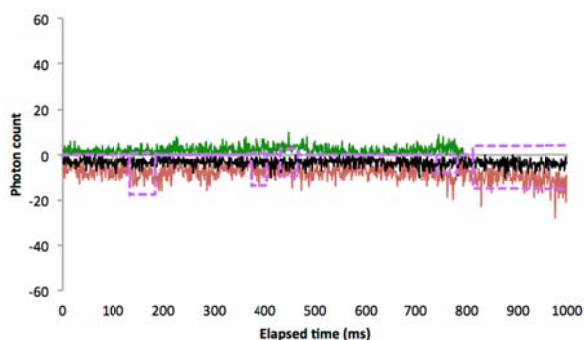

Figure 5: Stringent lower Gexc and AA thresholds still identify bursts in noise with 7/1000 burst searching.

Single molecule Hsc70 and Auxilin adaptor measurements

We collected data on monomeric site-specifically labelled Auxilin and Hsc70 adaptor proteins with the aim to monitor their interaction over the course of disassembly. Figure 6 shows the data typically collected for single molecule labelled DNA

oligomers (6a) compared with single molecule labelled Auxilin and Hsc70 (6b). We note the identification of bursts in noise for our collected data but further investigation is required to establish whether this is an analysis issue or a problem with the quality of the data.
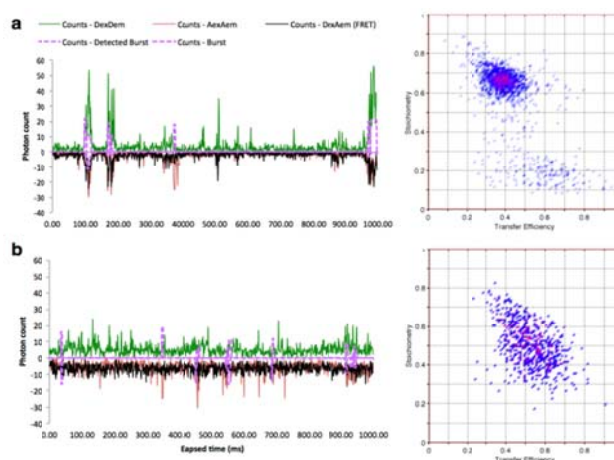

Figure 6: (a) Oxford collected data on labelled DNA oligomers (Cy3B-ATTO647) with corresponding burst searching and ES plot compared with (b) RAL collected data on single molecule labelled Aux-835 (Alexa546) and Hsc70-647 (Alexa647) witgh corresponding burst searching and ES plot. APBS, 7 nearest neighbours, 500µs arrival threshold.

In the sample shown in Figure 6b we would expect there to be no interaction between Hsc70–Auxilin, thus a negligible FRET signal and so in the same experimental set up sought to establish what the donor only and acceptor only populations for these proteins would look like. Figure 7 shows the raw data and corresponding ES plots for donor and acceptor only experiments of these labelled proteins. The donor only experiment reports a FRET efficiency of ~35%, highlighting a background $D_{exc}A_{em}$ fluorescence from the microscope leading to a spurious FRET baseline.
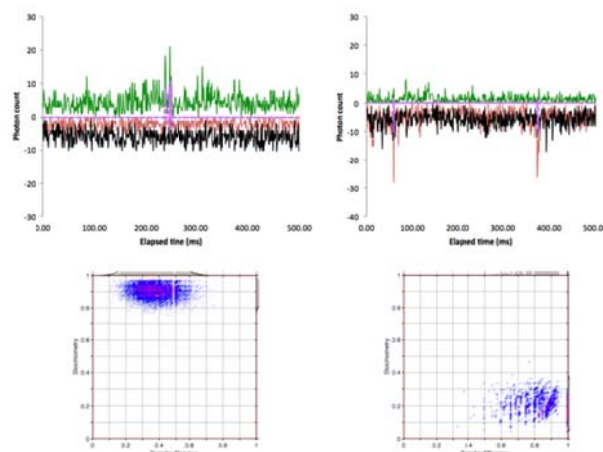

Figure 7: Donor-Auxilin only (left) and Acceptor-Hsc70 only (right).

Single molecule Hsc70 and Auxilin adaptor to labelled Clathrin preliminary measurements

Having established experimental parameters that appeared suitable for detecting single molecules of Hsc70 or auxilin, given the previously mentioned caveats, we sought to investigate how an experiment with donor labelled adaptor protein and acceptor labelled Clathrin would be expected to behave. The Clathrin cage bursts shown in Figures 1-3 use

relatively low laser powers (Donor 3.30 µW, Acceptor 4.00 µW) to cope with the intense fluorescent bursts from the multimeric multilabelled assembled structures. In contrast to record single molecule bursts from single labelled monomeric Auxilin or Hsc70, as shown in Figure 6b, higher laser powers are employed (Donor 119 µW, Acceptor 299 µW). For detecting single molecule donor labelled adaptor events with assembled acceptor labelled cages we explored using the high donor laser power for the adaptor with the low laser power for the acceptor (Donor 119 µW, Acceptor 4.00 µW). Figure 8 illustrates that in this experimental set up the donor laser is able to directly excite the acceptor fluorophore producing a false positive FRET value and also affecting the apparent stoichiometry in the produced ES plots. As previously described the burst searching on Clathrin data appears to be artifactual but here additionally we show that FRET may not be an appropriate metric for measuring the interaction between donor labelled adaptor and acceptor labelled clathrin cages. We instead propose that different metrics such as burst length and intensity may better indicate when single molecule adaptor proteins bind to Clathrin cages.
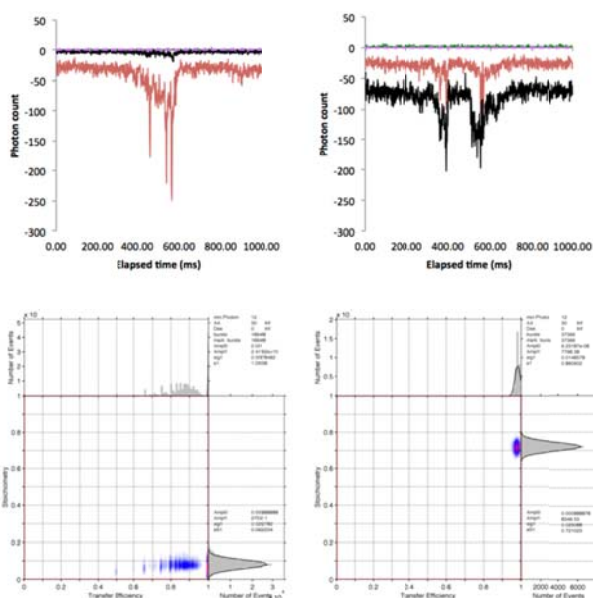
need to validate whether the burst searching is operating correctly for our single molecule data and whether development is required in the analysis methods or improvements made to the quality of our data. Further, the current analysis methods have not yet been able to reliably identify bursts from labelled Clathrin such that we may characterise number of FRET bursts per time point of the experiment. It may be more appropriate to analyse the data in new ways, being able to reliably and automatically quantify burst length, size and frequency may provide new metrics with which we can assess the assembly state of our labelled Clathrin cages.

References

1) Nam Ki Lee, Achillefs N. Kapanidis, You Wang, Xavier Michalet, Jayanta Mukhopadhyay, Richard H. Ebright, and Shimon Weiss (2005). "Accurate FRET Measurements within Single Diffusing Biomolecules Using Alternating-Laser Excitation". Biophys. J. **88**: 2939-2953. doi: **10.1529/biophysj.104.054114**.

2) Clarke DT, Botchway SW, Coles BC, Needham SR, Roberts SK, et al. (2011) Optics clustered to output unique solutions: a multi-laser facility for combined single molecule and ensemble microscopy. Rev. Sci. Inst. **82**: 093705.

3) Smith, CJ, Rothnie A, Clarke AR, Kuzmic P, Cameron A (2011) 'A sequential mechanism for clathrin cage disassembly by 70-kDa heat-shock cognate protein (Hsc70) and auxilin ' Proceedings Of The National Academy Of Sciences (USA) **108** (17), 6927 – 6932.

Figure 8: Donor laser direct excitation of acceptor fluorophore labelled Clathrin using Donor 119 µW Acceptor 4.00 µW laser powers (right).

Conclusions

We have performed the four experiments outlined in our original experimental proposal:

i) Clathrin cages composed of ALEXA FLUOR-647 and ALEXA FLUOR-546 labelled clathrin triskelions in the presence of unlabelled Hsc70 and auxilin with 1mM ATP.

ii) ALEXA FLUOR-546-labelled Hsc70 and ALEXA FLUOR-647–labelled clathrin cages in the presence of unlabelled auxilin with 1mM ATP

iii) ALEXA FLUOR-647-labelled auxilin and ALEXA FLUOR-546-labelled clathrin cages in the presence of unlabelled Hsc70 with 1mM ATP.

iv) ALEXA FLUOR-546-labelled Hsc70 and ALEXA FLUOR-647-labelled auxilin in the presence of unlabelled clathrin cages with 1mM ATP.

In light of the highlighted experimental data outlined here further work is needed to optimize the analysis. In particular we