Detection of platelet signalling using FRET biosensors and time-lapse fluorescence microscopy

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Introduction
Atherosclerosis is a major chronic disease that leads to life threatening conditions such as myocardial infarction and stroke. Atherosclerotic plaques break and their contents induce thrombus formation which results in stenosis. The mechanisms involved in thrombus formation are key to developing effective therapy, but are poorly understood. Here we use the FRET technique to quantitatively investigate platelet signalling during thrombus formation in transgenic mice.

Materials and Methods
Six subjects expressing ERK and PKA FRET biosensors, CFP and YFP fluorophores (FP), were prepared by transposon-mediated gene transfer method. The donor (CFP) and acceptor (YFP) FP s were joined with an optimised flexible Eevee linker (116 a.a.) ensuring a 1:1 FP concentration. Thrombi were induced by laser ablation of the endothelial and time lapse fluoroscopic images were captured using: Olympus IX81/FV1000 inverted microscope with built in PMT; oil immersed Olympus UPLSAPO 30xS objective lens; Spectra Physics Mai Tai DeepSee HP Ti:sapphire laser. This resulted in tiff image stacks of 512 × 512, 118 × 118 nm pixels, 2010 frames (1 frame per 1.1 secs) for each subject. To assess the detectability of FPs and FRET the SNR was determined for all subjects using a thrombus (signal) and a background (noise) 3D ROIs of equal size selected for smooth signal transition. The mean (std) number of ROI pixels per frame were 440 (143) and the number of frames were 116 (62). FRET was confirmed by ratiometric analysis on the six signal ROIs over all frames. The CFP and YFP SNR was estimated from the mean difference of signal and background intensities divided by the signal stdev. Analogously, novel FRET SNR measures were derived using the mean difference of donor and acceptor signals. To interpret temporal changes in the FRET signal a quantitative model based on uniform motion and the Förster energy transfer efficiency equations was determined.

Results
Ratiometric analysis identified several episodes of significant increase in FRET signal for each of the six subjects. The mean (std) SNR for YFP and CFP were 3.1 (0.7) and 2.4 (0.8) respectively. The corresponding FRET SNR was 1.6 (0.4). Our modelling indicated that changes in the CFP and YFP signal in the thrombus ROI over time were consistent with both increases in FP concentration as well as increases in the number of FP pairs close to the Förster distance which corresponds to signalling in both ERK and PKA pathways.

Conclusions
FP SNRs of 2.4 and 3.1, are below the detectability limits of many state-of-the art FP detection algorithms suggesting that accurate detection of FPs, and FRET even more so, may not be possible at the native resolution. The study demonstrated the considerable challenge of accurate automatic quantification of cell signalling processes \textit{in-vivo} at high resolution.