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Tissue Factor Oligomerization in Living Cells Using Förster Resonance Energy Transfer

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Tissue Factor (TF) is a transmembrane protein that is the main initiator of blood coagulation. The mechanism by which the complex of TF with activated coagulation factor VII (TF-FVIIa) activates factor X (FX) to FXa, ultimately leading to production of thrombin and fibrin clot formation, is well established [1]. However the regulation of TF procoagulant activity remains unclear. TF exists in two possible states: the cryptic form, in which TF is non-coagulant, and the decrypted form, in which TF is procoagulant [2]. Two mechanisms of activation have been proposed. The self-association hypothesis states that TF oligomerization is responsible for keeping TF in a cryptic state, and that oligomer dissociation allows the molecules to initiate coagulation [3]. In support of this hypothesis, oligomerization of TF has been observed in some cell types by chemical cross-linking assays that show the majority of TF molecules exist in dimers [4,5]. The competing allosteric disulfide bond hypothesis states that the redox state of TF is responsible for an activating conformational change [1]. No definitive evidence for either hypothesis is currently available. In this study, we have evaluated the TF self-association hypothesis in living cells.

We utilized Förster resonance energy transfer (FRET) using a spectrally resolved two-photon microscope [6] to examine the oligomerization of TF in stably transfected cells. FRET can detect molecular interactions of biomolecules when donor and acceptor fluorophores are less than 10 nm apart. We have engineered TF with monomeric turquoise (mTq) and monomeric super yellow fluorescent protein 2 (SYFP2) fusion proteins at the C-terminus (Figure 1) and transfected the TF-negative 300.19 murine pre-B cell line as our model. The fluorescent molecules mTq and SYFP2 were used as a donor and an acceptor, respectively.

Wet mounts of the cells were created and imaged at room temperature on a Nikon two photon microscope using a 100X oil immersion lens. The system contains a Ti:Sapphire laser for excitation, is spectrally resolved, and is equipped with an electron multiplying charged coupled device camera with single pixel resolution [7]. Software written in house using the programing environment Matlab was used to analyze data by generating elementary spectra of donors and acceptors and perform spectral unmixing at image-pixel level. Regions of interest were then chosen around the cell membrane to examine FRET efficiency for each pixel within the region of interest. Histograms showing the distribution of FRET efficiency across pixels within a segment were created, and the dominant peaks in these histograms were then assembled into meta-histograms to determine the dominant quaternary structure of the oligomers [8].

Our preliminary results indicate that TF does self-associate, as indicated by the presence of FRET signal all around the plasma membrane (Figure 2). The most likely structure is that of a dimer, as indicated by the presence of a prominent peak in the distribution of FRET efficiencies over several cells (data not shown). This gives us insight for our subsequent experiments using cell activating agents with lipopolysaccharide and phorbol myristate acetate. If the self-association hypothesis is correct, we expect to see a decrease in FRET upon cell activation.

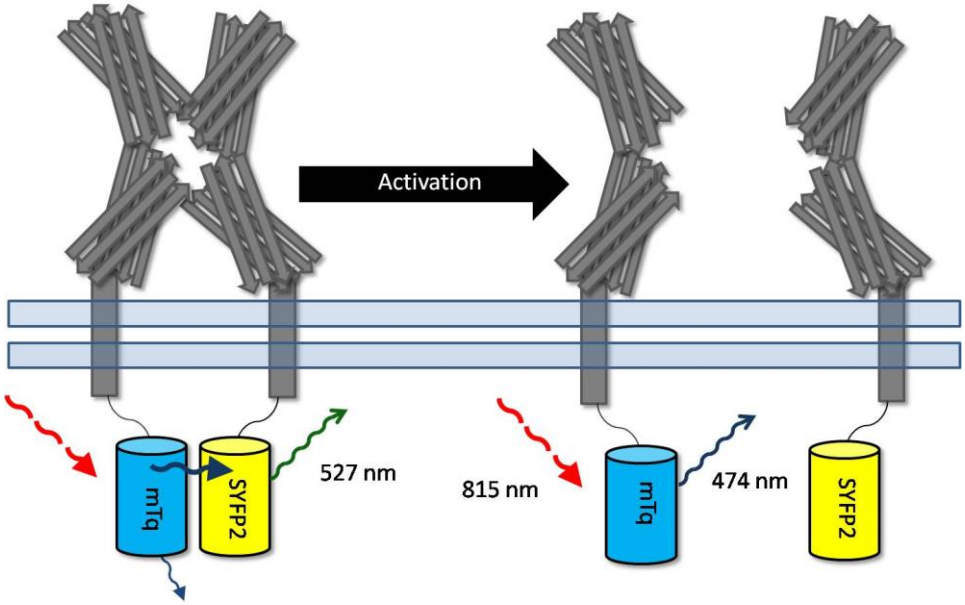

Figure 1. TF fusion proteins tagged with mTq and, separately, SYFP2. When the molecules are oligomerized, and therefore in a cryptic state, energy will be transferred from the donor to the acceptor fluorophore and FRET signal will be observed as emission from the acceptor fluorophore. When the molecules are dissociated due to cell activation, energy from the donor will be lost as fluorescence and TF will be able to initiate coagulation.

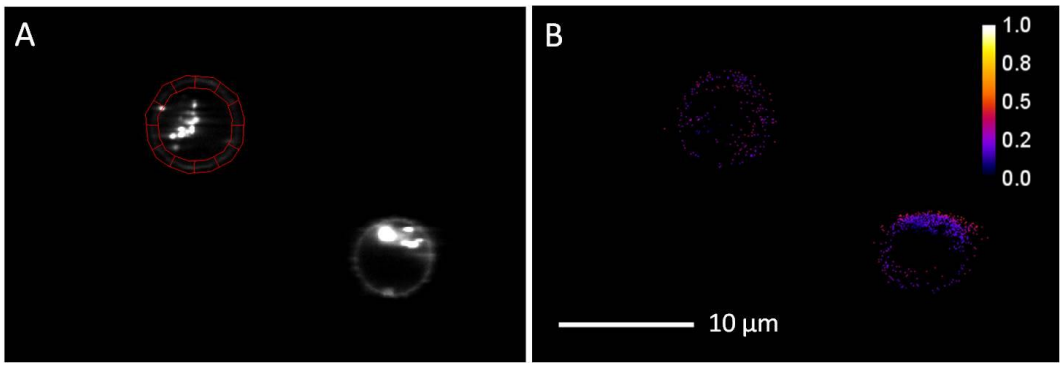

Figure 2. Representative images of cells expressing FRET efficiency. A. Image of donor molecules at 815 nm excitation with regions of interest selected around the cell membrane in the upper right. B. FRET efficiency map.

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