CH-413 Nanobiotechnology
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Problem Set 1
Fluorescence/FRET/TIRF

Exercise 1
'A typical tissue in the human body may contain many thousands of molecules, so there is not a need to perform single-molecule biology experiments to understand tissue behaviour.' Write a short paragraph discussing this statement.

Exercise 2
A single-molecule confocal microscopy experiment is performed where the focused laser volume had a lateral width measured at 270 nm and an axial width measured at 650 nm. An *E. coli* bacterial cell in the sample was measured as being 2.5 μm long and 0.9 μm wide, and had a shape approximated by a cylinder capped at either end by a hemisphere.

(a) What proportion of the cell could the focused laser excite in principle?
(b) If the focused laser beam is occupied by a single GFP-tagged protein in the cytoplasm of the cell for ~55% of the time, what is the molarity of the protein being examined?
(c) The laser beam was focused on the mid-point of the cell in the cytoplasm; from measuring the width of fluorescence pulses from a few fluorescently tagged molecules, the time taken to traverse the confocal spot in the sample plane was estimated at 0.5 ms. Assuming the cell is at 25 °C, and that the diffusion coefficient D can be approximated from Stokes law as $\frac{k_B T}{6\pi\eta r}$ where the cytoplasm viscosity (η) is ~0.001 Pa s and r is the effective radius of the GFP-tagged molecule, estimate the effective diameter of the protein. You may use the relationship $D = \frac{l^2}{2t}$, where l is the length travelled and t is the time elapsed.

Exercise 3
Suppose that a protein is genetically fused with two fluorescent proteins, CFP and YFP, at its two terminals. When activated, the protein will switch from bend confirmation to extended confirmation. This system is excited by a 436-nm laser. Two photomultiplier tubes (PMTs) are recording the CFP channel (488 nm) and the YFP channel (528 nm). The efficiency of the energy transfer is expressed as $E = \frac{I_{528}}{I_{488}+I_{528}}$. Most of the time the value of E is 0.7, but there are short periods of time when it drops to 0.1. If the Förster distance (R_0) for CFP and YFP is about 5 nm, estimate how far this protein is extended when activated.

Exercise 4

A protein is labelled with a donor and acceptor fluorophore to study a conformational change from state I to II using FRET. The FRET acceptor–donor pair has a known Förster radius of 5.5 nm, and the measured fluorescence lifetimes of the isolated donor and the acceptor fluorophores are 4.9 ns and 1.1 ns, respectively.

(a) Show that $E_{FRET} = 1 - \tau_{DA}/\tau_D$ where τ_{DA} and τ_D are the fluorescence lifetimes of the donor in the presence and absence of acceptor respectively.
(b) What is the distance between the donor and acceptor if the measured donor lifetime in conformation I is 50 ps? Previous structural data suggest that the fluorophore separation may change in a distinct step by ca. 0.9 nm when the protein makes a transition between conformations I and II.
(c) What is the associated donor fluorescence lifetime of conformation II?
(d) Using the same donor and acceptor, what could be done to measure this step change more clearly, and what is the maximum possible change in FRET efficiency that could be measured here?

Exercise 5

(a) Describe briefly the technique of total internal reflection fluorescence (TIRF) microscopy and give an example of its application in modern single-molecule cellular biophysics.
(b) Most TIRF live-cell applications tend to investigate membrane complexes, why is this?
(c) Would TIRF still be effective if there was a high concentration of autofluorescent proteins in the cytoplasm?

Exercise 6

Find a peer-reviewed research paper that investigates a scientific question using a single-molecule fluorescence microscopy experiment. Answer the following questions based on the study:

(a) What is the central research question?
(b) Why do the authors use a single-molecule approach instead of a bulk/ensemble experiment?
(c) How is the molecule of interest fluorescently labeled? What fluorophores are used, and where are they attached (e.g., site-specific labeling, FRET pairs, chemical modifications, or genetic tags)?
(d) What experimental techniques and microscopy setup are used? Specify the type of single-molecule fluorescence microscopy employed (e.g., TIRFM, confocal, smFRET).
(e) What type of data do they collect? Describe the single-molecule data obtained (e.g., fluorescence intensity traces, FRET efficiency distributions, diffusion trajectories, photon burst analysis).
(f) What are the key findings from the single-molecule experiment? How does single-molecule fluorescence contribute to answering the research question?