PRACTICAL MANUAL FOR FLUORESCENCE MICROSCOPY TECHNIQUES

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Foreword

Fluorescence describes the emission of photons upon excitation of a molecule by light or other electromagnetic radiation with certain well defined characteristics. These include: (i) the wavelength of excitation and emission being coupled, (ii) the process being time dependent in the region of ns, and (iii) photon emission being localized to within nm of the fluorescent molecule and influenced by its environment. Fluorescence has a long history dating back to the 1850s when George Stokes first analyzed the process using quinine and a prism in his treatise "On the Change of Refrangibility of Light". It was Albert Coons (1941) that linked fluorescein isothiocyanate (FITC) to antibodies and Gregorio Weber (1952) who developed dansyl chloride labeling of proteins that brought fluorescence into biology. Then in 1962, Osamu Shimoura and colleagues discovered the green fluorescent protein (GFP) of the jellyfish Aequorea victoria. GFP was cloned in 1992 by Douglas Prasher and expressed as an active fluorescent protein in E. coli and C. elegans by Martin Chalfie in 1994. Subsequent work by many people has developed GFP through side directed mutagenesis to optimize and diversify its uses. The use of GFP and its variants as genetically encoded fluorescent molecules has been key to using fluorescence to unravel the role of proteins in cell and molecular biology. Over the last few decades, these fundamental characteristics of fluorescence and the biology of "GFP" have been ingeniously used to probe protein dynamics, protein localisation and protein structure. In parallel to the development of fluorescent labeling techniques there has been significant developments in instruments to measure fluorescence inside biological specimens. These developments have included wide-field and confocal microscopy, two photon microscopy and most recently light sheet microscopy. In addition there has been significant improvements in lasers, detectors and software.

This practical manual has arisen through many courses and workshops that were run in Singapore between 2001 and 2016. The focus for this manual are the so-called F-techniques: FRET (fluorescence resonance energy transfer), FLIM (fluorescence lifetime imaging microscopy), FCS (fluorescence correlation spectroscopy) and FRAP (fluorescence recovery after photobleaching), and their use to probe protein structure and function. It is not our purpose here to describe the theory of the F-techniques in depth or all the discoveries that have been made with them. There are many excellent reviews and scientific papers that cover these topics. In this practical manual we seek to help scientists to implement and utilize the F-techniques. We also hope the manual will serve as a resource for anyone interested in the F-techniques. Here, we focus on the core F-techniques, but recognize the development of many interesting variants that are in current use, such as fluorescence cross correlation spectroscopy (FCCS).

FRET is the method of choice to measure protein-protein interaction in a cellular context. FRET is the process of energy transfer between two fluorescent molecules in close proximity (1-10 nm) to each other. If certain conditions are met FRET can be used as a molecular ruler. In Chapter 1 the relatively simple indirect technique, Acceptor Photobleaching-FRET (AP-FRET), to measure FRET is described. AP-FRET utilizes laser induced bleaching of an acceptor in a region of interest (ROI) and monitoring changes in fluorescence intensity of the donor molecule in the same ROI. These measurements are made in fixed cells. The major advantage of AP-FRET is its simplicity allowing wide application. Chapter 2 describes Sensitised Emission-FRET (SE-FRET), a ratiometric method for measuring FRET. In SE-FRET changes in fluorophore spectra are used to monitor FRET. SE-FRET has utility for rapid events occurring in live cells. Chapters 3 and 4 describe methods to measure the lifetimes of fluorophores, in the frequency domain (FD-FLIM) and the time domain (TD-FLIM). FD-FLIM is an indirect method for measuring fluorescent lifetimes and has utility for rapid measurements in live cells. In contrast, TD-FLIM is a direct method that relies on measuring the time between excitation and photon release. Although more time consuming than FD-FLIM, TD-FLIM gives higher resolution of fluorescent lifetimes. TD-FLIM can be used to measure fluorescent lifetimes at subcellular resolution. Both FD-FLIM and TD-FLIM can be used to measure FRET. With TD-FLIM being the "gold standard" for estimating quantitatively whether there is FRET occurring between two fluorophores. In addition, TD-FLIM allows the proportion of interacting molecules to be measured. Chapter 5 introduces FCS, a powerful technique for measuring the movement of molecules within a defined (confocal) volume. Using mathematical analysis of FCS data two parameters can be estimated: protein diffusion rates and protein concentration. FCS has applications to follow protein association and dissociation (affinity constants) and protein complex formation.

Thus, FCS and the FRET methods described above can be used in parallel to measure protein-protein interaction. Lastly, Chapter 6 describes FRAP, a technique which measures protein diffusion by bleaching fluorophores in a ROI and then looking for recovery of fluorescence in the same ROI. FRAP is a semi-quantitative method that can be used in parallel with FCS to examine protein diffusion. Both FRAP and FCS are carried out on live cells. In conclusion, it is important to realize that the F-techniques are a powerful set of techniques that can be used to interrogate protein behavior in cells and can be used together by complimenting and/or validating each other. The F-techniques are not restricted to following protein behaviour and can also be used to follow DNA/RNA, as well as protein-DNA and protein-RNA interactions (protein-ligand interactions). This practical manual serves to promote and facilitate the use of F-techniques together by describing the implementation of the core techniques in a single resource.

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