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BRAIN RESEARCH

Review

Studying inner ear protein–protein interactions using FRET and FLIM

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ABSTRACT

Molecular genetic studies of the inner ear have recently revealed a large number of previously undescribed proteins, but their functions remain unclear. Optical methods such as FRET and FLIM are just beginning to be applied to the study of functional interactions between novel inner ear proteins. This review discusses the various methods for employing FRET and FLIM in protein–protein interaction studies, their advantages and pitfalls, with examples drawn from inner ear studies.

1. Introduction

Concerted attention to mouse mutations with hearing phenotypes, combined with an energetic pursuit of inherited hearing loss loci in man, has brought us spectacular growth in the number of protein molecules found to be important in mammalian hearing (Di Palma et al., 2001; Friedman and Griffith, 2003; Steel and Kros, 2001; Zuo, 2002). A remarkable number of these proteins have been localized to hair cell stereocilia (Adato et al., 2005; Gillespie and Cyr, 2004). In retrospect, this abundance should not be surprising—upwards of 350 different proteins are estimated to be present in motile cilia (Pazour et al., 2005). There is no reason to believe that hair cell stereocilia are any less complex than motile cilia. Examples of protein pairs for which putative functional interactions exist include: between harmonin and cadherin23 in stereocilia (Boeda et al., 2002), between TRP channel subunits, between TRP channels and the proposed tip link protein cadherin 43 (Corey et al., 2004; Gillespie et al., 2005), and between spectrin and actin in the outer hair cell lateral wall (Holley and Ashmore, 1990). However, even as the pace of discovery quickens, demonstration of functional interactions between newly discovered proteins – an essential step in understanding structure–function relationships – has lagged.

Traditional approaches to studying protein–protein interactions such as Western-based pull-down assays or dual-label antibody labeling have severe limitations. Westerns are inherently denaturing and therefore may miss functionally important interactions, such as non-covalent quaternary structures. Co-localization by fluorescently coupled antibo-

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dies is diffraction-limited to 200 nm at best (in practice, somewhat more), which is large compared to the dimensions of proteins (1–3 nm). Immuno-electron microscopic co-localization greatly improves spatial resolution. However, the technique is difficult at best and impossible in cases in which epitopes are lost in the embedding process. Thus, there is a need for a method that can demonstrate co-localization of protein species on a nanometer scale, preferably in living cells. It would be even better if the technique could be used to study dynamic interactions.

Optical techniques relying on the property of fluorescence resonance energy transfer (FRET) between closely spaced fluorescent molecules are becoming more widely used for co-localization and functional interaction studies (Gordon et al., 1998). In this review, we survey the various approaches to FRET and show some results of an application of FRET to a specific problem in the auditory system. We here suggest that these merging methodologies have the potential to fulfill the above requirements, indeed are already beginning to do so in other systems. Advantages and disadvantage of the various approaches are incorporated. The application of non-linear microscopy techniques such as multiphoton excitation is highlighted. For more information, the reader is referred to an up-to-date sampling of the many reviews of this rapidly emerging field (Chen et al., 2003; Gryczynski et al., 2005; Peter and Ameer-Beg, 2004; van Munster and Gadella, 2005; Wallrabe and Periasamy, 2005).

2. FRET

2.1. *Introduction to FRET*

If the emission band of one fluorophore, called the donor, overlaps with the absorbance band of another fluorophore, the acceptor, and if they are sufficiently close to each other, non-radiative transmission of vibrational energy takes place from donor to acceptor when the donor is excited. Thus excitation of the donor alone will result in emission of a photon by the acceptor at the acceptor's wavelength (Fig. 1A). The enhanced emission of the acceptor after donor excitation is called FRET, and occurs only if the fluorophores are within 10 nm of each other. Thus, the detection of FRET is a strong indicator of the nanometer-range proximity of the two fluorophores, and thereby of the molecules to which they may be attached.

Donor–acceptor pairs that reliably exhibit FRET include FITC-TRITC, Cy3–Cy5, and Alexa Fluor 488-Alexa Fluor 555 (Chew et al., 2005). Antibodies coupled to fluorophores such as these have been used to observe FRET in several systems (Lichlyter et al., 2003; Maurel et al., 2004; Nagy et al., 2002). However, it is highly desirable, especially in living cells, to have fluorescent labels directly attached to the proteins in question. Modified versions of GFP (green fluorescent protein) family proteins have seen extensive recent use in FRET studies (Pollok and Heim, 1999). A popular fluorescent protein FRET pair is CFP–YFP, in which CFP (cyan fluorescent protein), which emits in the blue, interacts with YFP (yellow fluorescent protein), which emits in the yellow (Heim, 1999; Heim and Tsien, 1996). Two new variants of these popular

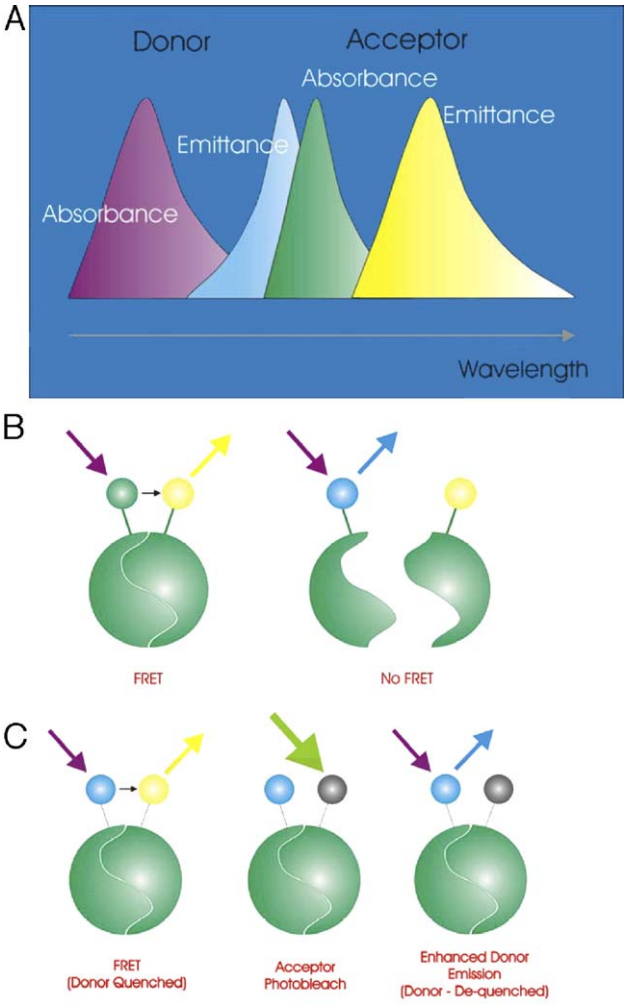

Fig. 1 – (A) Diagram of absorbance and emission bands for fluorophores that exhibit FRET, illustrating the overlap between the donor emission band and the acceptor absorbance band. (B) Diagram illustrating the sensitized emission FRET procedure. (C) Diagram illustrating the acceptor photobleach FRET procedure.

fluorescent proteins have recently been described: Cerulean (Rizzo et al., 2004), an improved CFP with higher quantum yield, and Venus (Nagai and Miyawaki, 2004), a brighter and more rapidly maturing YFP. Although it is best excited by UV light, CFP can be excited using the commonly available 456 nm line of the Argon laser, while the 514 nm Argon laser line is an excellent choice for YFP. Some deficiencies of these two fluorophores are described below. The pairing of enhanced GFP with the new monomeric form of dsRed has also been used (Day et al., 2001; Erickson et al., 2003; Peter et al., 2005) and would be advantageous in situations with high background.

Fusion proteins of CFP and YFP can readily be constructed in most laboratories and transfected into an expression system. For example, the important auditory protein prestin has already been widely transfected into HEK or CHO cells (Dallos and Fakler, 2002; Navaratnam et al., 2005). However, these cells do demonstrate some difficulty in proper membrane targeting. An alternative