Published in final edited form as:

Brain Res. 2006 May 26; 1091(1): 122–131.

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.

Keywords

Hearing; Cochlea; Hair Cell; FRET; FLIM; Confocal

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 antibodies 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

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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 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 choice, widely used in ion channel studies because of effective membrane targeting, is the frog oocyte (Riven et al., 2003; Zheng et al., 2002).

An emerging fluorescence technology is the application of semiconductor crystals (quantum dots) to biology and particularly to FRET (Michelet et al., 2005; Pinaud et al., 2006). Quantum dots have extraordinarily high quantum yields, very high photostability (they are practically unbleachable), wide absorbance bands but narrow emission bands (thus one laser may be used to excite several different dots simultaneously). Quantum dots with customized FRET spectra appear possible. However, batch to batch and within-batch variability and fluorescence intermittency (“blinking”) have so far limited their application to FRET studies (Pinaud et al., 2006).

2.2. Interpretation of FRET results

FRET efficiency E may be quantified from images of FRET pairs as follows:

$$E = 1 - (I_{DA} / I_D),$$

where I_{DA} and I_D are the donor emission intensity in the presence and absence of the acceptor, respectively. The donor and acceptor separation, r , may be calculated from the following relationship:

$$r = R_0 \{(1 / E) - 1\}^{1/6},$$

where R_0 is a coefficient called the Förster distance, which has been determined for many FRET pairs (Patterson et al., 2000).

The detection of FRET can therefore be used to infer that the fluorophores, and therefore the targeted proteins, are closely apposed to each other (although the absence of FRET should not be used to infer that the fluorophores are more widely separated). The calculation of donor–acceptor separation by the formulae given above must, however, be interpreted with caution. The stoichiometry of association of the candidate proteins may be something other than 1:1, and the expression levels of the proteins may differ substantially. Further, the relative brightness of the two fluorophores is often disparate (for example, YFP is about 5 times as bright as CFP Patterson et al., 2001). This may lead to mis-estimation of the donor–acceptor separation.

3. FRET methods

3.1. Sensitized emission FRET

In principle, sensitized emission FRET may be detected by acquiring just two images: one of donor alone excited by its excitation wavelength, and one of donor and acceptor excited by the same wavelength (Fig. 1B). A confocal microscope is not needed—excitation may be provided by the standard mercury source with the appropriate excitation and emission filters. Several companies now supply filter sets for the CFP–YFP combination (e.g., Chroma Technology Corp., Rockingham, VT; Semrock, Inc, Rochester, NY).

An ideal FRET pair for sensitized emission FRET would have extensive overlap of donor emission and acceptor absorbance bands to promote FRET, but no overlap of donor and acceptor absorbance bands, to eliminate cross excitation, and no overlap of donor and acceptor emission bands, to prevent crosstalk. Unfortunately, no FRET pair is ideal. Donor excitation often also excites the acceptor, and acceptor excitation often excites the donor. Further, donor and acceptor emission bands also overlap. Thus, it is difficult to design filters that completely separate donor and acceptor emission. YFP is particularly prone to this problem because its absorbance band overlaps the CFP absorbance band (Fig. 3A). A number of algorithms have been proposed to compensate for the expected artifacts (Chen et al., 2005; Gordon et al., 1998; Xia and Liu, 2001). Each algorithm requires several other control images to be obtained

and then digital subtraction applied to produce the result. The methods are complex and no one method has received universal acceptance.

Fluorescence-activated cell sorting (FACS) has also been used successfully to study protein–protein interactions (Szymczak et al., 2004). Fluorescence is quantified first for donor alone, acceptor alone, and for untransfected cells to establish background levels. Then doubly transfected cells are sorted, first for acceptor emission using acceptor excitation, then for donor and acceptor emission using donor excitation (Fig. 2). FACS reveals the percentage of cells showing FRET, which can be a useful means to establish that FRET interactions are present, and it can be established using a central university or hospital facility without the need for specialized equipment in the investigator's laboratory. More quantitative methods for the analysis of FRET in FACS experiments have also been described (Matko and Edidin, 1997).

3.2. Acceptor photobleach FRET

A widely used alternative procedure for obtaining FRET is acceptor photobleaching (Karpova et al., 2003). In this approach, donor emission is compared before and after the acceptor is bleached by intense acceptor excitation (Fig. 1C). The transfer of donor excitation to acceptor is blocked by the bleaching of the acceptor, causing the donor emission to be enhanced (de-quenched). Only donor emission with donor excitation is measured; thus cross contamination of the acceptor channel by donor emission is avoided (although contamination of the donor channel by acceptor emission is not). It is important that the acceptor bleach wavelength does not also bleach the donor (for the CFP–YFP pair, the 543 HeNe line is adequate). Acceptor photobleaching may be performed on an epifluorescence microscope with appropriate filters, although a confocal approach is preferable because bleaching may then be restricted to a region of interest (ROI). A disadvantage of acceptor photobleach is that it irreversibly damages the acceptor, which severely comprises the ability to perform time-series experiments. In addition, the intense excitation required for photobleaching may cause damage to or movement of living cells. The latter may in turn lead to misregistration artifacts.

When using CFP–YFP (and possibly other fluorophores), it is essential to use a control unbleached ROI in parallel with the test ROI. CFP has a short recovery time from bleaching by U–V or laser illumination (with a time constant of minutes). The usual inspection and selection of cells under U–V illumination can partly bleach CFP, and the subsequent recovery can be mistaken for positive FRET.

3.3. Spectral FRET methods

The difficulty of separating fluorophores in FRET studies has led some investigators to look at spectral methods (Dickinson et al., 2001; Nashmi et al., 2003; Zimmermann et al., 2003). The underlying principle behind the various spectral methods is the same: if the spectra of two fluorophores are known, the observed spectrum at any pixel is the sum of the spectra of the component fluorophores, weighted by linear coefficients:

$$\text{for wavelengths } \lambda = \lambda_1, \dots, \lambda_n$$

$$S(\lambda) = A \times \text{Donor}(\lambda) + B \times \text{Acceptor}(\lambda),$$

where $S(\lambda)$ is the spectrum observed at the pixel, $\text{Donor}(\lambda)$ and $\text{Acceptor}(\lambda)$ are the spectra for the donor and acceptor over the same wavelength range, and A and B are constants. The goal is to determine A and B from the set of linear equations above. This can be readily achieved by least-squares fitting and is referred to as linear unmixing (Dickinson et al., 2001). Zeiss offers on its confocal microscopes a dedicated bank of 32 detectors called the META (although only eight channels may be acquired in each pass). The bandwidth of the detectors is 10.7 nm (or integer multiples thereof) and the sampled range is from 400 to 700 nm. The spectrum acquired is not an accurate fluorophore spectrum, since it includes the effects of the objective,

filter and detector characteristics. However, if the acquisition parameters are kept constant, the fluorophore spectra can be reliably used for unmixing. Software for linear unmixing is supplied with the Zeiss confocal operating application. Other confocal vendors offer similar devices [e.g., the Olympus FV1000, the Leica SP2, and the Nikon C1Si]. Santos-Sacchi and colleagues (Navaratnam et al., 2005) have recently described the use of the META detector (without applying linear unmixing, however) to observe CFP–YFP FRET between prestin monomers in HEK cells, an important auditory problem. Zheng et al. (2002) have described a conceptually similar spectral (and much less expensive) approach to CFP–YFP FRET using an epi-illumination microscope, a digital camera, and a spectroscope.

Fig. 3B shows a spectrum of images of a HEK cell transfected with CFP–prestin and YFP–prestin constructs. Figs. 3C and D show the results of unmixing the spectral image series obtained from the cell before and after an acceptor photobleach experiment, respectively. Note the lower left panel (panel c) in each figure. This is the residual image, i.e., the pixel values that were not fit by the curve fitting procedure. This image possibly represents background fluorescence. It is important for successful linear unmixing that the residuals be small compare to the unmixed images. If necessary, a background image in an untransfected cell can be used to obtain a third “fluorophore” with which to separate background from fluorophore spectra.

FRET efficiency can then be calculated from the change in donor intensity in the ROI after photobleaching, corrected for any change in the control ROI. Thus, the FRET efficiency E is given by:

$$E = \frac{I_{\text{ROI Post}}}{I_{\text{ROI Pre}}} - \frac{I_{\text{Control ROI Post}}}{I_{\text{Control ROI Pre}}}$$

where I_{ROI} and $I_{\text{Control ROI}}$ represent the donor intensity in the test and control ROIs, and the suffixes Post and Pre indicate after and before acceptor photobleaching, respectively.

Error analyses indicate that the number of channels used influences the errors introduced by the unmixing procedure (Neher and Neher, 2004; Zimmermann et al., 2003). Intuitively, the more channels are available, the more accurate (or efficient) should be the unmixing. However, if the channel bands are too narrow, detector noise comes to dominate over signal in some of the channels, and such noise can significantly degrade the efficiency of unmixing. For this reason, removing background (possibly via the “third fluorophore” approach described above) is also important. Linear unmixing is typically used to separate closely spaced or overlapping spectra. Paradoxically, too much spectral separation also reduces unmixing efficiency, possibly because the signal levels in some channels are too low.

3.4. Fluorescence lifetime imaging

When a fluorophore is excited, it returns to its ground state by emitting a photon after an interval of picoseconds to nanoseconds. The statistics of the interval between absorbance and emission resemble a Poisson process, and the time constant of the interval histogram is referred to as the *fluorescence lifetime* of the fluorophore. An image composed of pixel-by-pixel fluorescence lifetimes, rather than photon counts, is referred to as a fluorescence lifetime image (FLIM).

Fluorescence lifetime is a sensitive measure of the fluorophore environment, and in particular is shortened by quenching processes such as FRET (Chen and Periasamy, 2004). Measurement of fluorescence lifetime is therefore a useful alternative approach to FRET detection. Fluorescence lifetime measurement has been performed using both frequency-domain and time-domain approaches. In the frequency-domain approach, sinusoidally modulated excitation from continuous-wave lasers or bright light-emitting diodes is used to excite the donor, and the phase change in emitted light intensity is detected and used to calculate

fluorescence lifetime. Dedicated instruments that use the frequency-domain approach are available (e.g., Lambert Instruments, Leutingewolde, The Netherlands) (Verveer et al., 2000, 2001). In the time domain approach, pulsed or pulse-modulated laser excitation or gated bright LEDs are used as the excitation source (Chen and Periasamy, 2004; Elangovan et al., 2002; Murata et al., 2001). A fast photomultiplier is used to derive the lifetime by accumulating a histogram of the intervals between pulse generation and photon emission. The approach is analogous to the familiar click post-stimulus time histogram of auditory nerve studies (Gerstein and Kiang, 1960). The fluorescence lifetime is then obtained as the exponential decay constant of the histogram (Fig. 4A). Excitation intensity and/or scan rates need to be such that on average no more than one photon per pulse is emitted (preferentially much less than one) to avoid double counting artifacts. Manufacturers of equipment for time-domain fluorescence lifetime measurement include LaVision Biotech GmbH (Göttingen, Germany) and PicoQuant GmbH (Berlin, Germany).

Many investigators have found that the tunable titanium:sapphire near infrared laser often used for two-photon confocal microscopy is also an excellent source of excitation for time-domain FLIM studies. Titanium:sapphire lasers are mode-locked lasers operating typically at a pulse rate of about 90 MHz with 100–200 femtosecond pulse widths. Optimum two-photon wavelengths for CFP and YFP excitation are 820 nm and 920 nm, respectively (Chen and Periasamy, 2004). The usual non-descanned detectors supplied with multi-photon confocal microscopes are not fast enough for FLIM, so faster detectors must be obtained, along with picosecond resolution timing gear. If scan timing signals are available from the confocal microscope, they can then be used to build up a lifetime image. Manufacturers of such equipment include Becker and Hickl GmbH (Berlin, Germany). An example of a FLIM image obtained by this method is shown in Fig. 4Ba.

FRET efficiency E may be calculated from fluorescence lifetime measurements using the relationship:

$$E = 1 - \left(\frac{\tau_{DA}}{\tau_D} \right),$$

where τ_{DA} and τ_D are the fluorescence lifetimes in the presence and absence of acceptor. The fluorescence lifetime for a fluorophore in a cell is obtained from a histogram of fluorescence lifetimes in the pixels in a region of a cell containing the fluorophore (Fig. 4Bb, c). Fluorophore separation may then be calculated using the relationship described earlier, with some but not all of the same caveats. Fluorescence lifetime measurement is insensitive to the relative concentrations of the fluorophores or their relative brightness, provided enough emitted photons can be obtained to distinguish separate peaks in the lifetime histogram.

Although implementation of a FLIM approach is complex, the procedure has a number of other advantages. Near infrared (two-photon) excitation is inherently less damaging to cells and at the same time has greater spatial resolution and depth of penetration than wide-field or confocal excitation. Thus, two-photon FLIM is the preferred method for living tissue or cells. Further, the dynamic nature of the approach means that FRET may be studied under changing stimulus conditions. Although data may need to be accumulated over seconds or minutes (unlike intensity-based approaches, which acquire snapshot images), lifetime acquisition may be acquired synchronously with a repetitive external event, such as a voltage pulse. Then the data may be “unpacked” from the data file and separately analyzed. This approach may be applicable to any protein or protein pair that undergoes a stimulus-dependent conformation change.

One disadvantage with some FLIM systems is an inability to obtain simultaneous lifetime and intensity data, which may then be used to estimate donor and acceptor concentrations and thereby study the influence on lifetime of their relative concentrations. Overall, in some ways,

FLIM analysis suffers from a surfeit of data: the reasons for the variation in measured lifetime from region to region in cells (as exemplified in Fig. 4Ba) are not yet clear. It is to be hoped that future improvements in the software will bring clarity to this issue.

3.5. Background

Anyone using fluorescence techniques in cells or tissue knows that the unwanted “background” fluorescence found in both fixed and living specimens constitutes a potential source of artifact or competition with the signal or signals of interest. The observation that background is diminished towards the red wavelengths has stimulated a push towards obtaining red-shifted fluorophores including fluorescent proteins (Zhang et al., 2002), and detectors. Significant improvement in this area will undoubtedly occur in the coming years. However, an interesting, if tangential, aspect of lifetime imaging is its application to, via lifetime measurements, the study of intrinsic fluorophores such as NADH, flavoproteins, and lipofuscin that might normally be considered “background”. Especially in the auditory system, where vulnerability to diminished oxygenation is of clinical significance, such metabolic imaging studies promise new understanding of metabolism in health and under challenge.

4. Conclusion

FRET detection and measurement are important new tools in structure–function studies. Application to proteins of interest to auditory investigators, just beginning, promises a wealth of new understanding of structure–function relationships. However, selection of the appropriate method for FRET determination is important and care must be taken with interpretation of the results. The development of applications of FLIM methods to the auditory system will be of particular importance for analysis of functional interactions in living tissues.

Acknowledgments

Supported by DC 06471, ALSAC, and CA21765 (to J.Z.), DC 02053 (to R.H.), RR17417-01 (to Creighton University), and NSF-EPSCoR EPS-0346476 (to RH). R.H. is the Director of the Nebraska Center for Cell Biology, where the images in this review were obtained. We thank LeAnn Tiede, Creighton University, for help in implementing FLIM, and Ammasi Periasamy and Richard Day, University of Virginia, for help in understanding FRET methods. We also thank Heather C. Jensen-Smith and Wade Bell for useful comments on the manuscript.

REFERENCES

- Adato A, Michel V, Kikkawa Y, Reiners J, Alagramam KN, Weil D, Yonekawa H, Wolfrum U, El-Amraoui A, Petit C. Interactions in the network of Usher syndrome type 1 proteins. *Hum. Mol. Genet* 2005;14:347–356. [PubMed: 15590703]
- Boeda B, El-Amraoui A, Bahloul A, Goodyear R, Daviet L, Blanchard S, Perfettini I, Fath KR, Shorte S, Reiners J, Houdusse A, Legrain P, Wolfrum U, Richardson G, Petit C. Myosin VIIa, harmonin and cadherin 23, three Usher I gene products that cooperate to shape the sensory hair cell bundle. *EMBO J* 2002;21:6689–6699. [PubMed: 12485990]
- Chen Y, Periasamy A. Characterization of two-photon excitation fluorescence lifetime imaging microscopy for protein localization. *Microsc. Res. Tech* 2004;63:72–80. [PubMed: 14677136]
- Chen Y, Mills JD, Periasamy A. Protein localization in living cells and tissues using FRET and FLIM. *Differentiation* 2003;71:528–541. [PubMed: 14686950]
- Chen, Y.; Elangovan, M.; Periasamy, A. FRET data analysis: the algorithm. In: Periasamy, A.; Day, RN., editors. *Molecular Imaging: FRET Microscopy and Spectroscopy*. Oxford: Oxford University Press; 2005. p. 126-145.
- Chew, TL.; Chisholm, RL. Monitoring protein dynamics using FRET-based biosensors. In: Spector, DL., editor. *Live Cell Imaging. A Laboratory Manual*. Cold Spring Harbor, New York: Cold Spring harbor Press; 2005. p. 145-157.
- Corey DP, Garcia-Anoveros J, Holt JR, Kwan KY, Lin SY, Vollrath MA, Amalfitano A, Cheung EL, Derfler BH, Duggan A, Geleoc GS, Gray PA, Hoffman MP, Rehm HL, Tamasauskas D, Zhang DS.

- TRPA1 is a candidate for the mechanosensitive transduction channel of vertebrate hair cells. *Nature* 2004;432:723–730. [PubMed: 15483558]
- Dallos P, Fakler B. Prestin, a new type of motor protein. *Nat. Rev., Mol. Cell Biol* 2002;3:104–111. [PubMed: 11836512]
- Day RN, Periasamy A, Schaufele F. Fluorescence resonance energy transfer microscopy of localized protein interactions in the living cell nucleus. *Methods* 2001;25:4–18. [PubMed: 11558993]
- Di Palma F, Holme RH, Bryda EC, Belyantseva IA, Pellegrino R, Kachar B, Steel KP, Noben-Trauth K. Mutations in *Cdh23*, encoding a new type of cadherin, cause stereocilia disorganization in waltzer, the mouse model for Usher syndrome type 1D. *Nat. Genet* 2001;27:103–107. [PubMed: 11138008]
- Dickinson ME, Bearman G, Tille S, Lansford R, Fraser SE. Multi-spectral imaging and linear unmixing add a whole new dimension to laser scanning fluorescence microscopy. *Biotechniques* 2001;31:1272, 1274–1276, 1278. [PubMed: 11768655]
- Elangovan M, Day RN, Periasamy A. Nanosecond fluorescence resonance energy transfer-fluorescence lifetime imaging microscopy to localize the protein interactions in a single living cell. *J. Microsc* 2002;205:3–14. [PubMed: 11856376]
- Erickson MG, Moon DL, Yue DT. DsRed as a potential FRET partner with CFP and GFP. *Biophys. J* 2003;85:599–611. [PubMed: 12829514]
- Friedman TB, Griffith AJ. Human nonsyndromic sensorineural deafness. *Annu. Rev Genomics Hum. Genet* 2003;4:341–402. [PubMed: 14527306]
- Gerstein GL, Kiang NY. An approach to the quantitative analysis of electrophysiological data from single neurons. *Biophys. J* 1960;1:15–28. [PubMed: 13704760]
- Gillespie PG, Cyr JL. Myosin-1c, the hair cell's adaptation motor. *Annu. Rev. Physiol* 2004;66:521–545. [PubMed: 14977412]
- Gillespie PG, Dumont RA, Kachar B. Have we found the tip link, transduction channel, and gating spring of the hair cell? *Curr. Opin. Neurobiol* 2005;15:389–396. [PubMed: 16009547]
- Gordon GW, Berry G, Liang XH, Levine B, Herman B. Quantitative fluorescence resonance energy transfer measurements using fluorescence microscopy. *Biophys. J* 1998;74:2702–2713. [PubMed: 9591694]
- Gryczynski, Z.; Gryczynski, I.; Lakowicz, JR. Basics of fluorescence and FRET. In: Periasamy, A.; Day, RN., editors. *Molecular Imaging*. New York, NY: Oxford University Press; 2005. p. 21–56.
- Heim R. Green fluorescent protein forms for energy transfer. *Methods Enzymol* 1999;302:408–423. [PubMed: 12876789]
- Heim R, Tsien RY. Engineering green fluorescent protein for improved brightness, longer wavelengths and fluorescence resonance energy transfer. *Curr. Biol* 1996;6:178–182. [PubMed: 8673464]
- Holley MC, Ashmore JF. A cytoskeletal spring for the control of cell shape in outer hair cells isolated from the guinea pig cochlea. *Eur. Arch. Otorhinolaryngol* 1990;247:4–7. [PubMed: 2310549]
- Karpova TS, Baumann CT, He L, Wu X, Grammer A, Lipsky P, Hager GL, McNally JG. Fluorescence resonance energy transfer from cyan to yellow fluorescent protein detected by acceptor photobleaching using confocal microscopy and a single laser. *J. Microsc* 2003;209:56–70. [PubMed: 12535185]
- Lichlyter DJ, Grant SA, Soykan O. Development of a novel FRET immunosensor technique. *Biosens. Bioelectron* 2003;19:219–226. [PubMed: 14611757]
- Matko J, Edidin M. Energy transfer methods for detecting molecular clusters on cell surfaces. *Methods Enzymol* 1997;278:444–462. [PubMed: 9170326]
- Maurel D, Kniazeff J, Mathis G, Trinquet E, Pin JP, Ansanay H. Cell surface detection of membrane protein interaction with homogeneous time-resolved fluorescence resonance energy transfer technology. *Anal. Biochem* 2004;329:253–262. [PubMed: 15158484]
- Michalet X, Pinaud FF, Bentolila LA, Tsay JM, Doose S, Li JJ, Sundaresan G, Wu AM, Gambhir SS, Weiss S. Quantum dots for live cells, in vivo imaging, and diagnostics. *Science* 2005;307:538–544. [PubMed: 15681376]
- Murata S, Herman P, Lakowicz JR. Texture analysis of fluorescence lifetime images of AT- and GC-rich regions in nuclei. *J. Histochem. Cytochem* 2001;49:1443–1451. [PubMed: 11668197]

- Nagai T, Miyawaki A. A high-throughput method for development of FRET-based indicators for proteolysis. *Biochem. Biophys. Res. Commun* 2004;319:72–77. [PubMed: 15158444]
- Nagy P, Vereb G, Sebestyen Z, Horvath G, Lockett SJ, Damjanovich S, Park JW, Jovin TM, Szollosi J. Lipid rafts and the local density of ErbB proteins influence the biological role of homo- and heteroassociations of ErbB2. *J. Cell Sci* 2002;115:4251–4262. [PubMed: 12376557]
- Nashmi R, Dickinson ME, McKinney S, Jareb M, Labarca C, Fraser SE, Lester HA. Assembly of alpha4beta2 nicotinic acetylcholine receptors assessed with functional fluorescently labeled subunits: effects of localization, trafficking, and nicotine-induced upregulation in clonal mammalian cells and in cultured midbrain neurons. *J. Neurosci* 2003;23:11554–11567. [PubMed: 14684858]
- Navaratnam D, Bai JP, Samaranayake H, Santos-Sacchi J. N-terminal mediated homo-multimerization of prestin, the outer hair cell motor protein. *Biophys. J.* 2005
- Neher R, Neher E. Optimizing imaging parameters for the separation of multiple labels in a fluorescence image. *J. Microsc* 2004;213:46–62. [PubMed: 14678512]
- Patterson GH, Piston DW, Barisas BG. Forster distances between green fluorescent protein pairs. *Anal. Biochem* 2000;284:438–440. [PubMed: 10964438]
- Patterson G, Day RN, Piston D. Fluorescent protein spectra. *J. Cell Sci* 2001;114:837–838. [PubMed: 11181166]
- Pazour GJ, Agrin N, Leszyk J, Witman GB. Proteomic analysis of a eukaryotic cilium. *J. Cell Biol* 2005;170:103–113. [PubMed: 15998802]
- Peter M, Ameer-Beg SM. Imaging molecular interactions by multiphoton FLIM. *Biol. Cell* 2004;96:231–236. [PubMed: 15182705]
- Peter M, Ameer-Beg SM, Hughes MK, Keppler MD, Prag S, Marsh M, Vojnovic B, Ng T. Multiphoton-FLIM quantification of the EGFP-mRFP1 FRET pair for localization of membrane receptor-kinase interactions. *Biophys. J* 2005;88:1224–1237. [PubMed: 15531633]
- Pinaud F, Michalet X, Bentolila LA, Tsay JM, Doose S, Li JJ, Iyer G, Weiss S. Advances in fluorescence imaging with quantum dot bio-probes. *Biomaterials* 2006;27:1679–1687. [PubMed: 16318871]
- Pollok BA, Heim R. Using GFP in FRET-based applications. *Trends Cell Biol* 1999;9:57–60. [PubMed: 10087619]
- Riven I, Kalmanzon E, Segev L, Reuveny E. Conformational rearrangements associated with the gating of the G protein-coupled potassium channel revealed by FRET microscopy. *Neuron* 2003;38:225–235. [PubMed: 12718857]
- Rizzo MA, Springer GH, Granada B, Piston DW. An improved cyan fluorescent protein variant useful for FRET. *Nat. Biotechnol* 2004;22:445–449. [PubMed: 14990965]
- Steel KP, Kros CJ. A genetic approach to understanding auditory function. *Nat. Genet* 2001;27:143–149. [PubMed: 11175778]
- Szymczak AL, Workman CJ, Wang Y, Vignali KM, Dilioglou S, Vanin EF, Vignali DA. Correction of multi-gene deficiency in vivo using a single 'self-cleaving' 2A peptide-based retroviral vector. *Nat. Biotechnol* 2004;22:589–594. [PubMed: 15064769]
- van Munster EB, Gadella TW. Fluorescence lifetime imaging microscopy (FLIM). *Adv. Biochem. Eng. Biotechnol* 2005;95:143–175. [PubMed: 16080268]
- Verveer PJ, Squire A, Bastiaens PI. Global analysis of fluorescence lifetime imaging microscopy data. *Biophys. J* 2000;78:2127–2137. [PubMed: 10733990]
- Verveer PJ, Squire A, Bastiaens PI. Improved spatial discrimination of protein reaction states in cells by global analysis and deconvolution of fluorescence lifetime imaging microscopy data. *J. Microsc* 2001;202:451–456. [PubMed: 11422665]
- Wallrabe H, Periasamy A. Imaging protein molecules using FRET and FLIM microscopy. *Curr. Opin. Biotechnol* 2005;16:19–27. [PubMed: 15722011]
- Xia Z, Liu Y. Reliable and global measurement of fluorescence resonance energy transfer using fluorescence microscopes. *Biophys. J* 2001;81:2395–2402. [PubMed: 11566809]
- Zhang J, Campbell RE, Ting AY, Tsien RY. Creating new fluorescent probes for cell biology. *Nat. Rev., Mol. Cell Biol* 2002;3:906–918. [PubMed: 12461557]
- Zheng J, Trudeau MC, Zagotta WN. Rod cyclic nucleotide-gated channels have a stoichiometry of three CNGA1 subunits and one CNGB1 subunit. *Neuron* 2002;36:891–896. [PubMed: 12467592]

- Zimmermann T, Rietdorf J, Pepperkok R. Spectral imaging and its applications in live cell microscopy. *FEBS Lett* 2003;546:87–92. [PubMed: 12829241]
- Zuo J. Transgenic and gene targeting studies of hair cell function in mouse inner ear. *J. Neurobiol* 2002;53:286–305. [PubMed: 12382282]

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.

Fig 2.
Principle of fluorescence-activated cell sorting detection of FRET by sensitized emission.

Fig 3.

(A) Absorbance and emission spectra of Cerulian CFP (cCFP) and Venus YFP (vYFP) determined using a Zeiss META detector for a 40x oil objective and expression in HEK cells. The spectra were obtained for illumination with 457 nm light over 13 bands each 10.7 nm wide centered between 479 nm and 586 nm, and are normalized to the peak. (B) Spectrally resolved images of an HEK cell co-transfected with prestin-CFP and prestin-YFP obtained using the same bands. The center wavelength of each band is given at the top left of each image. (C) Linear unmixed CFP (a) and YFP (b) images of the same cell; the calculated residuals (c); and the combined image (d). The unmixing was performed using the spectra shown in panel A. (D) Linear unmixed images of the same cell after acceptor photobleach by 543 nm light in a region

of interest, using the same key as panel C. The solid square outline represents the bleach area, while the dashed square outline represents the control area.

Fig 4.

(A) Principle of fluorescence lifetime measurement. A brief (femtosecond time scale) pulse of light ("Pulse", top) is repetitively presented and the latencies to the re-emission of a photon are measured (center). A histogram of re-emission latencies is accumulated over multiple presentations (bottom). The decay constant of the histogram (" τ ") is the lifetime of the fluorophore. (B)(a) Fluorescence lifetime image of a CHO cell transfected with prestin-YFP. The color key represents 2.3 ns (orange) to 2.7 ns (blue). (b) Histogram of lifetimes from the same image for pixels within the cell boundary. The fitted curve is a Gaussian with a maximum at 2.50 ps ($R^2 = 0.76$). (c) Latency distribution of photons observed in the brightest pixel of the

image in panel Ba. The latency distribution was well fit by a single lifetime of 2.49 ns ($\chi^2 = 1.17$).