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Intracellular FRET-based probes: a review

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Abstract

Probes that exploit Förster resonance energy transfer (FRET) in their feedback mechanism are touted for their sensitivity, robustness, and low background, and thanks to the exceptional distance dependence of the energy transfer process, they provide a means of probing lengthscales well below the resolution of light. These attributes make FRET-based probes superbly suited to an intracellular environment, and recent developments in biofunctionalization and expansion of imaging capabilities have put them at the forefront of intracellular studies. Here, we present an overview of the engineering and execution of a variety of recent intracellular FRET probes, highlighting the diversity of this class of materials and the breadth of application they have found in the intracellular environment.

1. Introduction

As the atom forms the fundamental building block in chemistry, so the cell is the unit on which life is built. Developing an understanding of cellular processes and the interplay of conditions and biomolecular species required for those processes to take place demands a window into the intracellular environment. The challenge, though, of probing an environment as small as the cell and of gleaning from it information on a subcellular length scale is a considerable one. The use of fluorescent probes designed to report conditions and analytes provides an incremental step towards the elucidation of the intracellular environment, and the paired use of fluorophores engaged in Förster resonance energy transfer (FRET), a nonradiative energy transfer process, adds sensitivity, reliability, and specificity that puts these probes at the forefront.
of intracellular detection [1–3]. Moreover, recent developments in the library of fluorophores available for FRET-based applications, including expansion of the application of quantum dots to biological systems and continued development of fluorescent proteins, have made FRET probes still more powerful tools [3–10]. Coupled with the increasingly widespread availability of instrumentation for fluorescence-based microscopy, these developments have translated to a surge in interest in using FRET-based probes for intracellular applications [11–18].

In this review, we discuss the benefits and challenges to using FRET in intracellular detection. We present an overview of the attributes required to design FRET-based probes suitable for use in intracellular environments and highlight the probes that have been developed to assess intracellular states, species, and processes. Throughout this review, we aim to emphasize the breadth of materials that are now available in intracellular FRET-based detection and, rather than provide an exhaustive review, instead survey the diversity that has developed in the field.

2. Designing and delivering intracellular FRET probes

From understanding how and when processes as fundamental to life as DNA replication occur to deciphering indicators of impending cell death, the benefits of intracellular sensing are both wide-ranging and profound. The extraordinary diversity of intracellular probes currently available and the breadth of the analytes and processes they detect is a testament to the scale of the interest in these materials, not to the ease with which they are developed. Indeed, the complexity of designing and delivering an intracellular probe requires addressing a number of intrinsic challenges, including both biocompatibility and toxicity concerns. Moreover, depending on the target analyte, probes may require the ability to target specific regions within a cell or require operation in size-restricting intracellular areas. In this section, we begin with a discussion of what advantages FRET offers over traditional, single fluorophore-based fluorescence detection schemes and subsequently touch on the universal considerations in the design of FRET-based probes, including the criteria that must be met in order for FRET to take place, the selection of suitable fluorophores, assembly of the probe, and delivery into the cell.

2.1. Why FRET over other detection methods?

There are a number of reasons that FRET-based probes are particularly well-suited to intracellular detection. The benefits of generating a fluorescence signal—applicable to both FRET and single fluorophore-based imaging—include the ability to look at broad resolution ranges, from the single cell level to imaging whole organs or more, with much greater sensitivity than that provided by simply color staining. Emission in the optimal spectral region (near-IR) is even able to penetrate several layers of cells, allowing detection of a signal in deeper tissue than color staining could permit [5]. However, traditional fluorescence imaging suffers from the limitations of the fluorophore. Background emission from excess fluorophore cannot be differentiated from the intended signal, limiting sensitivity. Emission intensity may well fluctuate with environmental factors such as temperature, pH, or the presence of exogenous ions [5, 12]. Inhomogeneous distribution throughout the cell of either the fluorophore or the analyte may be difficult to detect because of spatial averaging and the inherent wavelength-based limitations of light microscopy. Fluorophores may degrade over time in the cell or may suffer from photobleaching [6]. Even varying absorption by the cell can attenuate fluorescence signals in such a way as to make quantification of an analyte challenging, if not impossible [12]. Although significant advances have recently been made in super-resolution microscopy, with methods such as stimulated emission depletion (STED), stochastic optical reconstruction microscopy (STORM) and photo-activated localization microscopy (PALM), these techniques require complex and expensive instrumentation, sensitive optics, and advanced electronics [19].

Using FRET rather than traditional fluorescence measurements overcomes many of these limitations. Because the generation of the FRET signal is gated by the analyte, background from probes that fail to detect the analytic is mitigated, a condition that improves the signal/noise ratio and therefore the sensitivity and compensates for inhomogeneous distribution throughout the cell. For some FRET probes, luminescence is only activated in the presence of the analyte, which enhances sensitivity even further. Other FRET probes generate a ratiometric signal, which functions as an internal standard and allows for a correction to account for probe degradation and attenuation of photoluminescence (PL) by the cellular environment [1, 12]. Finally, FRET remains an accessible and cost-effective method for obtaining proximity information on the sub-twenty nanometer scale, and it may well find use paired with super resolution methods for an even more powerful detection scheme [11, 19].

2.2. FRET efficiency

Förster resonance energy transfer, FRET, was first described by Theodor Förster in 1946 [2]. FRET is a nonradiative energy transfer process between a photoexcited donor and a ground-state acceptor species. Subsequent to energy transfer, the now-excited or sensitized acceptor relaxes in its usual manner, either radiatively or through nonradiative processes. In order for FRET to take place, the donor and acceptor must be in close physical proximity, must be energetically well-matched, and must exhibit suitable dipole alignment [1, 8, 20]. The combination of these factors is taken into
account in the following equation describing FRET efficiency, $E$:

$$E = \frac{R_0}{R_0^6 + R^6}.$$  \hspace{1cm} (1)

Where $R_0$ is the Förster distance, or the distance at which energy transfer efficiency is 50%, and $R$ is the donor-acceptor separation distance, both expressed in angstroms. $R_0$ is proportional to the donor’s quantum yield ($Q_D$), the integral of the spectral overlap between the donor emission and acceptor absorbance ($I$), and the relative orientation of the dipoles of the donor and acceptor ($\kappa_p$) and inversely proportional to the refractive index of the material ($n_D$), as shown below:

$$R_0 = \left( \frac{Q_D[9000(ln 10)] \kappa_p^2}{128N_A \pi n_D^4} \right)^{1/6}. \hspace{1cm} (2)$$

$N_A$ is Avogadro’s number. For a more expansive discussion on the calculation of FRET efficiency, we refer the reader to an excellent review by Wu and Brand [20]. Medintz and Hildebrandt also provide an excellent primer on how to implement FRET formalism [21].

Förster radii of 3–6 nm are typical for most species, and because FRET efficiency falls off as $1/R^6$, observation of FRET is typically limited to donor/acceptor distances of about 10 nm [1, 6, 20]. Small changes in the donor/acceptor distance can thus give rise to substantial changes in FRET efficiency and therefore in excitation of (and emission from) the acceptor species. Probe design in FRET-based systems is based heavily on altering FRET efficiency, often by exploiting the distance dependence of the energy transfer. Alternatively, some FRET-based probes rely on environmental sensitivity of the acceptor’s absorption spectrum, a key selection criterion in choosing a suitable FRET pair (see section 2.3), to alter FRET efficiency [8, 22]. The overlap in dipole moments of the donor and acceptor provides a third way of manipulating FRET efficiency; however, ascertaining the relative dipole orientation would require precise structural/orientational knowledge of both fluorophores, and tuning that orientation would require substantial control over the system, limiting the effectiveness of this parameter in anything but the most specialized probe design [23].

It is worth noting that FRET differs both in length-scale and mechanism from Dexter energy transfer. The latter relies on electron transfer between the donor and acceptor and therefore does not require, for example, a specific dipole orientation. A result of the mechanism, however, the redox potential must be suitable for the electron transfer to take place, and the distance over which Dexter energy transfer takes place is less than 1.5 nm [24].

2.3. Fluorophore types

In many FRET systems, both the donor and acceptor are fluorophores, though pairing an emissive donor with a quenching acceptor (i.e. one that relaxes nonradiatively such as a ‘dark-quencher’) is equally possible. One of the key considerations in the selection of a FRET pair is the degree of spectral overlap between donor emission and acceptor absorption and spectral differentiation between donor and acceptor emission. While maximizing spectral overlap also maximizes FRET efficiency, the desire to spectrally resolve donor and acceptor emission is often crucial to the measurement; thus acceptors with a large Stokes shift are optimal [1, 6, 8, 20]. Systems relying on homo-FRET, in which both the donor and acceptor species are the same, provide an exception, preferring a minimal Stokes shift in order to maximize energy transfer [1, 25, 26]. Finally, if the acceptor absorbs in the spectral region in which the donor is excited, direct excitation of the acceptor occurs, confounding the FRET signal and requiring spectral deconvolution. Thus, except in time-gated systems and in FRET pairs that operate on a quenching mechanism, acceptors with discrete absorption bands (e.g. organic dyes) that do not overlap with the excitation wavelength are typically preferred to those with broad absorption bands (e.g. quantum dots) [1, 6, 8]. Here we provide an overview of the fluorophores that are discussed in the context of this review. For a more comprehensive review, we refer the reader to Hötzer et al [3].

Organic dyes constitute a broad range of organic small molecules, many of which are built on one of several common scaffolds, that take advantage of the energy differential between the highest occupied molecular orbital (HOMO) and lowest unoccupied molecular orbital (LUMO) [6, 8, 9, 27]. Upon electronic excitation of the molecule, typically accomplished by the absorption of a photon of suitable energy (i.e. photoexcitation), a ground-state electron is promoted to the LUMO. Return to the ground state results in the emission of a photon that is slightly lower in energy (spectrally redder) than the excitation wavelength, a phenomenon known as the Stokes shift [9]. Cyanine-based dyes, for example, can be structurally altered to emit anywhere from the ultraviolet to the infrared regions of the spectrum, with absorption occurring to the blue of the emission profile. By changing the physical structure, the electronic structure of the dye changes, permitting the tuning of the absorption and emission profiles. Cyanine 3 is shown as an example in table 1. For a survey of the structural and photophysical breadth of dyes, the review by Sapsford et al and Handbook of Fluorescent Probes and Research Products by Haugland are excellent resources [6, 28].

A key advantage to using dyes in FRET probes is the ability to tune absorption and emission bands using chemical substituents, a fact that translates to the commercial availability of derivatives adapted to every region of the visible spectrum and more limited availability in the infrared. An additional advantage, dyes are bright, typically exhibiting quantum yields between 50 and 100%, though those values are substantially diminished in the near-IR (2–25%) [9]. Disadvantages to dyes as fluorophores include their propensity to
photobleach, their low molar absorptivity, and their toxicity [9, 16]. For more information on dyes and their role in FRET-based probes, we refer the reader to reviews by Yuan et al and Resch-Genger et al [8, 9].

Quantum dots (QDs) are semiconducting materials exhibiting quantum confinement; that is, their size is smaller than the bulk semiconductor’s exciton Bohr diameter, the average spatial separation between an excited state electron and the ground state hole that excitation left behind [29]. As with other semiconductors, ground state electrons located in the valence band can be promoted through photoexcitation to the conduction band. Although the vocabulary is different, the driving principle is the same as in organic dyes: the excited state electron relaxes to the ground state through nonradiative or radiative pathways. In the latter case, the emission of a quantized photon is what gives rise to photoluminescence (PL). The energy of the bandgap for a bulk semiconductor is fixed, and the exciton Bohr radius, also intrinsic to the semiconductor, becomes important in materials at sizes below anywhere from several nm (e.g. ZnS at 4.4 nm) [30] to over 100 nm (e.g. InSb at 108 nm) [31]. As a result of quantum confinement, QDs exhibit a size-dependent blue-shift in their bandgap as compared to the bulk semiconductor [29]. In addition to the size-tunability of the bandgap (and therefore of the absorption and emission maxima), QDs have several properties that make them versatile fluorophores. Their molar absorption cross-sections are large (1–2 orders of magnitude greater than organic dyes), their low molar absorptivity, and their toxicity [9, 16]. For more information on dyes and their role in FRET-based probes, we refer the reader to reviews by Yuan et al and Resch-Genger et al [8, 9].

Table 1. Representative fluorophores.

<table>
<thead>
<tr>
<th>Example</th>
<th>Structure</th>
<th>Spectra</th>
<th>Extinction coefficient $\lambda_{\text{max}}$ (10$^5$ cm$^{-1}$ M$^{-1}$)</th>
<th>Quantum yield (%)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic Dye</td>
<td>Cyanine 3 (Cy3)</td>
<td><img src="image" alt="Cyanine 3" /></td>
<td><img src="image" alt="Excitation/Emission" /></td>
<td>1.36</td>
<td>15</td>
</tr>
<tr>
<td>Organic Dye</td>
<td>Fluorescein (FITC)</td>
<td><img src="image" alt="Fluorescein" /></td>
<td><img src="image" alt="Excitation/Emission" /></td>
<td>0.93</td>
<td>93</td>
</tr>
<tr>
<td>Quantum dot</td>
<td>CdSe/ZnS</td>
<td><img src="image" alt="CdSe/ZnS" /></td>
<td><img src="image" alt="Excitation/Emission" /></td>
<td>1–8$^a$</td>
<td>1–5 (core)$^a$</td>
</tr>
<tr>
<td>Polymer dot</td>
<td>PFBT</td>
<td><img src="image" alt="PFBT" /></td>
<td><img src="image" alt="Absorption/Emission" /></td>
<td>100</td>
<td>30</td>
</tr>
<tr>
<td>Fluorescent protein</td>
<td>Green fluorescent protein (GFP)</td>
<td><img src="image" alt="GFP" /></td>
<td><img src="image" alt="Excitation/Emission" /></td>
<td>0.56</td>
<td>60</td>
</tr>
<tr>
<td>Quencher (organic)</td>
<td>Dabcyl</td>
<td><img src="image" alt="Dabcyl" /></td>
<td><img src="image" alt="Excitation" /></td>
<td>0.32</td>
<td>NA</td>
</tr>
</tbody>
</table>

$^a$ TEM courtesy of E. Oh, NRL. Excitation/emission spectra and extinction coefficient depend on the size of the QD, and QY can vary.
dyes), and they absorb all wavelengths bluer than their first excitonic absorption peak, making it easy to excite them at wavelengths easily differentiable from their far redder emission profile (see table 1) [32]. Their quantum yields in the near-IR, a spectral region important because of the higher near-IR transparency of tissue, are better than organic dyes at 20–70% [9]. Additionally QDs have some of the largest two-photon action cross-sections known, which also allows them to function as unique multiphoton excited donors [33, 34].

In addition to their photophysics, the physical properties of QDs make them well-suited to tailored FRET-based applications [35]. For example, the ability to coordinate multiple molecules to their surfaces, thanks to their substantial surface area, affords the opportunity to tether multiple acceptors to the QD donor to increase energy transfer efficiency. This same principle can be exploited with multiple types of acceptors, opening the door to multiplexing acceptors with different sensitivities to a single donor [5, 36].

QDs, however, come with their own set of challenges: Their broad absorption bands limit their use as FRET acceptors because they are co-excited with the FRET donor. Moreover, their substantial size (~3–60 nm diameter) relative to molecular dyes and the hydrophobic ligands that typically passivate their surface make it challenging to integrate them into small, water-rich biological environments [7, 9]. While it might seem that opting for smaller QDs solves at least the problem of size, the size-tunable nature of the band-gap means that it is precisely the larger QDs that emit in the more biologically interesting spectral region.

Upconversion nanoparticles are non-semiconducting nanoscale materials doped with rare earths used in fluorescence imaging by exploiting upconversion, a process in which low-energy (redder) excitation is converted to higher-energy (bluer) emission through multi-photon absorption and the use of dopants (often Yb, Er, or Tm) as the emissive species [37, 38]. Although this process does not overcome the poor transparency of biological tissue in the visible region, it does substantially reduce background from cell autofluorescence by employing redder excitation energies. Moreover, the long radiative lifetimes of lanthanides allow for time-gated microscopy, in which detection occurs after excitation is completed, eliminating spectral overlap and background from the excitation source during detection of the emissive probe [39–43].

Semiconducting polymers operate on the same premise that QDs and organic dyes utilize: they take advantage of an energy gap between the ground and excited state across which an electron can be promoted, and the relaxation of that electron across the bandgap results in PL. Polymers aggregated to form nanoscale materials, known as polymer dots or Pdots, have recently caught on as fluorescent markers [44]. Unlike QDs, their PL is not the result of quantum confinement but rather originates from the bulk bandgap of the polymer, so Pdots can be synthesized on any size scale without altering their photophysical properties. Moreover, they are remarkably bright, with high quantum yields, high absorption cross-sections, and fast emission rates [44]. In FRET probes, they have been used in the past few years simultaneously as both a fluorophore and a substrate [45, 46].

Fluorescent proteins (FPs) have been amply developed in the past two decades. From the naturally occurring green fluorescent protein (GFP) found in Aequorea victoria, researchers have both discovered other FPs in marine organisms and engineered a variety of FP mutations with different emission profiles, giving rise to a range of materials exhibiting substantial spectral diversity (see figure 1) [10, 47–51]. For a description of the biochemical mechanism by which FPs photoluminesce, we refer the reader to Frommer et al [10]. Advantages of FPs include their noninvasiveness (after all, they are being produced intracellularly) and their inherent biocompatibility. Challenges to their application include broad excitation and emission spectra, which, when FPs are used in tandem as in FRET applications, can lead to cross-talk between the FPs. Further, the large size of FPs makes it difficult to bring them into proximity with each other, leading to low FRET efficiencies (discussed below) [13]. An additional drawback in FP use originates in the
fact that they are genetically expressed, making them useful in laboratory environments where cells or organisms are grown for study but limiting their utility in areas such as \textit{in vivo} diagnostics. Moreover, all FPs require some maturation time for the fluorophore to form from the reorganization of key amino acids in its structure.

\textbf{Quenchers} function in FRET-based probes as FRET acceptors that are non-emissive. Their precise role in FRET detection schemes will be expanded on in section 2.4, but here we discuss several types of quenchers in common use. As with fluorophores, there is a library of small molecule organic quenchers that are built on several basic scaffolds [6]. The addition of substituents changes the absorption spectrum of the quencher so that it can be tailored for maximum overlap with the donor’s emission. Gold nanoparticles (AuNPs), though fluorescent at very small sizes ($<5$ nm), function as effective quenchers above that size [52–54]. Moreover, colloidal gold has been used extensively in biological applications. Carbon allotropes, including C$_{60}$ and graphene sheets, have also come into use as quenchers more recently [55]. It is not, however, clear whether energy transfer interactions with gold colloids or carbon allotropes can be truly ascribed to FRET due to the nature of the latter materials and especially their high electron density [56–58].

\subsection*{2.4. Probe design}

FRET-based probes rely on a change in energy transfer efficiency between the donor and acceptor pair, which is reported as a change in PL intensity from the donor, the acceptor, or both. Changes in the distance between the donor/acceptor pair as well as changes in the spectral overlap between donor emission and acceptor absorption have been demonstrated as effective means of generating a probe response [5, 6, 8, 12, 20]. While the former is often utilized as an on/off reporter (i.e. either FRET is taking place or the donor and acceptor are too far apart for FRET to be observed), changes in spectral overlap are necessarily more subtle. Below, we cover the principle means of changing donor/acceptor distances, including conformational changes, cleavage, and binding/unbinding. Though addressed discreetly, substantial overlap exists among these categories, with some probes, for example, undergoing a conformational change as a result of a binding interaction. We further discuss the conditions under which changes in absorption can be observed and highlight some of the most commonly employed probe designs.

\textbf{Conformational changes} include rearrangements such as those that occur during micelle formation or protein folding and alter FRET signals by changing the distance between the donor and acceptor. Molecular beacons (MBs) represent an important class of FRET probes that rely on conformational changes and consist in their most basic form of a single strand of nucleic acids with complementary ends (see figure 2) [17, 66, 67]. While closed, the complementary nucleic acids are bound to form the ‘stem’ of the probe, and a donor and acceptor attached to either end of the strand are held in sufficiently close proximity to undergo FRET.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{mb.png}
\caption{(top) A molecular beacon consists of a nucleic acid strand with complementary ends that form a stem-loop or hairpin structure and is capped by a FRET donor/acceptor pair. The introduction of a strand of target DNA or a denaturing reagent causes the stem to melt and spatially separates the donor from the acceptor. (bottom) In a typical MB imaging experiment, ratiometric comparison between a control or reference probe (a and d) and the experimental probe (b and e) corrects for experimental variations, where dividing the MB image by the reference image (c and f) gives more reliable results than the MB alone. In the top panels, the analyte is absent from the cells and the MBs remain closed, while the bottom panels show cells in which the target analyte is present and the MBs are open. Reprinted from with permission from [67].}
\end{figure}
When the MB opens, for example when the ‘loop’ portion of the probe recognizes a complementary nucleic acid strand or the MB encounters a denaturing reagent, the donor and acceptor are spatially separated, and FRET ceases to take place. If two fluorophores serve as the donor and acceptor, this separation is observed as a cessation in PL from the acceptor. MBs commonly use a donor/quencher pair (often an organic dye and organic quencher), however, in which case the change in conformation is observed as the initiation of PL from the donor [66, 67]. Conformational changes are also exploited in other systems such as tethered small molecule dyes that are brought into proximity through complexation with a target analyte (e.g. chelation of a cation) or tethered FPs pulled together by the folding of a protein between them [8, 66, 67]. Conformational changes can be the result of the unbinding of complementary nucleic acids in the stem and can be initiated by binding of a complementary oligonucleotide in the loop.

Aptamers are single-stranded oligonucleotides with unique conformation that have been engineered for high affinity and selectivity in binding with target molecules, which range in type from proteins to metal ions to whole cells [71]. Thanks to the broad range of targets for which they are suited, aptamers can be used to deliver a probe to the cell or to a specific intracellular region or as a means of capturing and detecting...
For example, by labeling the aptamer with a fluorophore and introducing it into the cell with a loosely bound quencher, FRET prevents observation of PL until the aptamer dissociates from the quencher in order to bind the analyte, to which it has a higher affinity [73].

Modulation of the absorption of the FRET acceptor can arise from environmental sensitivity of the acceptor and can reflect changes in temperature, pH, potential, etc. [8]. For example, bromothymol blue exhibits a color change based on pH-dependent protonation or deprotonation of a hydroxyl group. The concomitant change in the compound’s absorption spectrum and thus its absorption coefficient at the donor emission wavelength has been exploited to change the probe’s energy transfer efficiency [22]. Absorption spectra of bromothymol blue in both protonated and deprotonated forms are shown in figure 5 [74].

2.5. Delivery of the FRET probe into the cell
Without a means of delivering a probe into a cell, detection of intracellular phenomena is a nonstarter. In addition to transferring the probe across the cell membrane, biocompatibility of the probe, toxicity, and, in some cases, intracellular targeting and probe size must be taken into consideration in the selection of appropriate fluorophores and scaffolding for the probe [75, 76].

Some of the probes already discussed have the advantage of intrinsic biocompatibility. MBs, because they are composed of nucleic acids, can readily exist in a biological environment without the need for additional functionalization, although their introduction across the cell membrane requires the use of pore-forming agents or cell-penetrating peptides [77]. FPs are often genetically programmed so that cells express them after plasmid or gene transfection, eliminating not only the need to functionalize them for biological compatibility but also the need to deliver them into the cell altogether. QDs, on the other hand, are commonly synthesized in nonaqueous solvents and passivated with long, aliphatic ligands. A wide range of biomolecules has been used to functionalize them for use in biological systems; for a comprehensive review of the current strategies we recommend the recent article by Sapsford et al [7]. Like QDs, Pdots, AuNPs, and graphene require special attention to their surface chemistry in order to render them biologically compatible [7, 44, 55, 78].

When a probe is designed for long-term use or with the intention of eventual use in vivo, toxicity becomes important. Toxicity studies vary widely in the cell line used, concentration of analyte, incubation time, etc., limiting the value of direct comparison. The examples cited here are not intended to serve as a comparison of one fluorophore to another but rather to give the reader a general sense of whether toxicity presents a substantial concern at concentrations comparable to those that might be used in intracellular detection. Many organic dyes, for example, result in extremely poor cellular viability (below 10%) within an hour of introduction into a cell [79]. In this vein, Bradburne et al performed a controlled study that demonstrated that under identical conditions, CdSe/ZnS QDs were substantially less toxic than the commercial live-cell labeling dyes tested (in many samples, cell viability was greater than 70% after 24 h), perhaps in part thanks to the wide bandgap inorganic ZnS shell used to improve QD quantum yield [79–82]. FPs have been shown to possess a measurable degree of cytotoxicity in spite of their biological (cellular) origin, with some engineered FPs actually used to generate controlled cytotoxicity [83]. The problem of phototoxicity and photo-induced cell death linked to lower (ultraviolet) wavelengths is also commonly discussed in the context of FPs and applies equally to other fluorophores that must be excited in this spectral region [84, 85]. AuNPs, which have received substantial

![Figure 5.](image-url)
attention for possible use in medical application, have been found to be relatively nontoxic, with many studies reporting 85% or greater cell viability after several days [86]. Pdots have been shown to have close to 100% cellular viability, even at loadings much higher than would be used in imaging, after incubation times of up to 24 h [87].

In cases where a specific organelle of the cell or a process that occurs in a specific region is being targeted, delivery of the probe becomes increasingly challenging. For example, one of the challenges in detecting changes in membrane potential lies in the delivery of voltage-sensitive dyes to the membrane. Without specific targeting of the dye to the membrane, substantial background can occur due to dye leaching into the cytosol, complicating detection of change in potential, a fact that underscores the advantage of relying on a FRET signal rather than on direct detection of a change in PL [88]. By using an acceptor that only fluoresces in hydrophobic environments like in the interior of a lipid membrane, the background problem is substantially mitigated [88, 89]. In other systems, such as the detection of analytes, the use of quenchers to eliminate background from poorly targeted probes provides another work-around.

Finally, the size of the probe becomes important under certain circumstances. While small molecular dyes largely skirt this problem, QDs and AuNPs can easily approach the tens-of-nanometers scale. Considering once again the example of imaging changes in cellular membrane potential and with the knowledge that the membrane dimension between phospholipid heads is approximately 5 nm, the challenge of matching the fluorophore size with the available space becomes apparent [90].

3. FRET-based probing of cellular physiology

Probing cellular physiology encompasses probing and measuring indicators of cellular homeostasis such as pH and temperature. Requirements for suitable probes depend on the intended application. Thermal probes that are intended to screen for cells with elevated metabolic rate, for example, require no specific targeting after introduction into the cell, and the rapidity of feedback they provide is only crucial in systems in which rapid temperature fluctuations are expected. In contrast to these somewhat lax requirements, a probe intended to measure the pH gradient across the endosomal trafficking pathway, for example, requires a relatively rapid feedback mechanism and benefits from additional means of targeting the correct cellular region [25, 91]. For detecting action potentials, which occurs on a millisecond timescale, rapid response becomes even more critical [92, 93]. In temperature and pH measurements, the probe must provide incremental feedback, whereas an on/off probe would be suitable for determining whether a cell is polarized or depolarized. Further, in addition to the basic biological compatibility required of all intracellular probes, the possibility of using these probes for long-term monitoring increases concern over cytotoxicity common in dyes [79]. Long-term monitoring also amplifies the value of ratiometric sensing, which provides an internal standard for validating the integrity of the probe over time.

3.1. Temperature

Because of the important role that temperature plays in kinetic rates and chemical equilibria, cellular temperature is inextricably linked to a wide range of intracellular processes [18, 94, 95]. For example, elevated cellular temperature, which correlates with an elevated metabolic rate, has been suggested as an indicator of carcinogenesis [95, 96]. Resulting interest has led to the development of thermometers to monitor temperature on a cellular and subcellular level, both to understand native processes and the effects of external stimuli such as thermal therapies aimed at cancer treatment [97–99]. While a wide range of intracellular thermometers have been reported, FRET-based temperature probes remain relatively rare in the literature in spite of the advantages they offer in sensitivity and reliability [18, 95, 100–104]. The FRET-based thermometers that have been reported to date exploit the intrinsic temperature sensitivity of some aspect of the probe, either in the donor or acceptor of the FRET pair or in temperature-dependent alterations to the scaffolding between the donor/acceptor pair.

The intrinsic temperature dependence of the melting point of double-stranded DNA, for example, suggests itself as a natural scaffold material for thermometry. Ke et al report an L-DNA molecular beacon with a donor and quencher tethered to the stem. The probe exploits the melting temperature of the hairpin structure to introduce separation between the FRET pair, resulting in an increase in donor (and therefore total) PL intensity with increasing temperatures, as illustrated in figure 6 [105]. The use of nucleic acids in the probe assembly ensures biological compatibility, while using the L-isomer rather than the naturally occurring D-isomer precludes interaction of the probe with the cellular environment (e.g. enzyme digestion, unintended hybridization with natural D-DNA). By varying the number of base pairs used in the hairpin assembly, the probe can be targeted to a specific temperature range, where resolution of 0.5 °C is achieved; or by mixing molecular beacons of varying lengths and using different fluorophores, a wider temperature range can be monitored. The probe’s lack of cytotoxicity was verified, and the durability of the probe was tested using cyclical heating experiments to determine the probe’s usable lifetime.

While this probe relies on a temperature-induced morphology change to disrupt FRET, a second strategy is to rely on constant energy transfer and instead exploit a native change in luminescence in one of the FRET participants. Ye et al reported a ratiometric temperature probe...
based on FRET between semiconductor polymer dots (Pdots) and rhodamine B (RhB) [46]. The RhB FRET acceptor in this system is a dye that exhibits decreasing PL emission intensity with increasing temperature. Because energy transfer between the Pdots and RhB is independent of the emissive state of RhB, PL from the Pdots remains constant, irrespective of the temperature of the system, while the emissive intensity from the acceptor serves as the ‘thermometer.’ In complex systems like cells and of particular importance in situations where monitoring is expected to be on-going, the ratio metric nature of the probe (here, the continuous PL of the Pdot as compared to the changing PL from RhB) serves as an important internal measure of the continued integrity of the probe.

3.2. pH
Because of its substantial relevance on both the cellular and subcellular level, determination of pH has been amply demonstrated in systems that rely on the pH sensitivity of organic dyes, charge transfer, or FRET [106–109]. Detection of acidification associated with cell apoptosis or carcinogenesis, for example, requires probes with similar attributes to those suited for intracellular thermometry; namely, they should be bright and nontoxic without the need for targeting specificity within the cell [110, 111]. For subcellular applications, however, both rapidity of probe response and targeting to specific intracellular environments become relevant. In either case, sensitivity to minor changes in pH is critical, with sensitivity of tenths of a pH unit demonstrated in the probes highlighted here.

Perhaps the most straightforward FRET-based pH probe is one that relies on a constant donor and a pH-sensitive acceptor. Such a mechanism is demonstrated in a coumarin fluorophore donor covalently coupled with a pH-sensitive amino-naphthalene derivative acceptor [112]. Under basic conditions, the amino-naphthalene group receives a lone pair of electrons via photoinduced electron transfer from another region of the molecule, a state in which it functions as a poor FRET acceptor, and the blue emission from the coumarin dominates. Under acidic conditions, protonation of the amino group restores energy transfer, and green emission from the acceptor dominates. The probe functions over a wide pH range (pH 4–10) with sensitivity of approximately 0.2 pH units and has been determined to preferentially localize in lysosomes. The same principle has also been developed using a Pdot FRET donor paired with a pH-sensitive fluorescein dye acceptor [113]. In this case, the dye was surface-loaded rather than embedded in the polymer, as was the case in the Pdot-based temperature probe.

A second strategy employs a pH-induced change in conformation to alter FRET rather than exploiting the inherent pH sensitivity of one of the FRET participants. As an example, closing of a dye-bearing block copolymer into a micelle at a targeted pH, driven by deprotonation of a tertiary amine and therefore an increase in hydrophobicity, brings pH-insensitive dyes loaded onto the polymeric matrix into close proximity and gives rise to homo-FRET, or energy transfer between identical dyes that results in quenching of dye emission [25, 26]. Unlike some of the other probes highlighted here, this one functions as an on/off switch, with emission either present below the pH transition or absent above it. By changing the pKₐ of the functional groups on the block copolymer, the authors demonstrate the ability to change the pH transition point, and using dyes of different emission wavelengths, they offer a means for a more complex measurement (i.e. a series of on/off switches), which they demonstrate across the pH gradient of the endocytic pathway, as shown in figure 7.

At the other end of the proverbial spectrum, Peng et al report a pH probe that relies on competitive FRET among three discrete dyes (bromothymol blue, Nile Red, and coumarin 6) loaded together into a hydrogel nanoparticle (NP) that allows for delivery into the cell (see figure 5) [22]. The pH sensitivity of bromothymol blue (BTB) arises from a color change (and therefore change in the absorption spectrum) from yellow below pH 6 to blue above pH 8. Selecting donor dyes with emission wavelengths corresponding to the absorption maxima of the yellow and blue forms of BTB, here Nile Red and coumarin 6, respectively, results in energy transfer from the dyes to BTB that depends on the absorption spectrum of the BTB. Thus at low pH, PL from coumarin 6 will be attenuated by the competing FRET process, while Nile Red relaxes primarily via radiative recombination; and at higher pH, the scenario reverses. The probe is found to respond to changes in environmental pH within several seconds and possesses sensitivity on par with the other probes described here.

In contrast to discrete donor/acceptor dye pairs, Dennis et al report a pH probe that employs as FRET acceptor a protein with a molar extinction coefficient that is maximized as pH approaches the protein’s pKₐ [91]. As a result, both the efficiency of energy transfer (and therefore attenuation of PL) from the QD donor and the protein’s emission intensity change with changing pH, while the isosbestic point of the emission data provides an internal reference. The authors demonstrate the probe over pH range 5–10, with the best sensitivity achieved between pH 6 and 8. One of the key advantages to this system lies in the ability to modify the protein, here mOrange, for intracellular targeting. In this case, a C-terminal polyarginine sequence facilitated endosomal uptake, evidenced by observing emission from the QDs; and a gradual decrease in FRET signal reflected a drop in pH from the endocytic vesicle to the late endosome (see figure 8).

3.3. Membrane potential
Imaging membrane potential is of particular relevance in neurons, where visualizing neuronal action potential is most pertinent to networks of neurons rather than to individual cells; the same principles are equally applicable to networks of muscle cells.
The seminal paper in this field employed oxonol, a charged molecule that emits only in hydrophobic environments, as the FRET acceptor and a Texas Red-labeled wheat germ agglutinin on the external surface of the membrane as the donor. The charged oxonol’s migration through the membrane upon depolarization resulted in a change in the donor/acceptor distance and in the emission of the acceptor, as shown in figure 9 [89]. Since then, the same principle has been updated with more responsive fluorophores, including genetically encoded FPs in the membrane that serve as the FRET donor [88, 93, 114].

Figure 6. (a) Temperature-sensitive probes in HeLa cells at 20 °C (left) become markedly brighter with an increase in temperature to 37 °C (right). (b) The basis for increased PL from the probe lies in an L-DNA MB. The complementary ends adopt a hairpin structure that holds a FRET donor/quencher pair in proximity below the melting temperature (Tm), while above the melting temperature, the DNA unwinds, resulting in emission from the donor. (c) A plot of the intensity of PL from the probe against cell temperature demonstrates the probe’s dynamic range, while its sensitivity is highlighted in the temperature resolution achieved over that range. Reprinted with permission from [103]. Copyright 2012. American Chemical Society.

Figure 7. Using a mixture of probes that transition from bright to dark through changes in conformation resulting in homo-FRET quenching, the pH gradient of the endocytic pathway was monitored as a function of the time. Reprinted with permission from [25]. Copyright 2012. American Chemical Society.
The use of so-called voltage sensitive fluorescent proteins (VSFPs) relies on changes in the conformation of the tether between FPs rather than on distance created through electrostatic interactions [48]. Conformation changes may be induced by the presence of Ca$^{2+}$, released upon depolarization and therefore an indirect indicator of neuronal action potential, whereas the alternative voltage-induced conformational change provides a more direct measure (figure 10) [115]. A wide range of FPs have been tested to achieve maximum changes in donor/acceptor PL ratios upon depolarization [48].

This section is divided into four parts, the first three of which focus on organic molecules, including biomicromolecules (4.1), endogenous biomacromolecules (4.2), and exogenous biomacromolecules (4.3). The final section covers inorganic ions and molecules (4.4). Note that we limit our discussion here to probes that target a single species at a time. Establishing the proximity and interaction of multiple species will be discussed in section 5, where we look at FRET-based probes designed to detect processes rather than presence.

4.1. Biomicromolecules

The first step in understanding the role of small molecules in intracellular processes is to understand whether and in what quantity they are present in a cell. Wang et al., for example, report a dye/quencher FRET mechanism based on a graphene oxide substrate with which they demonstrate the detection of intracellular ATP [73]. Capitalizing on the development of aptamers that have already been designed with extraordinary affinity for various target analytes, the researchers tether a fluorophore to, in this case, an ATP aptamer. The aptamer readily adsorbs to the surface of a graphene oxide nanosheet, and energy transfer between the fluorophore and the graphene oxide results in PL quenching. The graphene nanosheets and their payload are absorbed into the cell upon incubation, and the presence of ATP results in the release of the aptamer.

Figure 8. (a) QD/mOrange pH probes exhibit a decrease in FRET intensity as the pH drops from the endocytic vesicle through the endosome. (b) PL from QDs demonstrates endosomal uptake of the probe within two hours of introduction to the cell. Direct excitation of mOrange shows that the mOrange remains emissive. The dramatic decrease in FRET signal after two hours is indicative of the pH dropping. Reprinted with permission from [91]. Copyright 2012. American Chemical Society.
from the graphene in favor of the analyte. Once the aptamer/fluorophore/ATP complex is sufficiently distant from the graphene oxide that FRET no longer takes place, fluorescence in the system is observed. Not only does this system show excellent specificity for ATP, but the specificity demanded from aptamers more generally suggests that this probe configuration could be applied to a wide variety of analytes.

Deng et al. also rely on a quenching mechanism for intracellular detection of glutathione [116]. The probe consists of a NaYF₄ NP doped with Yb and Tm, the luminescence of which is quenched by energy transfer to MnO₂ nanosheets that decorate the surface of the NP, as shown in figure 11. The addition of glutathione to the system reduces the MnO₂ to Mn²⁺ through the oxidation of the sulfide moiety to form a disulfide bond. The resultant decomposition of the nanosheets leads to emission from the NPs. Testing with a variety of biomolecules and biologically-relevant inorganic ions demonstrated the specificity of the probe.

Cyclic guanosine monophosphate (cGMP) and cyclic adenosine monophosphate (cAMP) both act as second messengers in the cell, and their importance in triggering intracellular processes has made them frequent targets of FRET probes using FP donor/acceptor pairs [15, 68, 117, 118]. By using an isolated nucleotide binding domain with a FP donor on one end and FP acceptor on the other, the conformational change...
induced by recognition of the nucleotide of interest initiates the FRET response. Optimization of the nucleotide binding domain and the relative importance of second messengers in the intracellular environment has led to the continued development of these probes [118].

4.2. Endogenous biomacromolecules

Because molecular beacons (MBs) rely on recognition among nucleic acids to maintain or interrupt the integrity of the hairpin, they are particularly well-suited to the detection of analytes that are themselves composed of nucleic acids. Previously described in detail in section 2.4, a molecular beacon that recognizes a series of nucleic acids complementary to its own will bind the complementary strand, resulting in melting of the stem and spatial separation of the FRET donor and acceptor (often a dye and quencher). As a result of the facility and reliability of the nucleic acid recognition, MBs have been a staple of single-strand DNA and RNA detection for years [14, 17, 66, 67, 119, 120]. Protein recognition of specific nucleic acid sequences means that molecular beacons can also be used as probes for the presence of proteins [67]. Similarly, exploiting the usual action of telomerase on nucleic acid-based biomolecules has rendered MBs suitable for telomerase detection and quantification [121].

Building on the classic MB design, Qiao et al. recently reported two different molecular beacons loaded onto a AuNP, allowing for simultaneous detection of multiple mRNA strands [122]. The AuNP functions both as a delivery mechanism and a quencher on one end of the MB stem, while the other end of the MB stem holds one of two discrete fluorophores (fluorescein isothiocyanate, FITC, and cyanine 5, Cy5), each associated with a MB sensitive to a different analyte (see figure 12). Hybridization of the target analyte with the MB removes the fluorophore from proximity to the AuNP, and the emission wavelength can be used to ascertain which target is present. In this case, the researchers suggest that the detection of multiple types of tumor mRNA reduces false positives in cancer diagnostics, but the principle of multiplexed MBs can be applied much more broadly.

Qiu et al. demonstrate even more imagination in their design of an MB-based mRNA probe by adding...
components that target specific cell types, facilitate transport into the cell, and delay activation of the probe [72]. In order to accomplish this, their MB is designed to hybridize with an aptamer (AS1411) that exhibits specific binding to nucleolin, a protein that is overexpressed on tumor cells. When the nucleolin crosses the cell membrane, the aptamer with the MB in tow piggy-backs into the cell’s cytoplasm. By inserting photocleavable linkers into the aptamer in the region where it hybridizes with the MB, the researchers create a means of releasing or photodecaging the MB from the aptamer/protein complex. Once released, the MB adopts a hairpin structure in the absence of the target analyte, and the usual dye/quencher FRET process between cyanine 3 (Cy3) and Black Hole Quencher 2 (BHQ2) leaves the probe dark. In the presence of the analyte, however, the MB instead hybridizes with the target, and emission from the dye is observed. Terminating the MB-hybridized end of the aptamer with a second dye allows FRET-based verification that the

Figure 12. (a) MBs suited to different targets and tethered to different dyes (fluorescein isothiocyanate, FITC, and cyanine 5, Cy5) are loaded onto a AuNP, which acts as both the delivery mechanism of the probe into the cell and the FRET-based quencher. When the MBs recognize the target analyte, the resulting spatial separation between the quencher and fluorophore gives rise to emission, the color of which identifies which of the targets was recognized. (b) Increased fluorescence intensity from FITC as a result of the detection of target 1 by MB1 is observed over a wide concentration range, while changing even a single base pair in the target dramatically decreases the response, reducing false positives. (c) Detection of both targets, one resulting in red emission and the other in green emission, is obvious in cells containing the two target types, with a combined image showing overlap between the two targets. Reprinted with permission from [122].

Figure 13. (left) The aptamer (AS1411) and hybridized MB enter the cell through binding of the aptamer to a protein, nucleolin, on the cell membrane. Upon illumination, two photocleavable sites on the aptamer release the MB, which either hybridizes with the target analyte or adopts the inert hairpin structure. (right) Confocal microscopy images of cells after indicated length of exposure time to photocleavage stimulus, with MB-Cy3 fluorescence on the left and Cy5 on the right. Reprinted with permission from [72]. Copyright 2013. American Chemical Society.
MB/aptamer complex has remained intact in its entry into the cell, and termination of that FRET signal evidences the successful photodecaging of the MB from the aptamer. In spite of a complex sequence of events, which are illustrated in figure 13, the result is elegant: the probe is delivered into the targeted cells and activated only when illuminated.

4.3. Exogenous biomacromolecules

While endogenous biomacromolecules are interesting largely for understanding how normal cell operations proceed, tracking exogenous biomacromolecules is important in designing biologically-based therapies. For example, small interfering RNA (siRNA) exists both natively in the cell and can be used therapeutically in gene silencing. In the latter case, because siRNA must be delivered into the cell, understanding the release of the siRNA from the delivery system (e.g. NPs) and the subsequent migration of the analyte through the cell is critical to optimizing the therapy. One means of accomplishing this is to use an upconversion nanoparticle (UCN) as both the delivery system and the FRET donor, while intercalation of an emissive dye, BOBO-3, into the siRNA acts as the FRET acceptor [38]. By using NaYF4 UCNs doped with lanthanides (here, either Yb/Er or Yb/Tm), the system is designed to function using upconversion of a near-IR excitation source prior to energy transfer to the FRET acceptor, as shown in figure 14. By observing the FRET efficiency in the system as a function of time, detachment of the siRNA from the UCNs can be monitored, and direct excitation of the dye allows for tracking the intracellular location of the siRNA after release.

4.4. Inorganic species

Inorganic compounds found in cells range from naturally ubiquitous ions such as Ca\(^{2+}\), critical for cellular function, to Hg\(^{2+}\), which is toxic and thankfully rare in cells. Because of the wide variety of ions found in cells and because these probes are not all able to capitalize on the same biological affinity that is found, for example, in the detection of RNA (section 4.2), the FRET-based strategies for ion detection are wide-ranging.

Since single ions can readily engage in chemistry with small molecule fluorescent dyes, the use of two covalently bound dyes in which the acceptor is activated by the presence of the analyte has been recently applied in intracellular detection of Cr\(^{3+}\), Hg\(^{2+}\), HNO, and many other species [8, 123–126]. Activation of the acceptor may occur through a conformational change induced by multidentate coordination of the dye to the analyte [125] or through chemistry (rearrangement, cleavage, etc.) catalyzed by the analyte that activates PL in the acceptor, as shown in figure 15 [123, 124]. Huang et al recently reported an intracellular fluoride probe that, like many of the dye/dye probes, relies on the ability of the analyte to deprotect (and thereby activate PL from) a coumarin dye [45]. In this report, however, the dye is loaded into a Pdot, which serves not only as a FRET donor but also provides a suitable matrix for introduction of the probe into the cell via endocytosis while insulating the cell from any cytotoxic effects of the dye.

A cornerstone of FPs in FRET-based intracellular detection of inorganic compounds is Miyawaki’s et al seminal report of Ca\(^{2+}\) detection using two FPs tethered by a calcium-modulated protein (‘calmodulin’ or CaM) [127]. Thanks to the critical role of Ca\(^{2+}\) in intracellular signaling, the biological infrastructure for a conformation change in CaM triggered by the binding of four Ca\(^{2+}\) ions comes with built-in selectivity. The addition of FPs to either end of the CaM used in this example provides a straight-forward route to the observation of FRET in the presence of Ca\(^{2+}\), and mutations to the CaM tether to tune Ca\(^{2+}\) affinities gives rise to a wide dynamic detection range. A variety of suitable Ca\(^{2+}\) binding molecules and substantial interest has led to a wide array of subsequent probes for Ca\(^{2+}\) and other ions, such as Zn\(^{2+}\), based on the same principle [128, 129].

Rather than a conformational change in the tether between two FPs, Kuner et al report a change...
in the emission intensity of a yellow FP in response to Cl\textsuperscript{−} concentration \[130\]. Using a cyan FP as a donor that is coupled to the yellow FP by a flexible peptide linker, the quenching of the acceptor PL results in a change in the ratio of donor/acceptor signal. The probe also responds to other halides, to nitrate of sufficient concentrations, and to Cd\textsuperscript{2+}, as well as exhibiting some pH response, but in spite of these limitations, this chloride probe has been successfully demonstrated in imaging synaptic inhibition, of which the presence of Cl\textsuperscript{−} is an indicator \[131\]. More recently, this same principle has been modified to detect intracellular levels of O\textsubscript{2} \[132\].

5. Detecting processes

In their overview of the benefits and drawbacks of using FPs in FRET configurations, Piston and Kremers draw an analogy that bears repeating \[13\]. Keeping in mind not only the limitations of typical laboratory equipment but also the fundamental resolution limitations of visible light, traditional fluorescence experiments are limited in resolution to the several hundred nanometer scale. In an intracellular environment, knowing that two species are within a hundred nanometers of each other is similar to knowing that two students are both present in a large lecture hall; it leaves us no way of knowing whether the students are having a conversation \[13\]. In order to know with certainty that intracellular species are interacting, the resolution limitations of light are simply not enough, but the length scale indicated by energy transfer is.

Information on molecular proximity is critical for elucidating the nature and mechanism of cellular processes. Such processes are dynamic events, in which cellular location, microenvironment, and local concentration are in constant flux. Tracking the movement of species through the steps of a mechanistic pathway gives valuable insight into the nature of these interactions and provides a deeper understanding of the complex inner workings of the cell. Many of the aforementioned FRET probes have been used to study these dynamic processes, although fluorescent proteins remain the most widely used. In addition to their widespread availability and biocompatibility, FPs are able to be genetically encoded as a fusion with the protein in question \[133, 134\]. This not only guarantees attachment of the fluorescent tag to the protein, but also enables tracking of the protein in both a spatial and temporal manner.

This section will cover the use of FRET probes for the following biochemical and biophysical processes: protein–protein interactions, cellular uptake and unpacking, molecular trafficking and localization, and global processes.

5.1. Protein–protein interactions

As compared with more ‘static’ uses of FRET detailed above, protein–protein interactions introduce additional challenges for detection. They may be influenced by a variety of factors, including cellular location, expression, and complex pathways of regulation. As such, information regarding these interactions can provide crucial details of the mechanisms and interactions that make up cellular biochemical pathways. Monitoring protein–protein interactions using FPs has been the focus of a number of review articles \[10, 15, 68, 135, 136\]. A few notable examples of this comprehensive groundwork
highlight the diversity and capabilities of FRET-based interactions. Phillip et al. ascertained the association rate constant of β-lactamase to β-lactamase inhibitor protein [137]. Through transient transfection, β-lactamase was expressed in HeLa cells fused to a cyan donor FP. The protein inhibitor was fused to a yellow FP and introduced into the cell through microinjection. Association of the two was observed in the growing FRET signal (figure 16), and quantification and mathematical treatment of the data permitted the determination of the association constant of the protein and protein inhibitor.

Another example in HIV research used FRET to demonstrate the association of CCR5/CD4 receptors on the plasma membrane with the glycoprotein gp120 to initiate viral entry into the cell. Here, Yi et al. showed that these interactions are mediated by the presence of cholesterol [70]. By expressing each receptor as a fusion protein with one member of a FRET pair (CCR5-cyan FP and CD4-yellow FP), their membrane location could be monitored both separately and in relation to each other. Under typical conditions, CCR5-CFP and CD4-YFP receptors remained in separate microdomains uniformly distributed within the plasma membrane. The addition of an extracellular ligand that was able to bind both receptors, here the HIV protein gp120, brought the receptors into proximity to form aggregates, a process revealed by CFP-YFP FRET (figure 17). However, a subsequent addition of a cholesterol depletion detergent, methyl-β-cyclodextrin (MβCD), showed a marked...

Figure 16. (a) In a typical experiment to determine protein–protein interaction kinetics, one protein is expressed in the cell with a FP fused to it, while the second protein, also carrying a FP tag, is introduced into the cell through microinjection. FRET is initiated when the two proteins interact. Reprinted with permission from [135]. (b) Confocal microscopy shows the expressed protein (FRET donor), the introduced protein (FRET acceptor), and transmission images taken before introduction of the second protein and 1 s and 30 s after introduction. In this case, the region highlighted in red was used for quantitative analysis. Reproduced with permission from [137].

Figure 17. Yi and colleagues used FRET to determine the co-localization of two cellular receptors, CCR5-CFP and CD4-YFP, in the presence of gp120 and cholesterol. The removal of cholesterol with MβCD resulted in a low FRET baseline, and the baseline remained low when the cells were incubated with two gp120 variants. Only the re-addition of cholesterol showed a significant increase in FRET. Adapted from [70].
5.2. Cellular uptake and unpacking

While monitoring protein–protein interactions can shed light on individual molecular association, binding, and interaction, FRET also enables tracking of a target molecule as it proceeds through a biochemical pathway. This information is critical not only for determining the fate of the target molecule (e.g. confirming whether it reaches its projected intracellular destination) but also for ascertaining the route—intended or unintended—that was taken to get there. One of the most physiologically and pharmacologically important processes is that of cellular uptake and endocytosis. Elucidating the underlying biophysical mechanisms of this process has wide-ranging implications, from extracellular environmental sensing to cargo delivery for targeted chemotherapy. Using FRET to determine the major factors responsible for membrane binding and fusion has greatly expanded our knowledge of the endocytic process. It has played an instrumental role in differentiating among various endocytic mechanisms, including rab5 activity [138, 139], receptor-mediated [140], caveolin and between clatherin-dependent [141, 142] and independent pathways [143]. Understanding the molecular mechanisms of endocytosis also provides critical insight as to the molecular basis of disease and specific events such as the process of viral fusion. In one impressive demonstration, FRET was used to create a 3D video of HIV translocation through cell–cell adhesions called virological synapses [144]. Viral membrane fusion has been shown through FRET-based experiments for many viruses, including HIV [145], adenovirus [146], and hepatitis C [147], among others.

Many groups have also sought to utilize the endocytic process for the uptake and unpacking of synthetic complexes carrying molecular cargo. Predominant methods for cargo delivery include either the use of cationic liposomes (lipoplexes), cationic polymers (polyplexes), or the use of QDs. Cargo molecules have included plasmid DNA (pDNA) for non-viral gene therapy [148, 149], siRNA [150, 151], and chemo-therapeutic compounds. The use of QDs and other NPs as a platform for FRET-enabled tracking of cargo delivery and unpacking has been thoroughly reviewed [152–157]. Such complexes must be tracked to ensure efficient membrane fusion and internalization, proper unpacking in the endosome, and intracellular trafficking to the proper target organelle, while complexes vary based on the biochemical makeup of the delivery vector, cargo, and delivery location, as well as the biomolecular delivery pathway. FRET has been shown to be highly useful in determining these specific and localized interactions, necessary for the characterization of delivery of lipoplexes [158], as well as a comparison of lipoplex versus polyplex efficiency [159]. Vector delivery has been achieved through various mechanisms: For example, several groups have demonstrated the use of cell-penetrating peptides (CPPs) as a mechanism for introducing QDs into cell [160–163]. Alternative delivery pathways include clatherin-mediated endocytosis [164], multiple-membrane fusion [165], or physical delivery methods [166, 167].

In addition to the analysis and characterization of delivery vectors, FRET has also been used extensively to analyze various cargo delivery and targeting. Non-viral pDNA gene therapy has been one of the prominent beneficiaries of FRET-based analysis [168]. Characterization includes packing efficiency and fidelity [169, 170], decondensation kinetics [171], and determination of various internal processing steps of pDNA localization from the endosome/lysosome to nuclear compartments [172–175]. While most cellular analysis is done using fluorescence microscopy, flow cytometry has also been demonstrated [176]. The delivery of siRNA for anti-tumorigenic gene silencing therapy is another rapidly expanding field. Various QD-siRNA complexes have been tested, and the delivery and unpacking of these nanocomplexes has been extensively characterized with FRET [173, 177–179]. Finally, chemotherapeutic compounds have also been explored using QDs, NPs, and FRET. Here, the rate of release/unpacking of compounds from their NP complexes and therefore the delivery of the compound to the cell becomes critical. Many groups have explored these rates [180], characterizing various compounds and quantitating rate and nature of release such as doxorubicin (DOX) and DOX-aptamers [181, 182]. Combining technologies, such as the delivery of both siRNA and DOX, may also overcome limitations by synergistic augmentation of treatment strategies [183].

5.3. Molecular trafficking and localization

As we have seen in the previous section, FRET is highly useful for following the trafficking and localization of a cargo through the endocytic pathways. Given the diversity of the aforementioned literature, it is evident that even a single physiological function of a cell, in this case endocytosis, is a complex network of biochemical pathways carried out by numerous protein interactions. It is not surprising, therefore, that FRET has been widely used throughout the literature to track the movement and location of proteins in the cell for nearly all relevant pathways [184–187]. Here we examine some select examples to highlight the power and utility of FRET characterizations.

5.3.1. HIV virion assembly

As knowledge of viral protein pathways within the cell is essential to elucidate the mode of infection, replication, and transmission, FRET offers a straightforward means for interrogating the nature of these interactions. In one prominent example, FRET and related fluorescent techniques enabled researchers to follow the biogenesis
process of HIV virions in real-time. By monitoring the expression and location of the Gag protein, the major structural component of HIV, via a GFP fusion tag, Jouvenet et al showed that individual virions were released at the plasma membrane and demonstrated accelerated assembly as the intracellular Gag protein concentration increased, taking on average 5–6 min to form [188].

5.3.2. Nucleic acid detection
In order to directly monitor the cellular processes involving RNA, such as siRNA and mRNA, hybridization-based probes (i.e. MBs) are often most useful [14, 77, 119]. For a comprehensive review on the use of FRET-based probes for studying oligonucleotide-centered processes, we refer the reader to reviews by Didenko and Dirks and Tanke [14, 189]. However, many processes involve the interaction of DNA and proteins, which are uniquely challenging to detect. Whereas attaching FPs to proteins is simply a matter of gene expression, coordinating FPs with oligonucleotides requires a more creative approach. FPs fused to proteins that bind specific oligonucleotide sequences such as telomeres or centromeres, for example, provide a means of attaching the FP to an endogenous nucleic acid [189]. An alternative approach was shown by Cremazy et al, in which a DNA-binding protein was tagged with GFP, and FRET between the protein and DNA labeled with the intercalating dye SYTOX Orange demonstrated localization [190].

5.4. Global processes
5.4.1. GTPases
While FRET can be used to detect protein and nucleic acid-based interactions and trafficking and localization through individual cellular pathways, it has also been used in monitoring the activity of proteins in whole cell events, such as the cell cycle, cytokinesis, and cancer progression. Of particular interest is the activity of GTPases, which regulate many signal transduction pathways in these global processes and have been monitored using FRET probes by a number of groups [191–197]. For example, using genetically encoded Ras and interacting protein chimeric unit (Raichu) probes based on fluorescence activation indicator for Rho proteins (FLAIR) probes originally developed by Hahn and colleagues, the Matsuda group demonstrated in vivo monitoring of the activity of several GTPases [197–200]. These two domain probes enable the sensitive detection of structural and activity changes of the regulator molecules of guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs), and guanine nucleotide dissociation factors (GDIs), as well as the activity of the small GTPase itself. Moreover, these probes enable the spatiotemporal tracking of GTPase activity, offering insight into their complex role in these global processes, as shown in figure 18 [199].

5.4.2. Apoptosis
Another global process whose monitoring has been assisted by FRET analysis is apoptosis, an activated cellular response for programmed cell death. Once apoptosis is activated in the cell, typically through external activation of a cell’s death receptors such as TNF-α or Fas, procaspase proteins are cleaved to yield their activated caspase form, a process tightly regulated by the Bcl-2 family of proteins. Insight into this complex yet important cell process has been significantly aided by FRET probes. FRET has been used to demonstrate the pathway used in tumor apoptosis and to monitor variations in cellular response to TNF-α signals [202, 203]. Caspase activity has also been extensively probed with FRET [204–211]. More recently, various groups have explored NP-based FRET for the induction of apoptosis in cancer tumor cells [212, 213]. For example, endocytosis of peptide-labeled AuNPs has...
been targeted with the intention of inducing apoptosis. Using Rhodamine B as the FRET donor and AuNP as a quencher, FRET prevents emission from the donor until active caspase enzymes cleave the peptide bond between the fluorophore and the AuNP, as shown in figure 19 [214].

6. Outlook

By providing a more sensitive and robust means of detecting intracellular physiology, species, and processes, FRET-based probes have already handily demonstrated their utility. The complexity of the cellular environment, however, leaves ample room for further exploration. As some of the techniques highlighted above demonstrate, increasingly creative means of probe design have enabled expansion from simple detection schemes to more active and comprehensive systems that not only engage in analyte detection but also add the means to target specific kinds of cells or deliver drugs. Indeed, it seems likely that as more and more cellular processes are elucidated using increasingly advanced FRET-based probes, the incorporation of secondary and tertiary functionalities (i.e. targeting and detection; targeting, detection, and drug delivery) to the probe’s scaffold will become more prevalent [215, 216].

On a more fundamental level, the current status of intracellular FRET-based detection still leaves ample room for the development of new combinations of fluorophores. For example, conjugating FPs to QDs capitalizes on the biological compatibility of the FP, providing not only an avenue into the cell but building a degree of targeting into the probe, while simultaneously exploiting the excellent photophysical properties of the QD [217]. Indeed, in the quest for better probes, even the basic library of FRET fluorophores will likely change. The recent development of near-IR emitting fluorescent gold nanoclusters, for example, could well lead to uses for AuNPs in intracellular sensing that go beyond quenching [218]. Long-lifetime metal chelates and time-gated fluorimeters open the door to using QDs not as FRET donors but rather as acceptors, a role in which their broad absorption spectra and high absorption

Figure 19. Endocytosis of peptide-labeled AuNPs for the induction of apoptosis. Here, red fluorescence is achieved only when active caspase enzymes are able to cleave the peptide bond between the Rhodamine B fluorophore and the AuNP, resulting in a loss of FRET. AuNP entry is dependent on the appropriate cellular receptor, demonstrated in the KB cells (A, B) that contain the receptor, and no entry into the control COS7 cells (C, D) that lack the necessary receptor. Mitochondria are stained green. Reprinted with permission from [214].
cross sections can be used to full advantage \[219\]. The exploration of new donor/acceptor combinations and the expansion of the fluorophore library will ultimately result in FRET pairs that are less toxic, more easily introduced into cellular environment, better able to resist degradation over time, less susceptible to photobleaching, and even more sensitive and robust.

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Competing interest

The authors have declared that no competing interests exist.

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