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MILESTONES IN THE DEVELOPMENT AND IMPLEMENTATION OF FRET-BASED SENSORS OF INTRACELLULAR SIGNALS: A BIOLOGICAL PERSPECTIVE OF THE HISTORY OF FRET

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Abstract

Förster resonance energy transfer (FRET) has been described for more than a century. FRET has become a mainstay for the study of protein localization in living cells and tissues. It has also become widely used in the fields that comprise cellular signaling. FRET-based probes have been developed to monitor second messenger signals, the phosphorylation state of peptides and proteins, and subsequent cellular responses. Here, we discuss the milestones that led to FRET becoming a widely used tool for the study of biological systems: the theoretical description of FRET, the insight to use FRET as a molecular ruler, and the isolation and genetic modification of green fluorescent protein (GFP). Each of these milestones were critical to the development of a myriad of FRET-based probes and reporters in common use today. FRET-probes offer a unique opportunity to interrogate second messenger signals and subsequent protein phosphorylation – and perhaps the most effective approach for study of cAMP/PKA pathways. As such, FRET probes are widely used in the study of intracellular signaling pathways. Yet, somehow, the potential of FRET-based probes to provide windows through which we can visualize complex cellular signaling systems has not been fully reached. Hence we conclude by discussing the technical challenges to be overcome if FRET-based probes are to live up to their potential for the study of complex signaling networks.

Keywords

Förster resonance energy transfer; FRET; fluorescent proteins; second messenger signaling; cAMP; cGMP; calcium; phosphorylation; microscopy

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INTRODUCTION

The second half of the 20th century brought both discovery and advancement in our understanding of second messenger signaling systems. [1–4] Early biochemical and physiological experiments provided a framework for understanding the molecular pathways and complex interactions between these signaling systems. Advancements were facilitated by development, implementation, and standardization of technologies to measure enzyme and ion channel function in *in vitro* and *ex vivo* settings. [5–15] Toward the end of the 20th century, Roger Tsien and colleagues developed a Förster resonance energy transfer (FRET) based approach to detect changes in cAMP in living cells. [16] Based upon this achievement many investigators predicted that the 21st century would lead to an unprecedented utilization of FRET. Indeed, a PubMed search of the terms ‘FRET’ and ‘fluorescence’ reveals that in the year 2000 fewer than 1000 studies utilizing FRET were published, whereas in 2019 more than 13,000 studies utilizing FRET were published. However, in these two decades we have made little progress in the assessment of second messenger signaling pathways in three spatial dimensions (x, y, z) using FRET-probes expressed in *in vitro* cell systems, *ex vivo* tissue preparations, or *in vivo* animal models. Similarly, while great strides have been made in targeting FRET probes, the ability to assess changes in localized signals, rather than spatially averaged signals, has lagged behind. To understand the limitations of both FRET measurement and FRET-based probes in the study of cellular signaling systems, we must first examine the theoretical and technological advancements that led us to this point, and second, identify the significant hurdles that must be overcome to develop FRET-based approaches with which we can interrogate intracellular signaling systems.

FRET is a phenomenon in which a donor chromophore, the part of a compound responsible for color, in an excited state transfers energy non-radiatively to an acceptor chromophore through resonance coupling. The efficiency of this energy transfer is highly dependent upon the distance between chromophores as well as the overlapping emission and excitation spectra of donor and acceptor chromophores. To better understand this phenomenon, it is often useful to consider a common analogy used to visualize FRET. [17] Imagine one tuning fork struck sufficiently close to another. If the frequencies are the same, the second fork will begin to vibrate at the same frequency as the first. However, if the distance is too great or the frequencies are not the same, little to no energy transfer will be possible or detectable.

The theory of FRET was developed over the first half of the 20th century, but it was not until the early parts of the 21st century that FRET began to live up to its potential as a tool for the visualization of intracellular signaling pathways. [18–20] Recently, FRET has given us insight into localized signaling events [21–26], protein-protein interactions [27–29], protein localization [30–32], and even allowed us to detect synaptic plasticity. [33] We discuss the development of FRET theory, the implementation of FRET as a biological tool, current FRET uses, and speculate on the future of FRET technologies. We will see that three major scientific milestones were necessary for FRET to emerge as an invaluable tool in biological studies, including the theoretical description of FRET, the concept of FRET as a molecular ruler, and the isolation, cloning, and genetic modification of green fluorescent protein (GFP).

FIRST OBSERVED FLUORESCENCE ENERGY TRANSFER

The story of FRET begins at the first observation of energy transfer across miniscule distances. A classic experiment by Cario and Franck in 1922 explored the excitation of a vapor composed of mercury and thallium atoms. [34] Their vapor mixture was excited at a wavelength of 253.6 nm, which was sufficient to excite only the mercury atoms, but the observed fluorescence emission was that of thallium. To illustrate using the tuning fork analogy, when the mercury fork was struck, the thallium fork vibrated. Cario and Franck determined this to be a transfer of energy from the excited mercury atoms to the thallium atoms. This observation triggered a cascade of similar experimental models in which atoms, excitation wavelengths, and several other parameters were varied. Over the next decade, additional experimental data were collected without a solid theoretical explanation.

EARLY ATTEMPTS TO ESTABLISH A THEORY OF FRET

In the 1920's, the field of physics was ripe with new ideas. All of the foundation was available to explain FRET, but no one had solidified a comprehensive theory. Between 1925 and 1928, several scientists published works that asserted different aspects of the currently accepted theory, but no single proposed theory agreed with the available experimental data. A large part of the final theory was established when an initial description was presented in a paper by Kallmann and London in 1928. [34] Their equations fit the experimental data up to that point for atoms in vapor, but were insufficient when applied to energy transfer in solution.

In 1925, before even the initial vapor papers were published, Jean Baptiste Perrin published the first of two papers detailing a theory that would account for energy transfer, regardless of the medium. [34] Unfortunately for Perrin, the necessary quantum mechanics to describe energy transfer were still being developed. The largest error in Perrin's theory of FRET was the assumption of *exact* resonance. Using our illustration again, Perrin assumed that the tuning forks needed very specific location and alignment parameters to allow energy transfer. Without the new quantum mechanics, Perrin was left with an incorrect factor of distance dependence. In addition, Perrin's theory did not account for the solution medium itself. Though he considered a liquid solution as opposed to a vapor, he assumed that the solution acted more as a virtually inert transfer medium and ruled out molecular interactions with the solution itself as a factor in energy transfer. This assumption is analogous to ignoring the need for air between the tuning forks as a transfer medium for the sound waves.

In the mid 1920's Heisenberg, Schrodinger, and Dirac proposed novel versions of quantum mechanics, described in [35–37]. Nobel Prizes in Physics were awarded to Heisenberg (1932) 'for the creation of quantum mechanics', and Dirac and Schrodinger (1933) 'for the discovery of new productive forms of atomic theory'. Concepts from quantum mechanics allowed Francis Perrin, son of Jean Baptiste Perrin, to make the next theoretical breakthrough in the understanding of FRET. Francis Perrin was a pioneer in the field of fluorescence and had intimate knowledge of the newly developed quantum theory. Having written his dissertation on the subject of his father's Nobel Prize in Physics (1911), it is not surprising that he became involved in his father's work on FRET. His 1932 and 1933

attempts at a theory of FRET were based largely on his father's work. Being well versed in quantum theory, he factored in new concepts that affected small molecules. His description of FRET accounts for changes in overlapping spectra that take place during interactions with the solution, but the *exact* resonance idea persisted. The resultant theory still left the distance dependence too large and the transfer efficiency too high.

THE IMPACT OF WORLD WAR II ON DEVELOPING FRET THEORY

The war placed enormous amounts of stress on scientific research, including any progress on FRET. Several prominent physicists were fired, relocated, or had their research redirected. In 1933, non-Aryans in German provinces were removed from their work positions, no matter their academic status. This directly affected Kallmann and London, who lost their research positions at the Fritz Haber Institute and the University of Berlin, respectively. Franck moved his family from Germany to the United States. [38] In 1940, Jean Perrin was appointed president of a committee for scientific research by the French government to help the war effort. Perrin fled France when German forces invaded in 1941 and ultimately made his way to the United States where his son was a professor of physics and mathematics at Columbia University.

Theodore Förster, however, was free to carry out research on FRET theory, having joined the Nazi party in 1933. The vacancies left by the removal of other physicists along with his more prominent Aryan status allowed far less restricted research. By 1940, Förster became a professor at the Max-Planck-Institute for Physics in Göttingen. It was around this time that he published the first of several papers on his FRET theory. [39]

THEODORE FÖRSTER FINALIZES THE THEORY OF FRET

The final contribution to FRET theory was also by Förster. His original 1946 paper is largely influential and is one of the only papers in this field to boast an entire English translation. [40] Förster's initial interest was due to photosynthesis, as plants held a remarkable efficiency in energy absorption. Förster also showed unparalleled foresight when he considered that the same energy transfer he was considering could explain DNA damage from radiation or ultraviolet light. This is incredible, given that the structure of DNA was not discovered until 1953. Förster reasoned that this type of energy transfer was too far apart for atomic collisions to give the kind of efficiency observed, but too close together for the emission and reabsorption of an "assimilation unit" (i.e. a photon). [40]

Perhaps Clegg described Förster's 1946 contributions best when he narrowed them to three key aspects. [34] First, the quantum mechanical treatment of collisions accounted for the correct relationship of resonance between the donor and acceptor. That is, Förster was able to remove the need for *exact* resonance asserted by the Perrins. Next, Förster's comparison of different dipole models allowed for the description of the so-called overlap integral. Finally, he was able to consolidate the correct distance dependence between the donor and acceptor. It should be noted that Förster was keenly aware of the publications by the Perrins. He praised the explanation of the physical principles, but showed that only his equations agreed with all data.

While the consolidation of FRET theory was an important milestone in physics, it initially had little or no impact elsewhere. Though Förster mentions the use of FRET in biological systems in virtually every paper, photosynthesis as a model of efficient energy transfer was his primary consideration. The use of FRET as an experimental tool was not yet considered.

LUBERT STRYER RECOGNIZES FRET AS A MOLECULAR RULER

The second milestone would be reached by Lubert Stryer. Stryer worked as a waiter at the Quadrangle Club while at the University of Chicago in 1955. Among the patrons he served was James Franck of the initial energy transfer study. In *Exploring Light and Life* [41] Stryer recalled that Franck's consistent dining order allowed discussion of Franck's previous energy transfer studies and interest in photosynthesis. Franck encouraged Stryer to work on energy transfer.

Within two years, Stryer found himself studying Förster's theories. He began to learn about labeling proteins for fluorescence measurements and was particularly fascinated by the specific distance dependence in Förster's equations. In 1959 he attended a bioenergetics meeting at Brookhaven where he met Förster and suggested that energy transfer could be used as a probe to gain insight into the conformation of biological macromolecules. Stryer often considered using energy transfer, but was limited by the lack of experimental confirmation of Förster's predictions.

A breakthrough paper by R. Bruce Merrifield in 1963 introduced a way to synthesize peptides of specific lengths with beads on either end. [42] In 1967, Stryer used this idea with his own peptides coupled with known donor and acceptor fluorophores at either end. [43] By varying the length of the total peptide, Stryer and his graduate student Haugland confirmed that energy transfer occurs over Förster's predicted distance. As his paper title *Energy Transfer: A Spectroscopic Ruler* suggested, Stryer had discovered a ruler for molecular interactions. [43] Two years later, his group confirmed the dependence of energy transfer on the spectral overlap. [44] This was done by altering the salts in the solution to vary the amount of spectral overlap present. Over the next decade Stryer and colleagues went on to use FRET-based molecular rulers to interrogate structural relations in a variety of proteins, protein assemblies, and multiunit enzymes, reviewed in. [45] Together, these studies established FRET as a powerful tool in the study of protein structure and the interactions of enzyme systems. Stryer had taken a physics phenomenon and turned it into a biological tool. However, Stryer described three caveats that must be considered: the prior calibration of a series of donor-acceptor pairs; the selective attachment of a single donor and a single acceptor to the macromolecule; and the availability of information concerning the relative orientation of the donor-acceptor pair. [42–45]

EARLY FRET STUDIES IN BIOLOGICAL SYSTEMS

The caveats outlined by Stryer initially proved to be too complicated for use of FRET. It was often necessary to build the peptide of interest to couple with the chromophores. The chromophore-labeled proteins were often difficult to produce and unstable. [46, 47] These issues were overcome when Roger Tsien and colleagues demonstrated that FRET

measurements were possible in living cells. [16] The approach used by Tsien and colleagues utilized a FRET-based probe nicknamed 'FICRhR', that was comprised of fluorescein- and rhodamine-labeled regulatory and catalytic subunits of Protein Kinase A I (PKA I). FICRhR was then microinjected into cells for subsequent imaging and FRET measurement. The basic idea was that as cAMP rose in the cell it would bind to regulatory subunits of FICRhR, triggering a dissociation of labeled regulatory and catalytic subunits, resulting in a reduction in FRET efficiency. This initial study triggered a set of studies using the FICRhR probe. [16, 48–52] These studies tried to grapple with questions about signaling specificity and the spatial spread of cAMP signals in cells as well as the propagation of signals through cellular systems. As such, they changed the questions that could be asked at the time. However, limitations of both the FICRhR probe and the confocal microscope systems at the time limited any quantitative assessment of results from these studies. Even with well documented limitations [18, 53–55], these are considered landmark studies because they demonstrate the potential for FRET-based measurements to expand our understanding of intracellular signaling pathways in living cells and systems. [54] While the potential for use of FRET-based probes to visualize signals as they propagate through cells had been demonstrated, FRET needed at least two more advances to become a practical research tool for the quantitative study of cellular signaling systems. Fortunately, Osamu Shimomura was working on one critical component while Stryer was making his contribution.

ISOLATION OF A FLUORESCENT PROTEIN FROM JELLYFISH

Near the end of World War II, Shimomura was a boy working at a factory near Nagasaki when the atomic bomb was dropped. [56] He decided to continue his education and eventually made his way to Nagoya University where he was tasked with the crystallization of luciferin, a light emitting compound responsible for blue bioluminescence. [57] Upon Shimomura's arrival at Princeton, Dr. Frank Johnson recruited him to explore the bioluminescence of *Aequorea*, since all bioluminescence was thought to be due to the luciferin and luciferinase reaction. Shimomura had doubts about the origin of the luminescence and sought to isolate a new bioluminescent protein. He realized that if the reaction was caused by a protein, a change in pH should inhibit luminescence. Lowering pH proved to suppress the luminescence, which returned after addition of sodium bicarbonate. This experiment demonstrated that the luminescent substance could, at least in principle, be extracted. A surprise came when he saw a bright blue flash after discarding the extract in a sink that was shared with aquarium overflow. A few experiments using seawater's known composition revealed that calcium activated the luminescence. Soon his group devised an extraction method using the calcium chelator, EDTA. [58]

While purifying the blue luminescence protein aequorin, the group noticed a separate, bright green fluorescence. Though only present in trace amounts, they purified this protein too and referred to it as a green protein. [58] This protein was later named Green Fluorescent Protein (GFP) by Morin and Hastings in 1971. By 1979, Shimomura's group discovered and characterized the luminescence reaction. [59] Amazingly, it was found that GFP activation was due to energy transfer from aequorin.

In this same paper, Shimomura characterized and analyzed the molecular structure of GFP. He found that GFP contained a chromophore within the protein molecule; at the time most known fluorescent proteins were a complex of a protein and a fluorescent compound. This allowed him to isolate the three amino acids that made up the chromophore out of the total protein and opened the possibility of cloning GFP. [59]

GFP GIVES RISE TO RELIABLE PROBES

After the discovery of GFP, several developments happened relatively quickly. Cody confirmed the structure of the GFP chromophore in 1993. [60] GFP was cloned in 1992 by Prasher and expressed in *E. coli* and *C. elegans* in 1994. [61] Also in 1994, the first new color, blue fluorescent protein (BFP), was reported. [62] Various fluorescent proteins have since been discovered and isolated that have allowed for significantly more reliable FRET studies. [63] Perhaps most importantly, Tsien proposed the usefulness of mutated GFP pairs in FRET probes in 1994. [64] The contributions of Shimomura and Tsien led to the award of the 2008 Nobel Prize in Chemistry (also shared with Martin Chalfie) for ‘the green fluorescent protein: discovery, expression and development’. [47, 56]

FRET WITH FLUORESCENT PROTEINS

In 1997, Tsien’s idea became a reality when his group used fluorescent indicators to detect localized calcium signals. [65] The indicators were composed of GFP and BFP coupled to calmodulin, a calcium binding protein. When calcium binds to calmodulin a conformational change occurs, which decreases the distance between the chromophores and increases the energy transfer (FRET) between the flanking GFP mutants. Tsien outlined three reasons this method is superior to traditional labelling with fluorescent probes. First, the indicator is generated *in situ* by gene transfer, which eliminated the need for microinjection, large-scale overexpression, and additional labelling. Second, the geometries and orientations of the fusion sites are precisely defined. Finally, the chromophore of each GFP variant is fixed within the protein. Essentially, the use of fluorescent proteins for FRET satisfied the caveats described by Stryer. [43]

Tsien initially used GFP and BFP, but he tested several deletions, insertions, and amino-acid substitutions and reported the best splice sequences, ultimately choosing enhanced cyan and yellow mutants, so called eCFP and eYFP. [66] These mutants were designed to answer a series of questions focused on the utility of FRET measurements in mammalian cells. Tsien answered these questions by using FRET in nuclear localization studies, endoplasmic reticulum studies, and free cytosolic Ca^{2+} studies. Tsien also broke the FRET probe into two pieces, each containing its own GFP mutant and part of the binding protein. [65] The experiments were done with dissociation controls, which demonstrated the possibility of monitoring the dynamics of reversible interactions in live cells. Thus, Tsien established that fluorescent proteins allow for FRET studies inside living cells.

Tsien concluded this contribution by speculating on the usefulness of targeted eCFP/eYFP probes to allow Ca^{2+} measurements at previously inaccessible cellular locations. He summed up the advantages of FRET with the observations that FRET is non-destructive,

quantifiable with high spatiotemporal resolution, and probably applicable to any compartment of the cell and to many proteins other than calcium binding proteins. Tsien did caution that rare associations will be difficult to detect, and he put significant emphasis on the need for controls.

STATE OF THE ART FRET APPROACHES

The ability to insert FRET probes into a targeted location in the cell without significantly disturbing cell function has given us remarkable insight into the general functions of virtually any aspect of living cells. FRET is being used in studies of single molecule interactions [67], within living cells [68], and even whole tissues. [69] FRET probes have been developed to interrogate a wide variety of processes in cellular signaling. [70, 71] We can also use FRET to examine causes, treatments and potential diagnostic tools of disease [72–74] and in pharmaceutical studies in high throughput screening platforms for compounds or drug screening. [20, 75, 76]

FRET is also being paired with other detection methods for faster and more accurate acquisition of information. Flow cytometry methods allow for interaction studies in large numbers of cells at a time. [77–79] Microscopy studies can monitor spatial and temporal protein interaction information. [80, 81] Confocal microscopy, along with time-lapse studies, has allowed tracking of different complexes, such as receptor-ligand complexes, over time and through various cellular compartments. [82, 83]

Studies of localized signaling events in *in vitro* cell cultures have led investigators to explore signaling systems using intravital imaging approaches. These powerful approaches allow *in vivo* imaging of systems ranging from *C. elegans* to transgenic mouse models expressing FRET-based probes. [84–86] Imaging modalities to measure FRET in these model systems range from epifluorescence and confocal microscopy of *ex vivo* tissues, skin preparations, or surface imaging of isolated vessel and/or tissue preparations to multiphoton imaging within intact tissues [85–88], to multiphoton imaging- and fluorescence lifetime-based FRET measurements of the nervous system through brain stem windows in mouse models. [89] The potential for study of cellular signaling systems using FRET-based probes and *in vitro* cell cultures, *ex vivo* tissue preparations, and *in vivo* model systems is compelling. However, FRET measurements still have limitations that need to be overcome.

THE ADVANTAGES AND LIMITATIONS OF FRET-BASED PROBES IN THE STUDY OF INTRACELLULAR SIGNALING PATHWAYS

FRET probes offer perhaps the most effective tools with which to study second messenger signaling systems, including cAMP systems and protein phosphorylation. Yet, even with the marked advances in our understanding of FRET and in discovering and developing new fluorescent proteins suitable for FRET probes, it is fair to ask why FRET probes have not revolutionized the study of second messenger signaling pathways. The primary reason for this is that FRET probes have markedly lower signal-to-noise ratios than their parent fluorophores. [55] The low signal-to-noise ratios of FRET-based probes lead to several compounding limitations:

1. It is difficult to measure rapid signals using FRET probes. This limitation is exacerbated when monitoring additional fluorophores (or autofluorescence) and when imaging in three spatial dimensions (x, y, z).
2. It is difficult to infer signal localization when examining a two dimensional image (the standard in the field) of a three dimensional process (cAMP signals).
3. It is also difficult to quantitatively discriminate FRET signals from other fluorophores and autofluorescence in *in vitro* cell cultures, *ex vivo* tissue preparations, and, perhaps most challenging, in *in vivo* animal models.
4. Thus, it is difficult to quantitatively measure localized signals using soluble FRET probes and standard FRET imaging approaches.

An additional challenge for intravital imaging must also be considered, motion artifacts due to heartbeat and respiration as well as potential for tissue shift, and muscle contraction. Motion artifacts can be minimized by physical constraint of tissues and potentially by the use of gated imaging. Motion artifacts can also be partially corrected utilizing image registration approaches. [90, 91] However, localized fluorescence and FRET measurements are highly sensitive to motion artifacts – especially if investigators attempt to study localized signaling domains. [91]

The limitations summarized above have hampered both the use and interpretation of intracellular signals using FRET probes. Two general approaches have been used to overcome these limitations. The first approach is to target FRET probes to discrete cellular locations. These locations may be specific proteins (e.g., AKAPs [25, 92–94]) or subcellular domains (e.g., plasma membrane and nucleus [95–97]). The advantages of this approach are several-fold:

1. Targeted probes allow measurement of signals at specific subcellular locations. In theory, the measurement can be localized within tens of nanometers of the targeted probe/structure.
2. Targeted probes typically display higher signal intensities than soluble probes, indicating that targeted probes are at higher local concentrations than soluble probes. This increases local signal-to-noise ratio.
3. It may be possible to track signals localized to multiple intracellular locations as more fluorescent proteins and FRET pairs are developed.

However, targeted FRET probes also have limitations that must be considered when interpreting FRET measurements. These limitations include:

1. Targeted FRET probes appear to be expressed at higher local concentrations than their soluble counterparts; thus, the likelihood of intermolecular or bi-molecular FRET is increased. Similarly, high local concentrations could lead to self-quenching of the probe. It is difficult to estimate the impact of nonlinear optical effects on perceived changes in cAMP, Ca^{2+} , or phosphorylation levels.
2. The local subcellular environment may alter the fluorescence properties of FRET probes. For example, it is well documented that fluorophore properties can be

markedly altered by changes in hydrophobicity (movement near or within the plasma membrane). [98]

3. Typically, investigators measure FRET in specified (and comparatively large) regions of interest – often an entire cell. Thus, while targeted FRET-based probes are localized, measurements represent averaged responses throughout the cell, markedly reducing the utility of the approach.
4. Targeted probes do not typically assess the spatial distribution/spread of intracellular signals or signaling events within cells.
5. Further limiting the utility of both soluble and targeted FRET probes, measurements are typically made in two spatial dimensions (x,y), see above. A recent study by Annamdevula and colleagues demonstrates that agonist-induced cAMP gradients exist in three spatial dimensions. [99] Thus, standard FRET microscopy approaches may not provide sufficient information to evaluate the distributions of intracellular FRET or the underlying biological signal.

Thus, care must be taken in interpreting data obtained using localized FRET probes. That said, targeted probes still offer the highest potential spatial resolution of any FRET measurement. Such exquisitely localized measurements provide a powerful approach to interrogate signaling events within nanodomains.

While localized FRET probes offer the potential to measure intracellular signals in highly localized domains, the inherent limitations triggered our group to explore an alternate approach – spectral FRET measurements. [99, 100] Spectral FRET measurements have a higher signal-to-noise ratio than standard intensity-based FRET measurements. This in turn allows more accurate estimates of FRET efficiency on a voxel-by-voxel basis. We have recently used this approach to visualize three-dimensional cAMP gradients in living cells. [99] A typical experiment is depicted in Figure 1. Here, forskolin was used to trigger increases in cAMP (hot colors indicate high cAMP levels) in a pulmonary microvascular endothelial cell. Both the nucleus and the area above the nucleus were masked from the image to allow visualization of the interior of the cell. There appear to be an array of cAMP hotspots at the plasma membrane, presumably representing domains of localized adenylyl cyclase activity. Similarly, cAMP hotspots are visible in the interior of the cells. We believe that these hotspots represent areas of activated adenylyl cyclase associated with intracellular vesicles, as described by Von Zastrow and colleagues, summarized in [101]. To our knowledge, no studies using targeted probes offer this level of detail about the distribution of cAMP signals in three spatial dimensions. This is in large part because in general, targeted probes do not assess the spatial distributions of cAMP within cells. However, this comes at a cost. The acquisition times for a three-dimensional spectral image stack are approximately three minutes. [99] While our recent efforts have reduced the acquisition time for a three-dimensional image stack to approximately 30 seconds, the temporal sampling rate is still insufficient for accurate assessment of cAMP kinetics. Two-dimensional image stack sequences can be acquired in < 2 seconds, which in general is a sufficient sampling rate for cAMP signals. Thus, at present, multiple approaches must be considered to fully interrogate intracellular signals in single cells, including the use of *both soluble and targeted FRET*.

based probes measured using spectral or lifetime imaging approaches in two and three spatial dimensions.

CONCLUSIONS AND FUTURE DIRECTIONS

FRET owes its usefulness to the consolidation of the theory by Förster, Stryer's insight to use FRET as a molecular ruler, and the utilization of GFP by a host of researchers. Breakthroughs in other fields should also be considered in the progression of development and implementation of FRET approaches in the biological sciences, including cell isolation and culture, transfection, transgenic models, confocal microscopy, optics, detectors, as well as developments that enabled exponential growth in computational power. But it was Förster, Stryer, Shimomura, Tsien, and colleagues that made FRET the tool it is today. Current FRET technologies allow us to examine protein interactions at a nanoscopic level. Limitations are being overcome as novel technological approaches allow FRET studies to expand well beyond the initial spectroscopic approaches pioneered by Stryer. In fact, it is possible that we will soon have the capacity for rapid intravital FRET studies. [102] However, additional technical hurdles will need to be overcome for rapid quantitative intravital FRET measurements. These include the development of transgenic models that express FRET probes based on brighter fluorescent proteins; spectral or other approaches to separate FRET signals from autofluorescence; automated segmentation and image analysis approaches to analyze large five dimensional image stacks (x, y, z, λ , t); and, perhaps most importantly, fast gated imaging approaches to minimize movement artifacts.

Several of these key areas appear to be priorities in the field. First, new chromophore pairs are in development that will potentially increase the intensity of FRET-based probes as well as allow the measurement of multiple FRET probes in the same cell. Second, analytical methods are evolving that will allow better resolution and interpretation of mined image data. For example, maximum likelihood estimation allows the removal of bias in data analysis. [103] Third, development and implementation of improved imaging and microscopy approaches will provide higher signal-to-noise ratio FRET measurements, which in turn will reduce sampling time and increase the overall utility of three-dimensional imaging. Thus, we are entering another phase of rapid discovery in the areas of intracellular signaling. It is likely that in the next decade we will be able to simultaneously measure multiple second messenger signals, protein phosphorylation, and subsequent regulation of cellular functions in cells and tissues. It has been a long journey from early studies of vapors comprised of mercury and thallium atoms – studies conducted a century ago. And when viewed through a *retrospectroscope* [104], this journey provides yet another example of the circuitous path of biomedical discovery, a path through the fields of quantum physics, optics, chemistry, and biology.

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Figure 1:

Forskolin (50 μ M) induced cAMP accumulation in a pulmonary microvascular endothelial cell. cAMP was monitored using the soluble H188 FRET-based cAMP probe. FRET efficiencies were estimated from spectral image stacks and cAMP concentrations as described previously. Hot colors (orange and red) indicate high cAMP levels; cool colors (green, blue) indicate low cAMP levels. The areas of high cAMP are stretched in the vertical dimension due to under sampling in z and 3D smoothing. The scale bar represents 20 μ m. The figure is adapted with permission from Annamdevula et al., *Cytometry A*. 93 (2018) 1029–1038