

# Fluorescence resonance energy transfer in the study of cancer pathways

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Many different signaling pathways are involved in deregulation of cell proliferation leading to cancer. Although genomic approaches successfully identified a great variety of molecules associated with cancerogenesis, other strategies must be applied to elucidate complex interactions between these molecules. One promising approach is fluorescence resonance energy transfer, a proximity-dependent fluorescence phenomenon. With the development of spectrally different fluorescent proteins and improved technologies for fluorescence measurements, this approach gains an enormous potential for future research. The fluorescence resonance energy transfer principle can be applied for studying all kinds of interactions or conformational changes, and it can also be used for microscopic visualization and subcellular localization of biochemical reactions, thereby promoting the progress of cancer research. Moreover, it can be exploited to develop sensitive and efficient drug screening systems and to design valuable diagnostic tools. *Curr Opin Oncol* 2003, 15:55–64 © 2003

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This work was supported by a grant from the Austrian Science Foundation (Fonds zur Förderung der Wissenschaftlichen Forschung, Project P14509 to HHS), Project and by the Competence Center Bio-Molecular Therapeutics, Vienna.

**Current Opinion in Oncology** 2003, 15:55–64

## Abbreviations

<b>CFP</b>	cyan fluorescent protein
<b>DRAP</b>	donor recovery after acceptor photobleaching
<b>EGFR</b>	epidermal growth factor receptor
<b>EBFP,</b>	ECFP, EGFP, EYFP: enhanced blue, cyan, green and yellow fluorescent protein, respectively (as specified by BD Biosciences Clontech Inc., Palo Alto, USA)
<b>FITC</b>	fluorescein isothiocyanate
<b>FLIM</b>	fluorescence lifetime imaging microscopy
<b>FRET</b>	fluorescence resonance energy transfer
<b>GFP</b>	green fluorescent protein
<b>RFP</b>	red fluorescent protein
<b>TRITC</b>	Tetramethylrhodamine isothiocyanate
<b>YFP</b>	yellow fluorescent protein

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In the past century, the human race succeeded in fighting many infectious diseases that caused the untimely death of countless people throughout history. However, although the lifespan of humans was extended by improved medical treatments, other diseases that have deregulation of the complex balance in the human multicellular organism as a primary cause became much more critical. One of these diseases is cancer, actually the final manifestation of a great variety of different events leading to uncontrolled cell proliferation.

## Pathways leading to cancer

The diverse pathways leading to development of cancer have been comprehensively reviewed by Hanahan and Weinberg [1••]. A variety of mechanisms can lead to uncontrolled proliferation, such as acquired self-sufficiency in growth signals, the acquisition of limitless replicative potential, or insensitivity to antigrowth signals or mechanisms to evade apoptosis. Finally, the induction of angiogenesis and metastasis play important roles in the clinical manifestation of cancer. It is important to note that different tumor-promoting proteins often play a role in more than one of these pathways, based on the complex mutual regulation of signaling networks. Prototypically, the *myc* oncogene family may serve as an example of an integrated component in cell cycle regulation that can receive signals from several pathways (reviewed in [2]). In many cases, tumor-related genes were identified by differential display approaches (reviewed in [3]), in which mRNA of tumor tissue is compared with controls. In the last few years, more comprehensive approaches were applied using microarray techniques covering major portions of the human genome [4]. These techniques, combined with methods to isolate mRNA even from minute amounts of tissue or cells (such as laser microdissection [5]), allowed a very efficient and extensive identification of genes involved in cancer development. However, posttranslational modifications of proteins as causative events in cancerogenesis are usually not addressed in genetic approaches of target identification. Moreover, a hallmark in the onset of cancer is how proteins interact either with each other or with other biologic molecules in the context of intracellular or intercellular signaling networks. Therefore, means to investigate and characterize macromolecular interactions are crucial for further elucidation of cancer. One promising approach to detect and measure these fundamental interactions is the application of a quantum physical phe-

nomenon termed fluorescence resonance energy transfer (FRET).

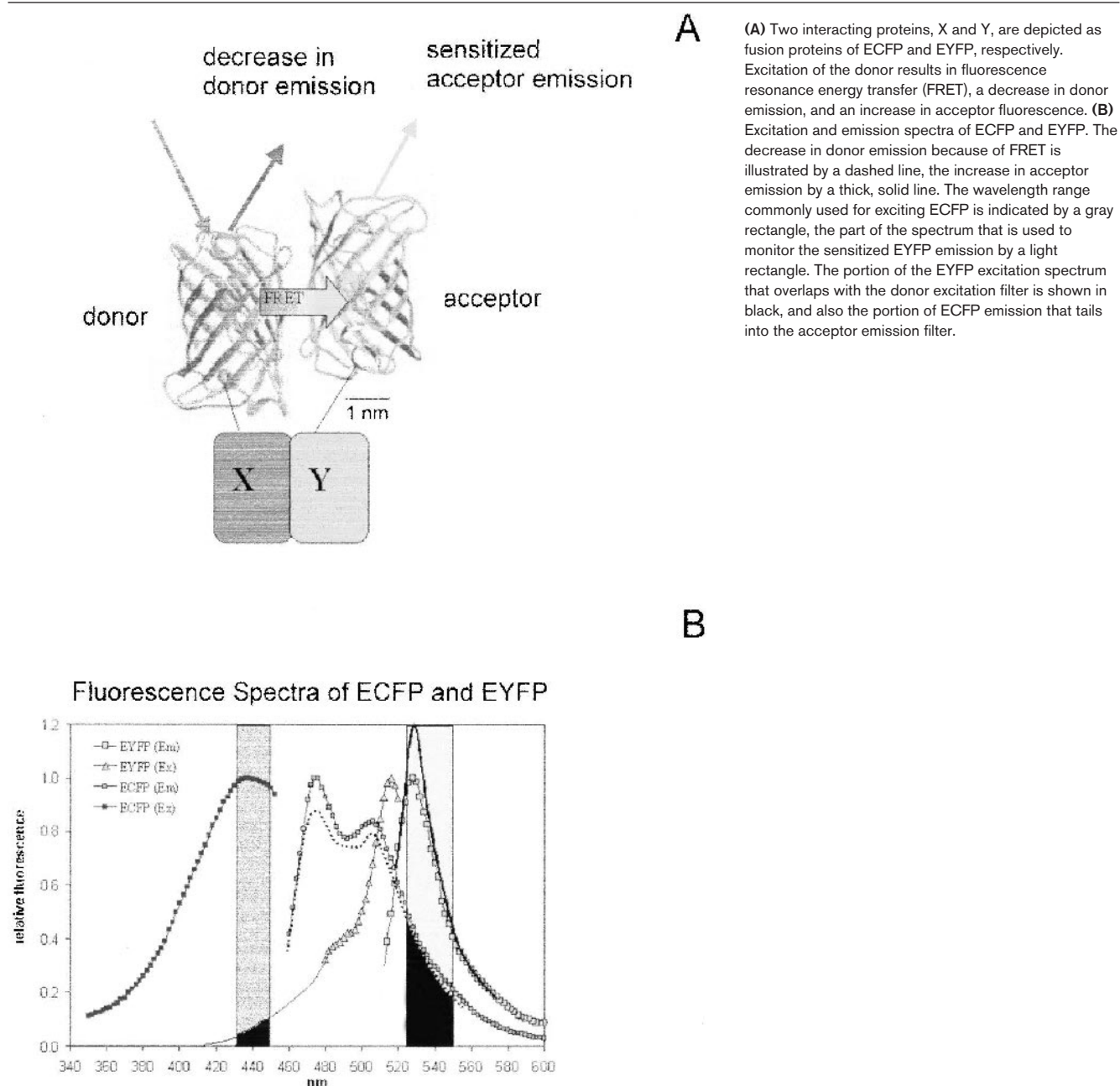
### Principle of fluorescence resonance energy transfer and appropriate fluorophores

Fluorescence resonance energy transfer is the transfer of energy from an excited fluorophore (the donor) to another fluorophore (the acceptor) [6]. It depends on three main conditions: (1) a close proximity (usually <10 nm), (2) an overlap between emission and excitation spectra of donor and acceptor, respectively, and (3) the orientation of the fluorophores (the dipole-moments), as reviewed in [7]. Energy is not transferred via photons but by dipole-

dipole interactions, and the efficiency of this transfer declines with the sixth power of the distance between the fluorophores. Those distinct proximity-dependent features provide a very useful tool for detecting macromolecular interactions, because FRET can be monitored by a decrease in donor emission and, usually, an additional increase of acceptor fluorescence (Fig. 1).

Fluorescent dyes suitable for both energy transfer and labeling of macromolecules have been available for a long time. Popular dyes used as FRET pairs are fluorescein and rhodamine, Cy3 and Cy5, and more recently, combinations of Alexa dyes (Molecular Probes, Eugene,

**Figure 1. Schematic illustration of fluorescence resonance energy transfer**



OR) [8]. Despite the availability of these labeling dyes, applications of FRET techniques had just begun to flourish when spectral variants of the green fluorescent protein (GFP) became accessible for studying protein interactions in living cells. Based on the originally discovered Aequorea protein [9,10] many variants of GFP were developed to improve the general fluorescence properties and to bring up spectrally distinct fluorescent proteins [11•,12] (Table 1).

Green fluorescent protein variants that are presently best suited as FRET pairs are cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP). After the discovery of a red fluorescent protein (designated DsRed [13]), many expectations were raised for its use as a FRET acceptor in combination with GFP. However, this protein requires tetramerization for being fluorescent and exhibits slow and complex maturation from green to red fluorescence, rendering it unusable for FRET applications. Reduced oligomerization was reported for the subsequently introduced DsRed2 (BD Biosciences Clontech, Palo Alto, CA) but later studies indicated that DsRed2 forms at least dimers [14]. A recently described monomeric form, although somewhat inferior in fluorescence, might guide the way to the desired FRET acceptor for GFP [11•].

## Techniques to measure fluorescence resonance energy transfer

One important issue for FRET measurements is that emission and excitation curves of many FRET pairs overlap in such a way that excitation of the donor leads to some coexcitation of the acceptor (resulting in non-FRET acceptor emission) and that the donor emission also tails into the wavelength spectrum of the acceptor emission, complicating simple filter-based detection strategies. Therefore, many pioneering studies of FRET were performed by spectrofluorometry, and algorithms were developed allowing calculation of FRET efficiencies based on spectral information [15,16]. Because fluorescence measurements principally are nondestructive, many efforts were undertaken to extend the powerful FRET approach to living cells. This goal was finally achieved for a variety of detection systems, the most prominent of them flow cytometry and microscopy. Flow analysis of FRET successfully proved clustering of receptors *in vivo* [17,18] and was also applied to cancer-related studies [19]. FRET between CFP and YFP can be monitored by fluorescence-activated cell sorter equipment with certain filter and laser adaptations [20–22,23•], and the authors envisage that the recent development of nonaggregating red fluorescent proteins will lead to a much broader application, because

**Table 1. Characteristics of commonly used and possible fluorescence resonance energy transfer pairs**

FRET pair, donor/acceptor	Donor excitation, extinction coefficient	Donor emission, quantum yield	Acceptor excitation, extinction coefficient	Acceptor emission, quantum yield	Comment
FITC/TRITC	494 (79,000)	518 (0.9)	547 (82,000)	572 (0.91)	Classic FRET pair for labeling proteins M(eg, antibodies)
Cy3/Cy5	550 (150,000)	570 (0.04)	649 (250,000)	670 (0.28)	Labeling dyes alternative to MFITC/TRITC
Alexa488/ Alexa546	494 (78,000)	517 [8]	554 (112,000)	570 [8]	Novel alternative as labeling dyes M(superior to FITC/TRITC)
EGFP/Alexa546	489 (55,000)	508 (0.06)	554 (112,000)	570 [8]	FRET between GFP and labeled Mantibody
EBFP/EGFP	380 (31,000)	440 (0.18)	489 (55,000)	508 (0.60)	BFP has inferior fluorescence Mproperties
EGFP/EYFP	489 (55,000)	508 (0.60)	514 (84,000)	527 (0.61)	Cannot be separated with filters (but Mcan be used in FLIM)
ECFP/EYFP	434 (26,000)	477 (0.40)	514 (84,000)	527 (0.61)	Good combination for normal FRET Mmicroscopy using mercury lamps as light source and special filters. CFP is poorly excited by argon lasers, but good excitation by blue laser diodes
EGFP/DsRed2	489	508	561	587	Problematic because of dimerization of DsRed2
EGFP/mRFP	489 (55,000)	508 (0.60)	584 (43,800)	607 (0.55)	Nonoptimal due to weak fluorescence of mRFP and lower spectral overlap with GFP (YFP as donor theoretically better)
	(55,000)	(0.60)	(44,000)	(0.25)	

CFP, cyan fluorescent protein; FITC, fluorescein; FLIM, fluorescence lifetime imaging microscopy; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; TRITC, rhodamine; YFP, yellow fluorescent protein. Data from [11•,93] and <http://www.clontech.com>.

standard cytometers are suited for the GFP and RFP combination.

Although flow cytometry has the advantage of generating good statistics, many studies on subcellular signaling events require microscopic evaluation. The technical progress in fluorescence microscopy stimulated the application of the FRET principle drastically during the last few years in this area. At first, simple filter-based methods were applied (such as imaging the acceptor fluorescence at the donor excitation), which suffer from major pitfalls such as spillover of non-FRET fluorescence to the FRET channel and concentration dependency, which can lead to false-positive FRET images. Subsequently, much better techniques were developed that compensate for the signal cross-talk. In general, this improvement was achieved by so-called “three-filter”

methods of image acquisition using filter sets for (1) the donor, (2) the acceptor, and (3) the acceptor at donor excitation (usually termed “FRET filter set”). In a first stage, the spillover of signal into the FRET channel (as determined by controls containing only donor or acceptor) was simply subtracted [24] (Table 2), which prevents false-positive results but does not normalize for different concentrations of fluorophores. This parameter was first considered by a numerical compensation method for calculation of a normalized FRET value [25]. Very recently, the three-filter approach was extended so that normalized FRET images can be derived [26••]. However, all these variations of the three-filter method are not suited to determine the FRET efficiency (which is necessary for computing distances between fluorophores). This determination can be achieved by FRET microscopy techniques that use either bleaching strategies or fluores-

**Table 2. Overview of fluorescence resonance energy transfer measurement techniques**

Study	FRET detection technique	Description	Equations
Clegg [15], Suzuki <i>et al.</i> [16]	Spectrofluorometry	Wavelength scanning at donor excitation and at acceptor excitation and analysis of the spectra	$E = (F_{A(ExD)}/F_{A(ExA)} - \varepsilon_{A_D}/\varepsilon_{A_A}) \times \varepsilon_{A_A}/\varepsilon_{D_D}$ $F_{A(ExD)}$ fluor. of acceptor at donor excitation; $F_{A(ExA)}$ fluor. of acceptor at acceptor excitation; $\varepsilon_{A_D}$ ext. coeff. of acceptor at donor excitation; $\varepsilon_{A_A}$ ext. coeff. of acceptor at acceptor excitation; $\varepsilon_{D_D}$ ext. coeff. of donor at donor excitation
Youvan <i>et al.</i> [24]	Three-filter FRET microscopy	Image acquisition with donor, acceptor, and FRET filter sets and digital image analysis	$\text{Corrected FRET} = FF - d \times DF - a \times AF$ $FF, \text{ FRET filter signal; } DF, \text{ donor filter signal; } AF, \text{ acceptor filter signal}$ $d = FF_d/DF_d \text{ (cross-talk correction 1)}$ $(FRET \text{ filter signal/donor filter signal for donor alone})$ $a = FF_a/AF_a \text{ (cross-talk correction 2)}$ $(FRET \text{ filter signal/acceptor filter signal for acceptor alone})$
Gordon <i>et al.</i> [25]	Three filter FRET microscopy	Image acquisition with donor, acceptor, and FRET filter sets and numerical calculation for regions of interest	$FRET N_G = (FF - d \times DF - a \times AF)/(DF \times AF)$ $\text{Normalized FRET according to Gordon } et al. [25]$
Xia and Liu [26••]	Three filter FRET microscopy	Image acquisition with donor, acceptor, and FRET filter sets and digital image analysis	$FRET N_x = (FF - d \times DF - a \times AF)/(DF \times AF)^{1/2}$ $\text{Normalized FRET according to Xia and Liu [26••]}$
Zal <i>et al.</i> [58]	Three-filter FRET microscopy	Image acquisition with donor, acceptor, and FRET filter sets and digital image analysis	$FRET N_z = (FF - a \times AF)/DF$ $\text{Normalized FRET according to Zal } et al. [58] \text{ (also designated as compensated FRET ratio } Fa_a/DF, \text{ meaning acceptor-compensated FRET filter set fluorescence related to donor fluorescence)}$
Miyawaki and Tsien [27]	Donor recovery after acceptor photobleaching	Image acquisition with the donor filter set, bleaching with the acceptor filter set, and second image with the donor filter set	$E = 1 - (F_{DA}/F_D)$ $F_{DA}, \text{ donor fluorescence before acceptor bleaching; } F_D, \text{ donor fluorescence after acceptor bleaching}$
Gadella and Jovin [28]	Donor bleaching kinetics	Recording of the donor bleaching at continuous excitation ( <i>eg</i> by time series of images) and calculation of the single exponential decay	$E = 1 - (\tau_D/\tau_{DA})$ $\tau_D, \text{ half time of donor bleaching alone; } \tau_{DA}, \text{ half time of donor bleaching in presence of acceptor}$ $\text{Single exponential decay: } y = A \times e^{-0.69 t/\tau} + B$
Bastiaens and Squire [31]	Fluorescence lifetime imaging microscopy	Monitoring the fluorescence lifetime of the donor in presence and absence of the acceptor	$E = 1 - (T_D/T_{DA})$ $T_D, \text{ fluorescence half-life of donor alone; } T_{DA}, \text{ fluorescence half-life of donor in presence of acceptor}$
Szollosi <i>et al.</i> [59]	Flow analysis	Cytometry of cells with donor excitation and recording of donor and acceptor with appropriate compensation	

CFP, cyan fluorescent protein; E, fluorescence resonance energy transfer efficiency; ext. coeff., extinction coefficient; fluor., fluorescence; FRET, fluorescence resonance energy transfer.

cence lifetime measurements. One of these is the so-called “donor recovery after acceptor photobleaching” technique, in which donor images are taken before and after photodestruction of the acceptor fluorophore by intense illumination with the acceptor filter set. In case of FRET, the second image is brighter, because energy that was previously transferred to the acceptor is now available for donor emission [27].

Another method suitable for deriving FRET efficiency values is based on the kinetics of donor bleaching, which is slower in the presence of FRET acceptors because bleaching energy is transferred to the acceptor. In this case, the donor bleaching is recorded (*eg*, by time series of images under continuous excitation) and compared with an external control without acceptor. This method was originally established for low molecular weight fluorophores [28], but the authors could recently show that it can be applied to the CFP and YFP FRET pair [29]. Finally, FRET can also be visualized by fluorescence lifetime imaging microscopy (FLIM) [30] (reviewed in [31]) based on the reduction of donor fluorescence lifetime in the presence of a FRET acceptor. For the future, the authors expect that recently developed technologies to resolve spectral information by confocal laser microscopy will be applied to FRET samples as they have been applied to samples *in vitro* using the established spectrofluorometry methods.

### Applications of fluorescence resonance energy transfer techniques in cancer research

Possibilities to apply FRET based methods for studying cancer signaling pathways are vast, especially considering that cancer-related proteins and their interactions can be particularly observed *in vivo*. The following section provides representative examples of applications in cancer research, with a more detailed enumeration of the most recent and important studies in Table 3.

The majority of current FRET applications are found in the area of protein–protein interaction studies. Although important protein interactions are often initially identified by screening techniques such as the yeast two-hybrid system, the FRET approach becomes increasingly valuable for the characterization of these interactions in mammalian cells. A cancer-related example is the investigation of epidermal growth factor receptor (EGFR) dimerization by donor-bleaching FRET microscopy [28], which has been further studied by single-molecule imaging using total internal reflection [32] and, most recently, by FLIM [33]. Furthermore, it has been shown that EGFR, Grb2, and Shc interact with each other on the surface of endosomes, and that active, GTP-bound Ras was observed both on endosomes and on the plasma membrane [34••]. Moreover, a fluorescent

reporter protein has been introduced recently to monitor tyrosine kinase activities of EGFR [35•].

These examples demonstrate that FRET microscopy is very useful to determine not only whether two proteins interact with each other but also the subcellular localization of the interaction and its dynamics in the course of signaling. With this respect, FRET microscopy can provide much better insights than classical methods such as coimmunoprecipitation studies. Other powerful techniques to characterize macromolecular interactions, like surface plasmon resonance (reviewed in [36]), have the disadvantage of using immobilized targets and hence cannot describe the interaction in solution or *in vivo*.

Another important field of FRET applications is the characterization of protein–DNA interactions [37], exemplified by studies performed by Ramirez-Carozzi and Kerppola [38•] providing detailed insights into binding of Jun-Fos-NFAT1 complexes to target DNA. The FRET technique was used to determine the orientation of the protein complex on the DNA, and it was shown that this orientation has an important impact for both promoter selectivity and efficiency of transcription. The authors' own studies proved the power of FRET measurements for the detailed kinetic characterization of nuclear factor  $\kappa$ B binding to its target DNA using stopped flow fluorometry [39]. Moreover, it has been demonstrated that movement of proteins relative to DNA is accurately measurable by changes in FRET [40•].

The strict dependence of FRET on the distance between the fluorophores is also an ideal basis for determining conformational changes of proteins. This concept is illustrated by a recent report [41] demonstrating different conformations for the MAP kinase-activated kinase 2 as a double fusion protein with BFP and GFP. FRET analysis proved this protein to exist in two forms: an inactive, closed, and an active, open one; these conformations were localized to different cellular compartments.

An additional, highly promising area for FRET applications is microscopic visualization of biochemical reactions, such as autophosphorylations. These approaches were pioneered by the group of Bastiaens (reviewed in [42••]), who used FLIM techniques for GFP fusion proteins of the ErbB1 receptor and Cy3-labeled antibodies against phosphotyrosine [43]. Although the latter bind to many different phosphorylated proteins, only those with the GFP tag result in a FRET signal because of the proximity of the GFP and the Cy3 label. Recently, these studies were extended to imaging of dephosphorylation [44•], and the general strategy might be applied to all biochemical processes that are detectable by antibodies. Apart from imaging biochemical events by binding of

**Table 3. Recent representative studies with fluorescence resonance energy transfer applications**

Study	FRET application field	Target	Fluorophores applied	Method used
Sato <i>et al.</i> [81]	Visualization of biochemical events	Phosphorylation (insulin R)	CFP/YFP	Ratio imaging, DRAP
Verveer <i>et al.</i> , [43] Haj <i>et al.</i> [44•]		Phosphorylation (ErbB1) Dephosphorylation	GFP/Cy3 GFP/Cy3	FLIM FLIM, DRAP
Rehm <i>et al.</i> [46], Tyes <i>et al.</i> [82•]	Biosensors	Caspase	CFP/YFP	Ratio imaging
Kalab <i>et al.</i> [83••]		Ran	CFP/YFP	Spectrofluorometry, three-filter method
Del Pozo <i>et al.</i> [84•]		Rac	GFP-Rac/Alexa546-PDB	Confocal FRET microscopy, ratio imaging
Zhang <i>et al.</i> [85•]		PKA	CFP/YFP	Ratio imaging
Ting <i>et al.</i> [35•]		PTK	CFP/YFP	Ratio imaging
Awaji <i>et al.</i> [86]		pH	GFP <sub>uv</sub> /EGFP (EYFP)	Ratio imaging
Mochizuk <i>et al.</i> [45••]		Ras, Rap1	CFP/YFP	Ratio imaging, spectrofluorometry
Honda <i>et al.</i> [87]		cGMP	CFP/YFP	Ratio imaging, spectrofluorometry
Miyawaki <i>et al.</i> [88]	Screening	Ca <sup>2+</sup>	CFP/YFP	FRET-ELISA
Berg <i>et al.</i> [49•]		Myc-Max	CFP/YFP	FRET-ELISA
Weatherman <i>et al.</i> [89]		Ligands/estrogen receptor	CFP/RFP, GFP/RFP (DsRed1)	Three-filter FRET microscopy
Mergny <i>et al.</i> [90], Riou <i>et al.</i> [91]		Telomerase	Fluorescein/rhodamine	FRET melting curve analysis
Eise <i>et al.</i> [53]	Diagnosics	mRNA; invasive cleavage assay	Donor/quencher	Fluorometry
Lopez-Crapez <i>et al.</i> [92]	Protein-protein interactions	SNPs	Europium/Cy5	Time-resolved fluorometry
Ruiz <i>et al.</i> [51]		FRET-real time PCR	Fluorescein/LC-Red	Real-time PCR
Westerman <i>et al.</i> [52]		FRET-real time PCR	Fluorescein/LC-Red	Real-time PCR
Legg <i>et al.</i> [60]		CD44/ezrin	GFP/Cy3	FLIM
Martin-Fernandez <i>et al.</i> [33]		EGFR homo-oligomerization	Rhodamine/erythrosine isothiocyanate	FLIM, anisotropy decay
Jlang and Sorkin [34••]		EGFR, Grb2, Shc, Ras	CFP/YFP	Three-filter FRET microscopy
Berg <i>et al.</i> [49•]	Protein-DNA interactions	Myc/Max	CFP/YFP	FRET-ELISA
Trinkle-Mulcahy <i>et al.</i> [61]		Protein phosphatase 1/inhibitor	CFP/YFP	Three-filter FRET microscopy
Fogg <i>et al.</i> [62]		PLCβ2/Gβγ	Coumarin/DABCYL	Spectrofluorometry
Mayr <i>et al.</i> [63]		CREB/CBP	CFP/YFP	Spectrofluorometry, three-filter microscopy, DRAP
Kraft <i>et al.</i> [64]	Protein-DNA interactions	CCR5/β-arrestin	CFP-YFP	Spectrofluorometry
Block <i>et al.</i> [65]		MHC/TCT/CD8/CD3	FITC, PE/allophycocyanine	Flow analysis
Triantafilou <i>et al.</i> [66]		LPS/hsp70/hsp90/CXCR4	Cy3/Cy5	DRAP
Sanetopoulos <i>et al.</i> [67••]		Gα/Gβ	CFP/YFP	Spectrofluorometry, three-filter microscopy
Wang <i>et al.</i> [68]	Protein-DNA interactions	Caspases (8,10)	CFP/YFP	Flow cytometry
KANE <i>et al.</i> [69]		p53/HDM2	XL665/Europium	Homogenous time-resolved fluorescence
Grinberg <i>et al.</i> [70]		tBID	CFP/YFP	Single-cell microscopy
Graham <i>et al.</i> [71]		Rac/Cdc42	BFP/GFP	Spectrofluorometry
Siegel <i>et al.</i> [23•]	Protein-DNA interactions	Fas homo-oligomerization	CFP/YFP	Spectrofluorometry, DRAP*
Mukhopadhyay <i>et al.</i> [40•]		σ <sup>70</sup> /RNA polymerase	Rhodamine/Cy5	PAGE-FRET ("in-gel FRET")
Lorenz and Diekmann [72]		IHF/DNA	Fluorescein/rhodamine	PAGE-FRET
Ramirez-Cavrozzi and Kerppola [38•]		Fos/Jun/NFAT	Fluorescein/Cy3/Texas red	Spectrofluorometry and kinetics
Ramirez-Cavrozzi and Kerppola [73]	Fos/Jun/NFAT	Fluorescein/Texas red	PAGE-FRET	
Kohler and Schepartz [74]	Fos/Jun	Fluorescein/rhodamine	Spectrofluorometry and kinetics (stopped-flow)	
Schmid <i>et al.</i> [39]	NF-κB/DNA	GFP/tetrachlorocarboxy-fluorescein	Spectrofluorometry and kinetics (stopped-flow)	
Hughes <i>et al.</i> [75]	Protein-phospholipid interactions	PLD/phosphatidylcholine	GFP/PLD/BODIPY-PC	FLIM
Blanchetot <i>et al.</i> [76]	Protein-conformation	RPTPs	CFP/YFP	Spectrofluorometry-microscopy

**Table 3. Recent representative studies with fluorescence resonance energy transfer applications (continued)**

Study	FRET application field	Target	Fluorophores applied	Method used
Baneyx <i>et al.</i> [77] Torok <i>et al.</i> [78]		Fibronectin Calmodulin/CaMK	Oregon green 488/TRITC AEDANS/DDP	Spectrofluorometry-microscopy Fluorescence lifetime analysis, stopped flow
Neininger <i>et al.</i> [41]		MK2	BFP/GFP	Spectrofluorometry, ratio imaging
Shih <i>et al.</i> [79]		Myosin	Oregon green 488/TRITC	Spectrofluorometry, stopped flow, fluorescence life time
Tuominen <i>et al.</i> [80]		Cytochrome c	Fluorescent Zn <sup>2+</sup> -cytochrome c	FLIM

CFP, cyan fluorescent protein; cGMP, cyclic GMP; DRAP, donor recovery after acceptor photobleaching; EGFR, epidermol growth factor receptor; ELISA, enzyme-linked immunosorbent assay; FITC, fluorescein; FLIM, fluorescence lifetime imaging microscopy; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; NF, nuclear factor; PCR, polymerase chain reaction; PKA, protein kinase A; TRITC, rhodamine; YFP, yellow fluorescent protein.

fluorescently labeled antibodies, many efforts were undertaken to visualize important cellular reactions by the design of FRET biosensors. The common principle for these is to generate fusion proteins of CFP and YFP (or another appropriate FRET pair) in which the fluorophores are separated by a sensory domain that reacts to a corresponding signal either by a conformational change or a cleavage. An important example is a recently developed biosensor for Ras that exhibits a GTP-Ras-dependent change in the FRET signal [45••]. An example for a cleavage-based principle is a biosensor for caspase activities using a corresponding cleavage site situated between CFP and YFP [46].

Another interesting FRET-based strategy to define and evaluate signaling pathways was recently described using a cell-permeable FRET dye cleaved by  $\beta$ -lactamase [47,48]. This approach is based on the transfection of a  $\beta$ -lactamase reporter construct, which can be either combined with various defined promoter elements or integrated into the genome, thereby substituting reporter gene assay systems commonly used for studies of signaling pathways.

The authors also anticipate the FRET principle to attain a wider application in high-throughput assay systems for drug screening. The usefulness of this application has been demonstrated by microtiter plate FRET-based screening of substances inhibiting Myc-Max dimerization [49•].

Furthermore, the sensitivity and specificity of the FRET signal is a very good basis for the development of diagnostic tools [50], realized, for instance, in the detection of cancer-related mRNAs using real-time polymerase chain reaction and FRET hybridization probes [51,52], or in quantification of RNA by invasive cleavage assays [53].

## Conclusions

The increasing application of the FRET principle in a vast variety of fields proves the general acceptance of this measurement principle and is an important basis for

further developments. We anticipate that some of the problems occurring in special cases, such as interactions of GFP proteins themselves at very high concentrations (reviewed in [54]), or nonphysiologic cellular situations triggered by overexpression of GFP fusion proteins, will be resolved by either controlled, moderate expression or the use of antibody-based FRET analyses of endogenous protein levels. Improved methods for direct delivery of proteins (such as fluorescently labeled antibodies) into cells (reviewed in [55]) may substitute more tedious microinjection approaches. The increasing use of biosensors, which can often be designed in such a way that they do not influence the cellular physiology but are well suited for monitoring the activity of endogenous signaling molecules, will be helpful in elucidating many of the cancer-related signaling pathways. Moreover, the application of FRET reporters or biosensors in transgenic animals combined with recently improved techniques of intravital microscopy [56] may provide innumerable insights into tumor biology *in situ*.

Additionally, we expect antibody-based FRET analysis of tissue sections to find its way into diagnostic strategies, and we expect that current developments in automated digital image analysis [57] will help bridge the gap between statistically highly relevant flow analysis and spatially exact microscopy.

## Acknowledgment

The authors are grateful to Renate Kroismayr and Veronika Sexl for reading the manuscript critically and to all their other colleagues for support and fruitful discussions.

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