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Analytical use of multi-protein fluorescence resonance energy transfer to demonstrate membrane-facilitated interactions within cytokine receptor complexes

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Abstract

Experiments measuring Fluorescence Resonance Energy Transfer (FRET) between cytokine receptor chains and their associated proteins led to hypotheses describing their organization in intact cells. These interactions occur within a larger protein complex or within a given nano-environment. To illustrate this complexity empirically, we developed a protocol to analyze FRET among more than two fluorescent proteins (multi-FRET). In multi-FRET, we model FRET among more than two fluorophores as the sum of all possible pairwise interactions within the complex. We validated our assumption by demonstrating that FRET among pairs within a fluorescent triplet resembled FRET between each pair measured in the absence of the third fluorophore. FRET between two receptor chains increases with increasing FRET between the ligand-binding chain (e.g., IFN- R1, IL-10R1 and IFN- R1) and an acylated fluorescent protein that preferentially resides within subsections of the plasma membrane. The interaction of IL-10R2 with IFN- R1 or IL-10R1 results in decreased FRET between IL-10R2 and the acylated fluorescent protein. Finally, we analyzed FRET among four fluorescent proteins to demonstrate that as FRET between IFN- R1 and IFN- R2 or between IFN- R1 and IFN- R2c increases, FRET among other pairs of proteins changes within each complex.

Keywords

FRET; Multi-protein; Interferon receptor; Lipid raft; Confocal fluorescence spectroscopy

1. Introduction

1.1. Multi-protein complexes

In cells, many biological complexes are composed of more than two proteins. Among these are DNA replication forks, RNA transcription complexes, ribosomes, proteosomes, histones, T cell, B cell, and NKT cell antigen receptor complexes, cytoskeletal focal adhesion

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complexes, nucleotide excision-repair complexes, RNA splicing complexes, and ASTRC that modifies RNA stability during the activation of immunocytes [1]. Although it is important to use chemically defined and well-controlled conditions to study interactions between two proteins in detail, these interactions occur in the context of entire complexes and in living cells, and may be modified in these more complex environments. The ability to analyze multiple interactions simultaneously and in cells is important to properly understand structures and functional dynamics of multi-protein complexes in cells.

1.2. Confocal fluorescence spectroscopy (CFS)

To visualize protein complexes noninvasively, we have utilized CFS and employed fluorescence resonance energy transfer (FRET) as an indicator of the juxtaposition of proteins within complexes in intact cells. We presented data supporting preassociation of the interferon-gamma (IFN- γ) receptor complex, discovered alterations that disrupt this preassociation, and showed that the interleukin-10 receptor complex is also preassociated [2–4]. Preassociation of the p37, p40 and Hsp27 components within the ASTRC has also been demonstrated with CFS [1,5].

To ensure an accurate analysis of biological systems, we developed a formal methodology to use CFS analytically. With this methodology, we estimated the energy flow through the donor:acceptor system through three possible paths: (1) into donor and out from donor, (2) into donor toward acceptor and out from acceptor, and (3) into acceptor and out from acceptor. Dissecting the energy flow allowed a determination of not only a more accurate FRET efficiency but also the acceptor:donor molar ratio, the relative levels of donors, of acceptors, and of donor:acceptor complexes, the coupling efficiencies of donors or acceptors into complexes, and the relative affinities of interactions within cells [6]. This methodology helped to explain a phenomenon in which (when coexpressing cDNAs encoding human IFN- γ R1 and IFN- γ R2c, the two transmembrane receptor chains of the Type I IFN receptor complex, in hamster cell lines) biologically active human Type I IFN receptors were much less easily generated in hamster cell lines than were IFN- γ receptors [7]. The efficient preassociation and the bioactivity of the Type I IFN receptor complexes depended on balanced expression of the two transmembrane receptor chains (IFN- γ R1 and IFN- γ R2c), and is aided by the coexpression of Janus kinases and/or the scaffolding protein RACK-1 bound to the intracellular domains of the receptor chains. Similar requirements may also exist for the IFN- γ receptor complex (composed of IFN- γ R1 and IL-10R2).

1.3. Lipid rafts

Many receptor complexes of neurological and immunological importance are found within subsections of the plasma membrane that are enriched in certain plasma membrane constituents such as cholesterol, sphingomyelins, ceramids, phosphatidylinositols, and glycerophospholipids with saturated long-chain fatty acids [8]; many nervous or immune receptor complexes must be present in these regions to have normal biological activity [9–14]. Extracting cholesterol from cells with methyl- β -cyclodextrins (MCD) or inhibiting cholesterol synthesis with statins, compromises cytoskeletal integrity, virus infection and release from cells, and signaling by many types of cytokines [15–23]. This may be a major reason why dissolution of cell membranes with detergents in order to perform co-immunoprecipitations of transmembrane receptor proteins results in the apparent dissociation of many receptor complexes in their resting state.

These membrane subsections enriched with cholesterol and the above-mentioned phospholipids are known as lipid rafts in intact cells. When they are isolated *in vitro* from cells, they are defined as detergent-resistant membranes. Lipid rafts have distinct biophysical properties (e.g., increased viscosity) and likely form a distinct physical phase in

the membrane from those of aggregates formed by other lipid molecules [11,24–30]. Lipid rafts are likely to be quasi-stable – although these lipid rafts are physically separated from other sections of the plasma membrane on a low-nanometer scale, individual rafts are not clearly resolved using visible-light optics nor are they stationary on a high-nanometer scale. Like oil droplets in turbulent water, individual rafts may fuse or dissociate. Individual rafts may also grow or shrink as many of their constituents partition into non-raft domains easily and thus readily diffuse between the membrane phases. Even with metastable lipid rafts, confinement of receptors to these lipid rafts will increase the frequency with which they encounter each other as well as interact. Using a fluorescent probe that localizes to these rafts with a technology that has a nanometer range of detection like FRET will allow one to measure the colocalization of proteins within these small and dynamic regions (nano-colocalization).

1.4. Measuring multiple interactions simultaneously by multi-FRET

We hypothesized that we could simultaneously monitor interactions among more than two proteins by simultaneously measuring FRET between more than two proteins (multi-FRET). In this manuscript, we devised a method to analyze FRET between three fluorophores by assuming a kinetic multi-path energy flow through multiple donors and acceptors and assuming that fluorescent interactions among three or more proteins are the sum of pairwise interactions within the ternary complex. We expanded our two-protein CFS protocol [6] to delineate energy flow through a three-protein or four-protein system from a two-protein system by exciting samples with not two but rather three or four lasers, each preferentially exciting one of the three or four proteins. After some algebraic manipulation, we determined various FRET efficiencies by estimating the fraction of energy that went through the 7 or 15 possible energy pathways.

2. Materials and methods

2.1. Reagents

All restriction endonucleases, shrimp alkaline phosphatase and T4 DNA Ligase were purchased from New England BioLabs. Recombinant Taq DNA polymerase was purified as described previously [31]. Turbo Pfu DNA polymerase was purchased from Stratagene. Polyethyleneimine (PEI) as the 25 kDa linear polymer, was purchased from Fluka as a powder. Solutions of PEI were prepared as described previously [32].

2.2. DNA constructions

All ligations were performed with T4 DNA Ligase. PCR using either genomic DNA or a human embryonic cDNA library (Stratagene, Inc.) as a template source was performed as described elsewhere [6,32]. Plasmids are purified using either a variant of the alkaline lysis method employing 7.5 M ammonium acetate instead of 5 M potassium acetate, or the MaxiPrep plasmid isolation kit (Qiagen, Inc., Valencia, CA). Plasmids were synthesized as described elsewhere [2,3,6].

2.3. Cell lines and transfections

Human kidney epithelial 293T cells and green monkey kidney epithelial COS-7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% (v:v) fetal bovine serum. Murine B16 murine melanoma cells were grown as described previously [32]. All cell lines were grown in HEPA-filtered tissue culture incubators under an environment of 37 °C, 5% CO₂, and 95% relative humidity.

Transfections were done using PEI as previously described [32]. Transiently transfected cell lines were used for experiments only when at least 15% of all cells express the required fluorescent proteins (judged visually using epifluorescence).

2.4. Fluorescence confocal spectroscopy

Details of the confocal microscope and spectrophotometer are described previously [1,3,5]. We generally used the 442 nm laser to excite ECFP, TFP, or EGFP; we used the 488 nm laser to excite EGFP, EYFP and CiFP; we used the 514 nm laser to excite CiFP; we used the 543 nm laser to excite ToFP, StFP, OFP, and ChFP; the 633 nm laser was used to excite the ChFP in 4-FRET experiments only.

2.5. Spectral deconvolution

Emission spectra, excitation spectra, extinction coefficients, and quantum yields were obtained either from members of the laboratories of Roger E. Campbell or Roger Y. Tsien or from published literature [33–35]. The process of adding scalable reference spectra to optimize a fit to an obtained cellular emission spectrum that was formatted to remove spectrometer noise and laser emission, has been described elsewhere [1,4]. This process was easily modified to allow the addition of more than two fluorescent proteins.

2.6. Analysis of deconvolved fluorescence intensities

The analysis of FRET between two fluorophores is described in detail elsewhere [6]. Supplementary Text 1 contains the detailed protocol to analyze FRET among three fluorophores. Supplementary Text 2 describes the derivation of the analysis of FRET among four fluorophores. In general, to analyze FRET among N fluorophores, one must use N lasers to excite the sample (each laser should optimally excite only one of the N proteins). FRET among the two most red-shifted proteins is analyzed first using the two spectra excited with the two most red-shifted lasers. FRET between the third most red-shifted protein and the two most red-shifted proteins is next analyzed using the three spectra generated with the three most red-shifted lasers. If a fourth protein is also excited, FRET between it and the other three proteins is analyzed with all four spectra. This process is continued until all N proteins have been analyzed.

3. Results

3.1. Empirical accuracy of our 3-FRET protocol to simultaneously ascertain three pairwise interactions

We based our analytical method to analyze three-protein FRET under the assumption that it is the sum of three pairwise FRET events, and derived equations to determine FRET efficiency between all three pairs in the triplet. We tested this assertion and our methodology by comparing cells coexpressing IFN- R1/TFP, IFN- R2/CiFP, and Lyn-ToFP with either those expressing IFN- R1/TFP, IFN- R2/CiFP, and pcDNA3 (lacking ToFP, Fig. 1A and B), those expressing IFN- R1/TFP, IFN- R2, and Lyn-ToFP (lacking CiFP, Fig. 1C and D), or those expressing IFN- R1, IFN- R2/CiFP, and Lyn-ToFP (lacking TFP, Fig. 1E and F). As described in Supplementary Text 3, all three fluorescent are simultaneously visible, even while the levels of all three proteins vary among cells. We compared the distribution of FRET efficiencies within the populations between the two fluorescent proteins that are common to both populations (TFP and CiFP, TFP and ToFP, and CiFP and ToFP respectively). In all cases, the datasets overlapped, demonstrating that our analysis of three-protein FRET can be used to obtain accurate pairwise FRET efficiencies in intact cells using our methodology. We observed here and elsewhere [6], that Lyn-ToFP exhibits FRET with IFN- R1/TFP at far lower numbers of complexes than Lyn-ToFP does with IFN- R2/CiFP;

inferring stronger restriction of IFN- R1 than IFN- R2 within membranes to where Lyn-ToFP nano-localizes.

3.2. Different patterns of three-protein FRET are observable in cells

To provide additional evidence that three-protein FRET can be measured in cells, we hypothesized that different patterns of three-protein FRET were observed when different fluorescent triplets were analyzed. To this end, we analyzed FRET and its efficiencies between the triplets {IFN- R1/TFP, IFN- R2/CiFP, Lyn-ToFP} (Fig. 2A–C), {RACK-1/TFP, IFN- R1/CiFP and IFN- R2c/StFP} (Fig. 2D–F), and {IFN- R2/ R2b/TFP, IFN- R1/CiFP, and IFN- R2/StFP} (Fig. 2G–I). We chose these triplets to determine (1) whether the localization of IFN- R1 or IFN- R2 to lipid raft subdomains of the membrane differ when the two chains are dissociated or when they interact, (2) whether the scaffolding protein RACK-1 [36–39] that stabilizes the interaction between IFN- R1 and IFN- R2c [7] interacts differently with IFN- R2c, IFN- R1, or IFN- R2c:IFN- R1 complexes, and (3) whether a dominant negative IFN- R2 chain [40,41] inhibits the assembly or the activation of the IFN- receptor complex. We confirmed that fusions of GFP variants to the COOH-termini of cytokine receptor chains did not inhibit their functionality [2,7], and that fusion of TFP to RACK-1 did not affect its ability to influence the interaction between IFN- R1 and IFN- R2c [7]; as this research was performed, a manuscript was published in which a COOH-terminal fusion of GFP to the nematode RACK-1 was successfully used [42].

The most intuitive way to distinguish differences among populations of fluorescent triplets is to display in three figures the FRET efficiency of one pair as a function of the FRET efficiency of a second pair. With this display, we observed distinct patterns of FRET among each triplet (Fig. 2), suggesting that multi-FRET can be used in cells to discriminate interactions among distinct protein complexes in cells.

While no obvious correlation existed between FRET between IFN- R2 and Lyn-ToFP and FRET between IFN- R1 and Lyn-ToFP (Fig. 2A) and only a weak correlation existed between increased FRET between IFN- R2 and Lyn-ToFP and increased FRET between IFN- R1 and IFN- R2 (Fig. 2B), increasing FRET between IFN- R1 and IFN- R2 was consistently seen with increasing FRET between IFN- R1 and Lyn-ToFP (Fig. 2C). This implies that the interaction between IFN- R1 and IFN- R2 is aided by (if not requires) the localization of IFN- R1 within subsections of the membrane to which Lyn binds. It is reasonable to speculate that confinement of IFN- R1 within lipid rafts facilitates its interaction with IFN- R2.

Although there was no strong correlation between FRET between IFN- R1 and IFN- R2c and FRET between RACK-1 and IFN- R2c (Fig. 2D), increased FRET between IFN- R1 and RACK-1 correlated with increasing FRET either between IFN- R1 and IFN- R2c (Fig. 2E) or increasing FRET between RACK-1 and IFN- R2c (Fig. 2F). Altogether, these data suggest that RACK-1 (that directly binds IFN- R2c, Jak1, and Tyk2, but only indirectly binds IFN- R1 via its interaction with Tyk2 [36–38]) increases its FRET with IFN- R1 as a consequence of IFN- R2c interacting simultaneously with IFN- R1 and RACK-1. The cooperative interaction among the three proteins may explain how RACK-1 stabilizes the interaction between IFN- R1 and IFN- R2c [7].

Although FRET between IFN- R2/ R2b and IFN- R2 did not correlate with FRET between IFN- R1 and either IFN- R2 or IFN- R2/ R2b (Fig. 2G, I, respectively), FRET between IFN- R2/ R2b and IFN- R1 increases with increasing FRET between IFN- R1 and IFN- R2 (Fig. 2H). These data suggest that IFN- R1 interacts with IFN- R2/ R2b only when it can also interact with IFN- R2, implying that IFN- R2 and IFN- R2/ R2b interact with IFN- R1 under similar conditions and may compete with each other directly for IFN- R1.

IFN- decreased the FRET efficiency between IFN- R1/CiFP and either IFN- R2/StFP or IFN- R2/ R2b/TFP to no more than 35% and 20% respectively (Fig. 2G, I, open circles), but only when all three chains were present (data not shown). Additional unpublished experiments revealed that in the absence of IFN- , IFN- R2/ R2b/TFP did not alter the affinity between IFN- R2/StFP and IFN- R1/CiFP, suggesting that it does not inhibit the assembly of the IFN- receptor. We attempt to interpret these results in Supplementary Text 4.

3.3. Nanolocalization of other cytokine receptor complexes

We extended our observations on Fig. 2A–C to see whether the IFN- , IL-10, and Type I IFN receptor complexes also require a specific nanolocalization of the ligand binding chain (IFN- R1, IL-10R1, and IFN- R2c) into nanodomains that harbor Lyn-ToFP, to interact with its partner chain (IL-10R2, IL-10R2, and IFN- R1, respectively). We analyzed FRET among the following fluorescent triplets: {IFN- R1/TFP, IL-10R2/CiFP, Lyn-ToFP} (Fig. 3A–C), {IL-10R1/TFP, IL-10R2/CiFP, Lyn-ToFP} (Fig. 3D–F), and {IFN- R1/TFP, IFN- R2c/CiFP, Lyn-ToFP} (Fig. 3G–I).

When FRET between either IFN- R1 or IL-10R1 and Lyn-ToFP increased, FRET between IL-10R2/CiFP and Lyn-ToFP decreased (Fig. 3A and D). More interestingly, as FRET between IFN- R1 or IL-10R1 and IL-10R2 increases, FRET between IL-10R2 and Lyn-ToFP decreases (Fig. 3B and E). This suggests that a conformational change in IL-10R2 that disfavors FRET with Lyn-ToFP occurs when IL-10R2 interacts with IL-10R1 or IFN- R1. Finally, increasing FRET between IFN- R1 and IL-10R2 correlates with increasing FRET between IFN- R1 and Lyn-ToFP in most cells (Fig. 3C). This suggests that, like IFN- R1, IFN- R1 must colocalize within lipid rafts to interact with IL-10R2. The same pattern was observed with IL-10R1, IL-10R2 and Lyn-ToFP (Fig. 3F), implying that IL-10R1 must also be present in lipid rafts to interact with IL-10R2. Because IFN- R1 and IL-10R1 are homologous to IFN- R1 [43], we believe that we are observing a general requirement of inclusion of the ligand-binding receptor chain within lipid rafts to interact effectively with the accessory receptor chain. In Supplementary Text 5, we investigate whether Jak1, that binds IFN- R1, IFN- R1, IL-10R1 and IFN- R2c, influences the interaction between IFN- R1 and either lipid rafts or IFN- R2.

In contrast, IFN- R1 and IFN- R2c exhibit distinct spectral characteristics with Lyn-ToFP. We first observed that IFN- R1 exhibits higher FRET with Lyn-ToFP than its homologous chains IFN- R2 or IL-10R2 did (cf. Fig. 3G, H with Figs. 2A, B, 3A, B, D and E). As seen with the IFN- receptor chains, there was no correlation of FRET between Lyn-ToFP and IFN- R1 and FRET between Lyn-ToFP and IFN- R2c (Fig. 3G). There also appeared initially to be no correlation between FRET between IFN- R1 and IFN- R2c and FRET between IFN- R1 and Lyn-ToFP (Fig. 3H). Interestingly, when FRET between IFN- R2c and Lyn-ToFP was measured as a function of FRET between IFN- R1 and IFN- R2c, the data fell into two distinct groups: The typical pattern of FRET between IFN- R2c and Lyn-ToFP increasing with increasing FRET between IFN- R1 and IFN- R2c was seen in one subgroup (Fig. 3I, filled black circles); in the second subpopulation, FRET between IFN- R2c and Lyn-ToFP remained poor even while FRET between IFN- R1 and IFN- R2c increased (Fig. 3I, unfilled circles). When we identified these cells in Fig. 3G and H, we found that FRET between Lyn-ToFP and IFN- R1 increases as FRET between IFN- R1 and IFN- R2c increased in most of the cells in this subpopulation (Fig. 3H, unfilled black circles). Excluding this subpopulation, there is a tendency for FRET between IFN- R1 and Lyn-ToFP to decrease as FRET between IFN- R1 and IFN- R2c increases (Fig. 3H, filled black circles). We hypothesize that in some cells a component interacting with both IFN- R1 and IFN- R2c alters the conformation of both chains so that FRET between one receptor chain and Lyn-ToFP is favored while FRET between the other receptor chain and

Lyn-ToFP is disfavored. In Supplementary Text 6, we provide evidence that this component is RACK-1.

3.4. Four-protein FRET

The assumption that we made to create a tractable model for multi-FRET should apply to four fluorescent proteins just as it did with three proteins. Supplementary Text 3 contains the derivation of formulae to determine the FRET efficiency among each of the six possible pairs, the relative levels of each fluorophore, the amounts of light present in the 15 possible pathways, and the relative levels of various pairs, triplet, or quartets. Supplementary Text 3 describes our ability to detect all four proteins simultaneously when exciting TFP, the most blue-shifted fluorescent protein.

3.4.1. Changes in pairwise FRET as receptor chains interact—IFN receptor chains are constitutively associated with Janus kinases in cells [44,45]; thus IFN- R1 and Jak1, IFN- R2 and Jak2, IFN- R1 and Tyk2, and IFN- R2c and Jak1 are constitutively bound together in cells. We hypothesized that when FRET between IFN- R1 and IFN- R2 (or between IFN- R1 and IFN- R2c) increases as the two chains interact, that FRET between proteins “on opposite sides” (i.e., Jak1 and Tyk2, IFN- R1 and Jak2) should also increase. To test this, we analyzed FRET among four proteins of the IFN- receptor complex, and among the four proteins of the IFN- receptor complex as a function of FRET between IFN- R1 and IFN- R2 or between IFN- R1 and IFN- R2c. We fused TFP and CiFP to the COOH-termini of the Janus kinases because the greatest sequence variation occurs there, the NH₂ terminus is involved in interactions with the receptor chain; also, it was shown that a fusion of GFP to the COOH terminus of Tyk2 is biologically functional [46].

As IFN- R1/OFP and IFN- R2/ChFP exhibited FRET, we found that the FRET increased between Jak1/TFP and Jak2/CiFP (Fig. 4A), between Jak1/TFP and IFN- R1/OFP (Fig. 4B), and between Jak2/CiFP and IFN- R1/OFP (Fig. 4C). FRET between Jak2/CiFP and IFN- R2/ChFP increased in a third of the measured cells as IFN- R1/OFP and IFN- R2/ChFP exhibited FRET, and no change was observed in the poor FRET observed between Jak1/TFP and IFN- R2/ChFP (data not shown).

As IFN- R1/OFP and IFN- R2c/ChFP exhibited FRET, FRET increased between Jak1/TFP and Tyk2/CiFP (Fig. 4D) and between Tyk2/CiFP and IFN- R1/OFP (Fig. 4E) in nearly all cells. Interestingly, only in some cells did FRET between Jak1/TFP and IFN- R1/OFP increase (Fig. 4F, gray circles); in other cells, no FRET between the two proteins was seen (Fig. 4F, black circles). Because no FRET between Jak1/TFP and IFN- R1/OFP was seen when no FRET between IFN- R1/OFP and IFN- R2c/ChFP was seen (Fig. 4F, unfilled black circles), we suspect that the increase in FRET between Jak1 and IFN- R1 denotes the presence of an additional interaction that can occur within the preassembled Type I IFN receptor complex. Most cells did not show FRET between either Jak1/TFP or Tyk2/CiFP and IFN- R2c/ChFP, especially when IFN- R1/OFP and IFN- R2c/ChFP demonstrated FRET (data not shown).

3.4.2. Changes in pairwise FRET as tetrameric receptor complexes form—We saw differences in apparent affinity between interactions mediated by prolonged protein:protein interactions and those mediated by transient diffusion-mediated collisions [6]. We hypothesized that affinity differences exist between pairwise interactions within fluorescent quartets. To test this, we compared the FRET efficiencies between various pairs as a function of the relative number of fluorescent quartets formed per cell. We found that with both IFN- receptor complexes (Fig. 5A) and IFN- receptor complexes (Fig. 5B),

FRET between the cytokine receptor chains (pink circles) was nearly optimal before FRET changed among other pairs of proteins, such as between the Janus kinases (black circles). We hypothesize that some interactions within protein complexes (such as those between IFN receptor chains) are of high affinity or long duration while other interactions occur transiently.

3.4.3. Changes in interactions within higher-order complexes—To further demonstrate that interactions between protein pairs are modified by inclusion within higher-order complexes, we compared the interaction observed between pairs within the quartet with the pair expressed in triplets or in isolation. Because the fluorescent proteins conjugated to each pair expressed in isolation or in the triplet were different than the fluorescent proteins conjugated to the pair within the quartet, we adjusted the FRET efficiency to correspond to the FRET efficiency of the pair used within the quartet.

FRET between Jak2 and IFN- R2 (Fig. 5A, blue circles), that was significant in the absence of quartets or in low numbers of quartets, disappeared when high numbers of quartets formed. These data supported our observations in the first manuscript in this series in which FRET between IFN- R2 and Jak2 was greatly reduced in a few cells that also expressed IFN- R1. FRET between Jak1 and IFN- R2 (Fig. 5A, green circles), that was significant only in a few triplets, was further decreased in fluorescent quartets when Jak2 was also coexpressed.

While FRET between IFN- R2c and Tyk2 (Fig. 5B, blue circles) was observed in isolated pairs, it was rarely observed in any fluorescent quartet; FRET between the pair rapidly disappeared as fluorescent quartets were formed. In some cells expressing Jak1 and IFN- R2c (Fig. 5B, green circles) as isolated pairs, FRET was observed; in other cells expressing the isolated pair and in fluorescent quartets, no significant FRET was observed. Most cells expressing IFN- R1 and Tyk2 (Fig. 5B, yellow circles) in isolation exhibited poor FRET efficiency; as fluorescent quartets formed, the FRET between IFN- R1 and Tyk2 increased. Finally, FRET between Jak1 and IFN- R1 (Fig. 5B, red circles) increased as fluorescent quartets formed, however, the range of FRET seen was similar to that seen in the pair expressed in isolation.

We observed above that the ectopically expressed Type I IFN receptor complex can assume two forms in cells, each with different conformations and a different affinity between IFN- R1 and IFN- R2c. Hypothesizing that the one of these populations exhibit FRET between IFN- R1 and Jak1 while the other population lacks this FRET, we compared the relative affinity of the interaction between IFN- R1 and IFN- R2c within the quartets. Cells exhibiting FRET between IFN- R1 and Jak1 (Fig. 5B, right panel, filled circles) have a stronger affinity between IFN- R1 and IFN- R2c than cells lacking FRET between IFN- R1 and Jak1 (Fig. 5B, right panel, unfilled circles). Somehow the conformation (that may contain RACK-1) with increased apparent affinity between IFN- R1 and IFN- R2c and altered conformations of IFN- R1 and IFN- R2c allows FRET between IFN- R1 and Jak1 to be measurable.

4. Discussion

4.1. Theory of multi-FRET

We analyze pairwise FRET by separating the various energy pathway flows through a single donor and a single acceptor [6]. Because molecules rotate and translate freely through space in most cases, the orientation, distance, and coupling between the electromagnetic fields of the donor and acceptor fluorophores are not constant. Based on this, we modeled electromagnetic interactions among three or four fluorophores by as the sum of FRET

between the donor and the intermediate fluorophores, between any intermediates, between the intermediate and the acceptor fluorophores, and between the donor and acceptor fluorophores (Supp. Figs. 1 and 4, top).

Because fluorescence may proceed among multiple paths in a multi-fluorophore system, we assumed a set of kinetic competitive pathways, in which any pathway that leads from a higher-energy fluorophore to a lower-energy fluorophore is utilized at a given rate. From the pathways in a three-fluorophore and a four-fluorophore system, we derived solutions to differential equations to determine the amounts of fluorescence that would be observed through each light pathway given a set of kinetic rate constants. By altering the kinetic constants among three fluorophores, fluorescent emission models predicted distinct emission patterns, implying that multi-FRET should be visible spectroscopically (Supp. Fig. 2). By exciting the three-fluorophore system with three lasers, we can determine the amount of light going through up to seven pathways originating at FP1 (Supp. Fig. 3). Alternatively, light through 15 pathways can be discriminated by exciting a four-fluorophore system with four lasers (Supp. Fig. 8). By evaluating differential equations for final fluorescent products (i.e., evaluated at infinite time) and after some algebraic manipulations, we calculated various pairwise FRET efficiencies.

4.1.1. Other attempts to perform multi-FRET—As this research was being performed, a paper was published that also used a kinetic model, three distinct excitations, and evaluation of differential equations based on the kinetic scheme at infinite time to quantify FRET among three fluorophores using an imaging protocol that reconstructs emission spectra based on a set of images using diverse emission filters that operate throughout the observable spectrum [47]. Although this approach is similar to ours, they did not account for internal conversion in their determination of FRET efficiencies. Aside from incorrectly defining FRET efficiency, this results in an overestimation of FRET efficiencies by reducing the magnitude of the denominator. Additionally, they define FRET from FP1 directly and only from the 3-FRET definition; these produced data with FRET from FP1 being lower within the triplet than in the isolated pairs [47]; inferred distances from FP1 to other proteins will therefore be overestimated. We ultimately determined FRET efficiency between FP1 and other proteins using a standard two-fluorophore definition and corrected these systematic errors.

4.1.2. Limits to multi-FRET—Logically and mathematically, multi-FRET can be extended to measure FRET among any number of fluorophores; however, for practical reasons, no more than four fluorophores can be easily resolved by FCS. This practical limitation is set by the width of fluorescence emission from each fluorophore, and by the restricted spectral window that can be used for spectroscopy in cells (500–700 nm): this window is limited on the blue side by cellular autofluorescence induced by visible light or by cellular quenching of desired fluorescence, and on the red side by intrinsically poor fluorescence of low-energy fluorophores. If autofluorescence can be significantly reduced (via strong quenching of bluish fluorophores by reddish fluorophores), one may be able to utilize a wider 450–700 nm window, resolve five fluorophores, and obtain biophysically accurate data.

Supplementary Text 7 contains discussions about the conditions necessary to most easily observe multi-FRET, and when it is preferential to analyze pairwise interactions in isolation by 2-FRET instead of within higher-order complexes containing other fluorescent proteins by 3-FRET or 4-FRET.

4.2. Empirical demonstration of multi-FRET

We chose an initial three-fluorophore system in which FRET between the three pairs of the triplet will be considerable, so that fluorescence from all three proteins will easily be observed. Because both IFN- R1 and IFN- R2 reside within the plasma membrane, we chose as a third fluorophore a fatty acylated peptide conjugated to ToFP, and chose to investigate the nano-localization of IFN- receptors within plasma membranes. Because we have little empirical control over how efficiently IFN- R1 and IFN- R2 interact in cells or how well either chain associates with lipid rafts, a technology like multi-FRET that can distinguish among the interaction of Lyn-ToFP and either IFN- R1, IFN- R2, or an IFN- R1:IFN- R2 complex, is ideally suited to address our interests.

Expressing the fluorescent triplet {IFN- R1/TFP, IFN- R2/CiFP, Lyn-ToFP}, we observed fluorescence from all three fluorophores; not surprisingly, the emission spectrum varies from cell to cell (Supp. Fig. 10). We tested our multi-FRET model by comparing the FRET efficiency calculated between each pair of the above fluorescent triplet {IFN- R1/TFP, IFN- R2/CiFP, Lyn-ToFP}, with the three pairwise FRET efficiencies among triplets in which only two proteins are fluorescent. The data from the each fluorescent doublet completely overlapped with data from the same pair within the fluorescent triplet (Fig. 1).

We further confirmed the utility of the multi-FRET system by demonstrating that different triplets exhibited different fluorescence emission patterns; this demonstration is most easily shown as three parametric plots of FRET efficiencies of one pair as a function of FRET between a second pair (Fig. 2). Analyzing various triplets in this fashion allowed us to observe circumstances in which particular pairs of proteins interact. In one triplet, we clearly observed that considerable FRET between IFN- R1 and IFN- R2 correlated with considerable FRET between IFN- R1 and Lyn-ToFP (Fig. 2C). This implied that IFN- R2 interacts with IFN- R1 only when IFN- R1 is found within membrane subregions to which Lyn interacts, such as lipid rafts. In another triplet, we discovered that FRET between RACK-1 and IFN- R1 (that do not directly interact [36–38]) increased with increasing FRET between IFN- R1 and IFN- R2c (Fig. 2E) or with increasing FRET between RACK-1 and IFN- R2c (Fig. 2F). This result is consistent with a model in which RACK-1 associated more strongly with complexes of IFN- R1 and IFN- R2c than with either chain in isolation. In a third triplet, we observed that the dominant negative receptor chain IFN- R2/ R2b interacted with IFN- R1 only when IFN- R2 also interacted with IFN- R1 (Fig. 2H), implying that IFN- R2 and IFN- R2/ R2b interact with IFN- R1 by the same mechanism.

4.3. Observations borne out with three-protein FRET

We hypothesized that chains of other cytokine receptor complexes interact under similar environmental restrictions as did those for IFN- . Consistent with this hypothesis, there was a clear correlation between increased FRET between Lyn-ToFP and IFN- R1 or IL-10R1 and increased FRET between IL-10R2 and either IFN- R1 or IL-10R1 (Fig. 3F and I). Interestingly, FRET between IL-10R2 and Lyn-ToFP decreased with increasing FRET between IL-10R2 and either IFN- R1 or IL-10R1 (Fig. 3E and H). Because IL-10R2 is bound to a receptor chain that exhibits strong FRET with Lyn-ToFP, we interpret the decreased FRET from IL-10R2 and Lyn-ToFP upon its association with IFN- R1 or IL-10R1 as a conformational change upon IL-10R2 brought about by its interaction with IFN- R1 or IL-10R1 that disfavored FRET.

Interpreting the nanolocalization of IFN- R1 and IFN- R2c with Lyn-ToFP was more complicated. First, FRET between IFN- R1 and Lyn-ToFP was more efficient than FRET between Lyn-ToFP and either IFN- R2 and IL-10R2. These data implied that IFN- R1

partitions into lipid rafts. This implication is consistent with a report describing the palmitoylation of IFN- R2c but especially of IFN- R1 [48]; this may constrain both chains to lipid rafts. Second, in addition to the expected population in which FRET between IFN- R2 and IFN- R1 increased with increasing FRET between IFN- R2c and Lyn-ToFP (Fig. 3I, filled circles), there was a population in which FRET between IFN- R2c and Lyn remained poor irrespective of FRET between IFN- R1 and IFN- R2c (Fig. 3I, unfilled circles). These two populations had different conformations of IFN- R1 as IFN- R1 and IFN- R2 interacted (Fig. 3H), and also had differing affinities between IFN- R1 and IFN- R2c (Supp. Fig. 14).

As described in Supplementary Text 5, we also observed changes in FRET between Jak1 and IFN- R1 as the numbers of IFN- R1:Jak1 pairs increased in cells (Supp. Fig. 13). FRET between the two proteins increased as the two proteins interacted, but decreased subsequently with large numbers of IFN- R1:Jak1 pairs (Supp. Fig. 13). In a few cells, where Jak1 exhibited FRET with either Lyn-ToFP or IFN- R2, IFN- R1 efficiently coupled to IFN- R2 or Lyn-ToFP as well as to Jak1; otherwise, IFN- R1 poorly coupled with Lyn-ToFP or IFN- R1 and Jak1 did not exhibit FRET with Lyn-ToFP or IFN- R2. We hypothesized that the conformational change in the IFN- R1:Jak1 pair was caused by the lipid raft nanoenvironment because similar changes were seen with the partner receptor chain (IFN- R2) that were seen with a fluorescent protein with no recognizable protein interaction motif (Lyn-ToFP).

4.4. Requirements for cytokine receptor chains to partition into lipid rafts

Previously and recently published research is largely in accord with our data supporting that interactions of cytokine receptor chains requires their assembly within lipid rafts. The co-sedimentation of both IFN- R1 and IFN- R2 with GM₁ *in vitro* has been shown by a few groups [49–51], inferring their presence within lipid rafts in intact cells. One study showed that the association of IFN- R1 with detergent-resistant membranes (scored by binding of cholera toxin B to the ganglioside GM₁) is only partial prior to ligand addition, and increases significantly after treatment of cells with ligand [50]. This result agrees with data stating that photocrosslinking of sphingomyelins with IFN- R1 increases after IFN- treatment [52]. Another study found strong colocalization of IFN- R1 with lipid rafts in intact cells as well as in detergent-resistant membranes *in vitro* that did not increase after binding IFN- ; the localization of IFN- R2 with lipid rafts as well as its cosedimentation with detergent-resistant membranes was partial prior to ligand treatment but increased after IFN- treatment [51]. These studies uniformly use nonneutralizing antibodies raised against IFN- R1 or against IFN- R2. In contrast, the incorporation of IFN- R1 as well as IFN- binding proteins (likely IFN- R2) with detergent-resistant membranes remained poor after IFN- addition [50]. A preassociation between IFN- R1 and IFN- R2 (within a population of paraformaldehyde-fixed cells, measured by FRET of the cellular suspension in a cuvette) that increased with ligand treatment, is inhibited by -MCD or by *Leishmania* infection that quenched cholesterol, but is restored by liposomal delivery of cholesterol to cells [51]. In fact, when cholesterol is quenched from cells, IFN- R1 and IFN- R2 co-sediment with GM₁ *in vitro*, bind IFN- with similar kinetics as if cholesterol were present, but do not initiate signaling. Surprisingly, cholesterol depletion by *Leishmania* infection disrupted IFN- signaling but did not inhibit IL-10-induced activation of Stat3. Were our data averaged as if taken throughout a population of cells, we would likely conclude also that the association of IFN- R1 with lipid rafts, as well as of IFN- R1 to IFN- R2, is partial. Because potential sequences were identified that correlated with cholesterol and sphingomyelin binding to - helices [52,53], we analyzed the intracellular domains of Class II receptor chains for these consensus sequence, as well as obvious patterns of conserved amino acid residues within the transmembrane receptor chains of Class II cytokine receptor chains. Our analysis of

cholesterol-binding and sphingomyelin-binding sites like those found in the IFN- R1 transmembrane segment is described in Supplementary Text 8. Altogether, these data agree with ours in that disruption of lipid rafts (that often occurs with dissolution of membranes) will disrupt ligand-independent interactions between cytokine receptor chains as well as potentially alter the nanolocalization of cytokine receptor chains.

4.5. Observations borne out with four-protein FRET

To further demonstrate the validity of the physical interaction between cytokine receptor chains, we wanted to correlate FRET between cytokine receptor chains with FRET between protein pairs within the tetrameric complexes. With both IFN- receptor chains and IFN- receptor chains, FRET between the several pairs of proteins increased with increasing FRET between the cytokine receptor chains (Fig. 4). In some cells, FRET between IFN- R1 and Jak1 increases as FRET between IFN- R1 and IFN- R2c increases (Fig. 4F). Cells with this additional interaction have a lower apparent affinity between IFN- R1 and IFN- R2c (Fig. 4B).

4.6. Two conformers of the Type I IFN Receptor Complex in cells?

Analysis of the interaction between IFN- R1 and IFN- R2c in the context of extra proteins revealed that cells can allow the receptor pair to interact in two distinct ways (Figs. 2G–I, 4F, and 5). The two entities have differing apparent affinities between IFN- R2c and IFN-

R1, different conformations of IFN- R1 and IFN- R2c, and a different spectroscopic interaction between Jak1 and IFN- R1. The affinity of the lower-affinity entity resembles that seen in the absence of RACK-1 (Supp. Fig. 14), while the affinity of the higher-affinity entity resembles that seen in the presence of RACK-1. The sixfold difference in affinity between the two entities resembles the fivefold difference seen in the tetrameric complexes. We hypothesize that RACK-1 is the present in both the high-affinity entity and in the high-affinity complex.

Although we observed both entities in experimental conditions, we do not believe that both entities exist under in physiological conditions. RACK-1 is critical to the function of many signaling complexes [39], is ubiquitously expressed, and interacts with the Type I IFN receptor complex in untransfected cells [36]. We hypothesize that under physiological conditions, that only the high-affinity complex that contains RACK-1, and where Jak1 and IFN- R1 exhibit FRET, exists in cells.

4.7. Structural model of the assembly of interferon receptor complexes

We forward the following structural models to explain the conformations that occur as the components of IFN receptor complexes interact in cells. IFN- R1 (red) binds Jak1 (yellow–green) with high affinity. The IFN- R1:Jak1 complex partitions strongly into subdomains of the plasma membrane (light brown) to which Lyn-ToFP associates (Fig. 6A), that we predict are lipid rafts (dark brown). Their nano-localization within lipid rafts at low levels allows IFN- R2 (green, in a complex with Jak2, in cyan) to interact (Fig. 6B). Although our data show that the IFN- R1:IFN- R2 complex remains tightly associated within rafts (Fig. 2C), we do not know if the tetrameric receptor complex is found on the edge of the raft (left side) or within the raft (right side) because we do not know whether IFN- R2 is within or outside the lipid raft while bound to IFN- R1. Logically, however, the inverse symmetry of the IFN- :IFN- receptor complex (see panel F) places physical hindrances against maintaining nanolocalization of both IFN- R2 chains along raft edges. We therefore hypothesize that IFN- R2 can nanolocalize within lipid rafts even while bound to IFN- R1. We believe that IFN- R1 and IL-10R1 interact with IL-10R2, and that IFN- R2c interacts with IFN- R1, by a similar mechanism and are similarly restricted in the membrane.

We propose that the primary contact between the IFN- R1:Jak1 complex and the IFN- R2:Jak2 complex (middle panels) as well as between the IFN- R2c:Jak1 complex and the IFN- R1:Tyk2 complex (bottom panels), is through the extracellular domains of the transmembrane receptor chains (Fig. 6C); this is sensible as this underlies the specificity of the association of receptor chains. The secondary contact is mediated by elements in the intracellular domain (Fig. 6D); based on data presented elsewhere [7], we believe that Jak1 makes the most important contact, though Jak2, Tyk2 and especially RACK-1 (with IFN- R2c) play roles in the association of the intracellular domains. These roles coincide with spectroscopically observable conformational changes. Contacts among intracellular domains and proteins help maintain preassociation of the complex even while (as hypothesized in the other two companion manuscripts) the extracellular domains frequently dissociate (Fig. 6E); we believe that this frequent dissociation allows ligand to interact with the dissociated extracellular domains of the complex and activate it (Fig. 6F).

4.8. Overall summary

Overall, we developed a protocol to identify and quantify electromagnetic interactions among three or four fluorescent proteins in intact cells. This protocol allows a detailed analysis of energy flow through the donor:intermediate(s):acceptor system. We used this multi-FRET system to show the membrane nanolocalization of two cytokine receptor chains (or their complex), to show the cooperative interaction of three proteins, to show the modification of an interaction by environmental factors, and to show protein rearrangements that occur in the assembly of multi-protein receptor complexes. All of this was done in intact cells, and demonstrates that multi-FRET is a useful technique to use to apply structural biology to live cells, within which pharmacologically sensitive and dynamic protein complexes exist.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CFS	confocal fluorescence spectroscopy
ChFP	mCherry/cherry fluorescent protein
CiFP	mCitrine/monomeric citrine fluorescent protein
EGFP	enhanced green fluorescent protein
EYFP	enhanced yellow fluorescent protein
FP	fluorescent protein
FRET	fluorescence resonance energy transfer
IFN	interferon
IFN-	interferon-
IFN-	interferon-

IFN-	interferon-
OFP	mOrange/orange fluorescent protein
ORF	open reading frame
PEI	polyethyleneimine
StFP	mStrawberry/strawberry fluorescent protein
TFP	mTeal/teal fluorescent protein
ToFP	tdTomato/tomato fluorescent protein

References

- [1]. Sinsimer KS, Gratacos FM, Knapinska AM, Lu J, Krause CD, Wierzbowski AV, et al. Chaperone Hsp27, a novel subunit of AUF1 protein complexes, functions in AU-rich element-mediated mRNA decay. *Mol Cell Biol.* 2008; 28:5223–37. [PubMed: 18573886]
- [2]. Krause CD, Mei E, Xie J, Jia Y, Bopp MA, Hochstrasser RM, et al. Seeing the light: preassembly and ligand-induced changes of the interferon receptor complex in cells. *Mol Cell Proteomics.* 2002; 1:805–15. [PubMed: 12438563]
- [3]. Krause CD, Lavnikova N, Xie J, Mei E, Mirochnitchenko OV, Jia Y, et al. Preassembly and ligand-induced restructuring of the chains of the IFN- receptor complex: the roles of Jak kinases, Stat1 and the receptor chains. *Cell Res.* 2006; 16:55–69. [PubMed: 16467876]
- [4]. Krause CD, Mei E, Mirochnitchenko O, Lavnikova N, Xie J, Jia Y, et al. Interactions among the components of the interleukin-10 receptor complex. *Biochem Biophys Res Commun.* 2006; 340:377–85. [PubMed: 16364239]
- [5]. Knapinska AM, Gratacos FM, Krause CD, Hernandez K, Jensen AG, Bradley JJ, et al. Chaperone Hsp27 modulates AUF1 proteolysis and AU-rich element-mediated mRNA degradation. *Mol Cell Biol.* 2011; 31:1419–31. [PubMed: 21245386]
- [6]. Krause CD, Digioia G, Izotova LS, Pestka S. Improving the spectral analysis of fluorescence resonance energy transfer in live cells: application to interferon receptors and Janus Kinases. *Cytokine.* 2013 <http://dx.doi.org/10.1016/j.cyto.2013.05.026>.
- [7]. Krause CD, Digioia G, Izotova LS, Xie J, Kim YS, Schwartz B, et al. Ligand-independent interaction of the type I interferon receptor complex is necessary to observe its biological activity. *Cytokine.* 2013
- [8]. Jain MK, White HB III. Long-range order in biomembranes. *Adv Lipid Res.* 1977; 15:1–60. [PubMed: 335851]
- [9]. Bi K, Altman A. Membrane lipid microdomains and the role of PKC in T cell activation. *Semin Immunol.* 2001; 13:139–46. [PubMed: 11308297]
- [10]. Miceli MC, Moran M, Chung CD, Patel VP, Low T, Zinnanti W. Co-stimulation and counter-stimulation: lipid raft clustering controls TCR signaling and functional outcomes. *Semin Immunol.* 2001; 13:115–28. [PubMed: 11308295]
- [11]. Dietrich C, Bagatolli LA, Volovyk ZN, Thompson NL, Levi M, Jacobson K, et al. Lipid rafts reconstituted in model membranes. *Biophys J.* 2001; 80:1417–28. [PubMed: 11222302]
- [12]. Gombos I, Kiss E, Detre C, Laszlo G, Matko J. Cholesterol and sphingolipids as lipid organizers of the immune cells' plasma membrane: their impact on the functions of MHC molecules, effector T-lymphocytes and T-cell death. *Immunol Lett.* 2006; 104:59–69. [PubMed: 16388855]
- [13]. Kamiguchi H. The region-specific activities of lipid rafts during axon growth and guidance. *J Neurochem.* 2006; 98:330–5. [PubMed: 16805828]
- [14]. Allen JA, Halverson-Tamboli RA, Rasenick MM. Lipid raft microdomains and neurotransmitter signalling. *Nat Rev Neurosci.* 2007; 8:128–40. [PubMed: 17195035]
- [15]. Meiri KF. Membrane/cytoskeleton communication. *Subcell Biochem.* 2004; 37:247–82. [PubMed: 15376624]

- [16]. Ciana A, Balduini C, Minetti G. Detergent-resistant membranes in human erythrocytes and their connection to the membrane-skeleton. *J Biosci.* 2005; 30:317–28. [PubMed: 16052070]
- [17]. Choi KS, Aizaki H, Lai MM. Murine coronavirus requires lipid rafts for virus entry and cell–cell fusion but not for virus release. *J Virol.* 2005; 79:9862–71. [PubMed: 16014947]
- [18]. Laliberte JP, McGinnes LW, Peeples ME, Morrison TG. Integrity of membrane lipid rafts is necessary for the ordered assembly and release of infectious Newcastle disease virus particles. *J Virol.* 2006; 80:10652–62. [PubMed: 17041223]
- [19]. Laliberte JP, McGinnes LW, Morrison TG. Incorporation of functional HN-F glycoprotein-containing complexes into newcastle disease virus is dependent on cholesterol and membrane lipid raft integrity. *J Virol.* 2007; 81:10636–48. [PubMed: 17652393]
- [20]. Desplanques AS, Nauwynck HJ, Vercauteren D, Geens T, Favoreel HW. Plasma membrane cholesterol is required for efficient pseudorabies virus entry. *Virology.* 2008; 376:339–45. [PubMed: 18471850]
- [21]. Aizaki H, Morikawa K, Fukasawa M, Hara H, Inoue Y, Tani H, et al. Critical role of virion-associated cholesterol and sphingolipid in hepatitis C virus infection. *J Virol.* 2008; 82:5715–24. [PubMed: 18367533]
- [22]. Sehgal PB, Guo GG, Shah M, Kumar V, Patel K. Cytokine signaling: STATS in plasma membrane rafts. *J Biol Chem.* 2002; 277:12067–74. [PubMed: 11815625]
- [23]. Griner LN, McGraw KL, Johnson JO, List AF, Reuther GW. JAK2-V617F-mediated signalling is dependent on lipid rafts and statins inhibit JAK2-V617F-dependent cell growth. *Br J Haematol.* 2013; 160:177–87. [PubMed: 23157224]
- [24]. Loura LM, Fedorov A, Prieto M. Partition of membrane probes in a gel/fluid two-component lipid system: a fluorescence resonance energy transfer study. *Biochim Biophys Acta.* 2000; 1467:101–12. [PubMed: 10930513]
- [25]. Wang TY, Leventis R, Silvius JR. Partitioning of lipidated peptide sequences into liquid-ordered lipid domains in model and biological membranes. *Biochemistry.* 2001; 40:13031–40. [PubMed: 11669641]
- [26]. de Almeida RF, Loura LM, Fedorov A, Prieto M. Nonequilibrium phenomena in the phase separation of a two-component lipid bilayer. *Biophys J.* 2002; 82:823–34. [PubMed: 11806924]
- [27]. Kusumi A, Nakada C, Ritchie K, Murase K, Suzuki K, Murakoshi H, et al. Paradigm shift of the plasma membrane concept from the two-dimensional continuum fluid to the partitioned fluid: high-speed single-molecule tracking of membrane molecules. *Annu Rev Biophys Biomol Struct.* 2005; 34:351–78. [PubMed: 15869394]
- [28]. Orädd G, Westerman PW, Lindblom G. Lateral diffusion coefficients of separate lipid species in a ternary raft-forming bilayer: a Pfg-NMR multinuclear study. *Biophys J.* 2005; 89:315–20. [PubMed: 15863478]
- [29]. Kusumi A, Ike H, Nakada C, Murase K, Fujiwara T. Single-molecule tracking of membrane molecules: plasma membrane compartmentalization and dynamic assembly of raft-philic signaling molecules. *Semin Immunol.* 2005; 17:3–21. [PubMed: 15582485]
- [30]. Kuo C, Hochstrasser RM. Super-resolution microscopy of lipid bilayer phases. *J Am Chem Soc.* 2011
- [31]. Pluthero FG. Rapid purification of high-activity Taq DNA polymerase. *Nucleic Acids Res.* 1993; 21:4850–1. [PubMed: 8233838]
- [32]. Krause CD, Izotova LS, Ren G, Yuan ZR, Shi Y, Chen CC, et al. Efficient coexpression of bicistronic proteins in mesenchymal stem cells by development and optimization of a multifunctional plasmid. *Stem Cell Res Ther.* 2011; 2:15. [PubMed: 21401924]
- [33]. Shaner NC, Campbell RE, Steinbach PA, Giepmans BN, Palmer AE, Tsien RY. Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nat Biotechnol.* 2004; 22:1567–72. [PubMed: 15558047]
- [34]. Shaner NC, Steinbach PA, Tsien RY. A guide to choosing fluorescent proteins. *Nat Method.* 2005; 2:905–9.
- [35]. Ai HW, Henderson JN, Remington SJ, Campbell RE. Directed evolution of a monomeric, bright and photostable version of *Clavularia* cyan fluorescent protein: structural characterization and applications in fluorescence imaging. *Biochem J.* 2006; 400:531–40. [PubMed: 16859491]

- [36]. Croze E, Usacheva A, Asarnow D, Minshall RD, Perez HD, Colamonici O. Receptor for activated C-kinase (RACK-1), a WD motif-containing protein, specifically associates with the human type I IFN receptor. *J Immunol.* 2000; 165:5127–32. [PubMed: 11046044]
- [37]. Usacheva A, Smith R, Minshall R, Baida G, Seng S, Croze E, et al. The WD motif-containing protein receptor for activated protein kinase C (RACK1) is required for recruitment and activation of signal transducer and activator of transcription 1 through the type I interferon receptor. *J Biol Chem.* 2001; 276:22948–53. [PubMed: 11301323]
- [38]. Usacheva A, Tian X, Sandoval R, Salvi D, Levy D, Colamonici OR. The WD motif-containing protein RACK-1 functions as a scaffold protein within the type I IFN receptor-signaling complex. *J Immunol.* 2003; 171:2989–94. [PubMed: 12960323]
- [39]. Adams DR, Ron D, Kiely PA. RACK1, A multifaceted scaffolding protein: Structure and function. *Cell Commun Signal.* 2011; 9:22. [PubMed: 21978545]
- [40]. Kotenko SV, Izotova LS, Pollack BP, Muthukumaran G, Paukku K, Silvennoinen O, et al. Other kinases can substitute for Jak2 in signal transduction by interferon- γ . *J Biol Chem.* 1996; 271:17174–82. [PubMed: 8663414]
- [41]. Krause CD, He W, Kotenko S, Pestka S. Modulation of the activation of Stat1 by the interferon-receptor complex. *Cell Res.* 2006; 16:113–23. [PubMed: 16467883]
- [42]. Demarco RS, Lundquist EA. RACK-1 acts with Rac GTPase signaling and UNC-115/abLIM in *Caenorhabditis elegans* axon pathfinding and cell migration. *PLoS Genet.* 2010; 6:e1001215. [PubMed: 21124943]
- [43]. Krause CD, Pestka S. Evolution of the Class 2 cytokines and receptors, and discovery of new friends and relatives. *Pharmacol Ther.* 2005; 106:299–346. [PubMed: 15922016]
- [44]. Pestka S. The interferon receptors. *Semin Oncol.* 1997; 24:S9.
- [45]. Kotenko SV, Pestka S. Jak-Stat signal transduction pathway through the eyes of cytokine class II receptor complexes. *Oncogene.* 2000; 19:2557–65. [PubMed: 10851054]
- [46]. Ragimbeau J, Dondi E, Vasserot A, Romero P, Uze G, Pellegrini S. The receptor interaction region of Tyk2 contains a motif required for its nuclear localization. *J Biol Chem.* 2001; 276:30812–8. [PubMed: 11399767]
- [47]. Sun Y, Wallrabe H, Booker CF, Day RN, Periasamy A. Three-color spectral FRET microscopy localizes three interacting proteins in living cells. *Biophys J.* 2010; 99:1274–83. [PubMed: 20713013]
- [48]. Claudinon J, Gonnord P, Beslard E, Marchetti M, Mitchell K, Boularan C, et al. Palmitoylation of interferon- γ (IFN- γ) receptor subunit IFNAR1 is required for the activation of Stat1 and Stat2 by IFN- γ . *J Biol Chem.* 2009; 284:24328–40. [PubMed: 19561067]
- [49]. Subramaniam PS, Johnson HM. Lipid microdomains are required sites for the selective endocytosis and nuclear translocation of IFN- γ , its receptor chain IFN- γ receptor-1, and the phosphorylation and nuclear translocation of STAT1. *J Immunol.* 2002; 169:1959–69. [PubMed: 12165521]
- [50]. Marchetti M, Monier MN, Fradagrada A, Mitchell K, Baychelier F, Eid P, et al. Stat-mediated signaling induced by type I and type II interferons (IFNs) is differentially controlled through lipid microdomain association and clathrin-dependent endocytosis of IFN receptors. *Mol Biol Cell.* 2006; 17:2896–909. [PubMed: 16624862]
- [51]. Sen S, Roy K, Mukherjee S, Mukhopadhyay R, Roy S. Restoration of IFN γ R subunit assembly, IFN γ signaling and parasite clearance in *Leishmania donovani* infected macrophages: role of membrane cholesterol. *PLoS Pathog.* 2011; 7:e1002229. [PubMed: 21931549]
- [52]. Contreras FX, Ernst AM, Haberkant P, Bjorkholm P, Lindahl E, Gonen B, et al. Molecular recognition of a single sphingolipid species by a protein's transmembrane domain. *Nature.* 2012; 481:525–9. [PubMed: 22230960]
- [53]. Fantini J, Barrantes FJ. How cholesterol interacts with membrane proteins: an exploration of cholesterol-binding sites including CRAC, CARC, and tilted domains. *Front Physiol.* 2013; 4:31. [PubMed: 23450735]

Fig. 1.

Comparison of pairwise FRET efficiencies between two-protein FRET and three-protein FRET. IFN- R1/TFP or IFN- R1, IFN- R2/CFP or IFN- R2, and Lyn-ToFP or pcDNA3 were expressed so that cells had either all three fluorescent proteins (red circles), or two fluorescent proteins and the nonfluorescent cognate of the third protein (black circles). Data are plotted as the FRET efficiency as a function of either the number of donor:acceptor complexes formed (A, C and E) or the molar ratio of the donor to acceptor that are common between the datasets (B, D and F). (A and B) The top graphs compared FRET between TFP and CFP. (C and D) The middle graphs compared FRET between TFP and ToFP. (E and F) The lower graphs compared the FRET efficiency between CFP and ToFP. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 2.

Differing FRET among different complexes containing TFP, CiFP and ToFP. The following triplets of proteins were coexpressed and pairwise FRET efficiencies were calculated from each set of spectra in the population: (A–C) FRET among IFN- R1/TFP, IFN- R2/CiFP, and Lyn-ToFP, (D–F) FRET among RACK-1/TFP, IFN- R1/CiFP, and IFN- R2c/StFP, and (G–I) FRET among IFN- R2/ R2b/TFP, IFN- R1/CiFP, and IFN- R2/StFP. Data was analyzed in the absence of IFN- (filled circles), and after 1700 U/mL IFN- was added for at least 10 min (unfilled circles). Pairwise FRET efficiencies were plotted as follows, described here as *x*-axis vs. *y*-axis: (A, D and G) FRET between TFP and either ToFP or StFP vs. FRET between CiFP and either ToFP or StFP, (B, E and H) FRET between TFP and CiFP vs. FRET between CiFP and either ToFP or StFP, and (C, F and I) FRET between TFP and CiFP vs. FRET between TFP and either ToFP or StFP.

Fig. 3.

Nano-localization of receptor chain pairs and Lyn-ToFP. The following triplets were coexpressed and FRET among various fluorescent pairs was plotted pairwise (as y-axis vs. x-axis). (A–C) IFN- γ R1/TFP, IL-10R2/CiFP, and Lyn-ToFP, (D–F) IL-10R1/TFP, IL-10R2/CiFP, and Lyn-ToFP and (G–I) IFN- α R1/TFP, IFN- α R2c/CiFP, and Lyn-ToFP. The data from this population of cells fell into two groups: those with the expected data distribution (filled circles), and those with a distinct distribution of data (unfilled circles). (A, D and G) Pairwise plots of FRET between the helper chain (IL-10R2/CiFP, IL-10R2/CiFP, or IFN- γ R1/TFP) and Lyn-ToFP as a function of FRET between the matched ligand-binding chain (IFN- γ R1/TFP, IL-10R1/TFP, or IFN- α R2c/CiFP, respectively) and Lyn-ToFP. (B, E and H) Pairwise plots of FRET between the helper chain and Lyn-ToFP as a function of FRET between the helper chain and its matched ligand-binding chain. (C, F and I) Pairwise plots of FRET between the ligand-binding chain and Lyn-ToFP as a function of FRET between the helper chain and its matched ligand-binding chain.

Fig. 4.

Pairwise FRET relationships within fluorescent quartets. The fluorescent quartets {Jak1/TFP, Jak2/CiFP, IFN- R1/OFP, IFN- R2/ChFP} (A–C) and {Jak1/TFP, Tyk2/CiFP, IFN- R1/OFP, IFN- R2c/ChFP} (D–F) were coexpressed in 293T cells and were excited with 442 nm, 514, 543, and 633 nm lasers. The four spectra were deconvolved and pairwise FRET efficiencies within each pair calculated as a function of FRET between IFN- R1 and IFN- R2 (A–C) or between IFN- R1 and IFN- R2c (D–F). (A) FRET between Jak1 and Jak2. (B) FRET between Jak1 and IFN- R1. (C) FRET between Jak2 and IFN- R1. (D) FRET between Jak1 and Tyk2. (E) FRET between Tyk2 and IFN- R1. (F) FRET between Jak1 and IFN- R1. Within (D–F) filled gray circles are from cells exhibiting FRET between both IFN- R1 and IFN- R2c and Jak1 and IFN- R1; filled black circles are from cells with FRET between IFN- R1 and IFN- R2c but not between IFN- R1 and Jak1; unfilled black circles are from cells with poor FRET between IFN- R1 and IFN- R2c and no FRET between Jak1 and IFN- R1.

Fig. 5.

FRET between pairs within increasing numbers of quartets. FRET among the fluorescent quartets {Jak1/TFP, Jak2/CiFP, IFN- R1/OFP, IFN- R2/ChFP} (A) and {Jak1/TFP, Tyk2/CiFP, IFN- R1/OFP, IFN- R2c/ChFP} (B) was determined, and plotted as a function of the relative number of fluorescent quartets present in the imaged area. All circles on the right-hand side of subfigure A are filled. Unfilled circles on the right-hand side of (B) represent data from cells in which no interaction between Jak1 and IFN- R1 was observed, while filled circles on the right-hand side of (B) represent data from cells in which an interaction between Jak1 and IFN- R1 was observed. Black, FRET between Jak1 and either Jak2 or Tyk2. Red, FRET between Jak1 and either IFN- R1 or IFN- R1. Green, FRET between Jak1 and either IFN- R2 or IFN- R2c. Yellow, FRET between either Jak2 and IFN- R1 or Tyk1 and IFN- R1. Blue, FRET between either Jak2 and IFN- R2 or Tyk2 and IFN- R2c. Magenta, FRET between IFN- R1 and IFN- R2, or IFN- R1 and IFN- R2c. The unfilled circles on the left-hand side of the figure come from FRET between isolated fluorescent pairs (Jak2/CiFP and IFN- R2/StFP, Jak1/StFP and IFN- R1/EGFP, IFN- R1/CiFP and IFN- R2/StFP, Jak1/CiFP and IFN- R1/StFP, Jak1/CiFP and IFN- R2c/StFP, Tyk2/CiFP and IFN- R1/StFP, Tyk2/CiFP and IFN- R2c/StFP, IFN- R1/CiFP and IFN- R2c/StFP) or from the fluorescent triplet {Jak1/TFP, IFN- R1/CiFP, and IFN- R2/ToFP}. These data are color-coded to correspond to the given pair within the quartet. Because in most cases FRET in the isolated pair was determined using a different pair of fluorescent proteins than used within the quartet, the FRET efficiencies of the isolated pairs or triplets were recalculated to match those of the relevant pair within the quartet. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 6.

Association of the IFN- and Type I IFN receptor complexes in cells. (A) IFN- R1 (red) and Jak1 (green-yellow) interact at very low levels, and associate with lipid rafts (dark brown) within plasma membranes (light brown) at similarly low levels. (B) The lipid-raft-associated IFN- R1:Jak1 complex interacts with a complex of IFN- R2 (green) and Jak2 (cyan) to form the tetrameric complex, confined to either the edge (left) or the middle (right) of lipid rafts. Hypothesizing intra-raft localization of the IFN- receptor complex (middle panels) or of the Type I IFN receptor complex (bottom panels), we schematically model their assembly. (C) The interaction of the extracellular domains of IFN- R1 and IFN- R2 or of IFN- R1 (dark green) and IFN- R2c (dark red) is the primary contact that forms the four-chain complex, although it is of low affinity. (D) Interactions between Jak1 and Jak2 occur that help hold together the tetrameric IFN- receptor complex, while interactions among Jak1, Tyk2 (dark blue) and RACK-1 (purple) assist in keeping IFN- R1 and IFN- R2c together. (E) The dissociation of the extracellular domains does not disrupt the complex due to the intracellular interactions. (F) The ligand (brown) binds to the receptor while the extracellular domains are dissociated; the activation of the receptor complex results in tyrosine phosphorylations (black dots) of IFN- R1, IFN- R2c, IFN- R1 and the Janus kinases. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)