The unraveling of cellular mechanisms relies heavily on understanding protein–protein interactions (PPIs). For example, interactions between proteins play a crucial role in host–pathogen interactions and are therefore potential therapeutic targets. To better understand the role of protein interactions in diverse processes, more powerful techniques to identify and confirm interactors of membrane and membrane-associated proteins have to be developed.

The majority of identified PPIs have been found using the yeast two-hybrid (Y2H) screen because it is a very powerful tool for identifying unknown interaction partners. A Y2H screen always yields a varying number of false-positive candidates. Therefore, results from Y2H screens must be confirmed by independent methods (1,2). Detecting protein complexes between two membrane proteins by co-immunoprecipitation is extremely difficult, if sometimes not impossible, due to their unstable interaction when being extracted from membranes. The most common technique used to circumvent this problem is to measure fluorescence (Förster) resonance energy transfer (FRET) between two fluorophores such as cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) fused to the interacting proteins. It is also possible to confirm PPIs with a proximity ligation assay (PLA) (3).

In this study, we present an approach that combines the well-established FRET technique and the recently introduced PLA. Combining FRET with PLA analysis allows visualization of YFP- and CFP-tagged interacting proteins and therefore detects cells that are co-expressing interactors. FRET-PLA can be further used to locate PPIs with an increased sensitivity and with a high spatial resolution. Intensity-based FRET analyses have a lower signal-to-noise ratio than PLA analyses, which detect PPI as a bright fluorescent dot (4–6). The introduction of the PLA technique during FRET measurements simultaneously allows an additional readout of PPIs based on recognition of epitopes that are in close proximity. By combining the advantages of the two methods, we were able to precisely determine the molecular proximity between two membrane proteins at nanometer distances.

Materials and methods

Molecular cloning

All primers used in the cloning steps (Supplementary Table S1) were obtained from Sigma Aldrich (Steinheim, Germany), and an overview of the cloning strategy is displayed in Supplementary Figure S2. The CD63 sequence was amplified using PfuUltra HF polymerase (Agilent Technologies, Böblingen, Germany) from the Y2H vector pPR3-N-CD63 (containing the full-length human CD63 sequence, codons 1 to 238, Genebank accession no. KF998086) in the pCMV-Tag2B vector (Stratagene, Heidelberg, Germany) using the primer pair DI001/D002 (containing a 5’ BamHI linker and a 3’ HindIII linker). The amplicon was digested with the indicated restriction enzymes (New England Biolabs, Frankfurt, Germany) and was ligated with T4 DNA ligase (New England Biolabs) into the BamHI/HindIII digested vector pCMV-Tag2B. Likewise, the cDNA (gp160 of HIV-1) was obtained from the pCMV-Tag2B vector (Stratagene, Heidelberg, Germany) using the primer pair DI001/D002 (containing a 5’ BamHI linker and a 3’ HindIII linker). The amplicon was digested with the indicated restriction enzymes (New England Biolabs, Frankfurt, Germany) and was ligated with T4 DNA ligase (New England Biolabs) into the BamHI/HindIII digested vector pCMV-Tag2B. The resulting plasmids were transformed into E. coli DH5α (Invitrogen, Carlsbad, CA, USA) and selected for insertion of the insertions using LB medium supplemented with kanamycin (50 μg/ml). The correct DNA sequence was confirmed by Sanger sequencing (Eurofins MWG Operon, Ebersberg, Germany).

METHOD SUMMARY

We combined FRET and PLA techniques in order to investigate protein–protein interactions between membrane proteins. We then demonstrated that PLA strongly correlates with FRET values. An important feature of the combined FRET-PLA approach is the additional readout of high spatial resolution from the PLA signals.
were washed twice with PBS and incubated for 48 h and fixed for 20 min in 2% paraformaldehyde dissolved in PBS. After the fixation step, cells were blocked for 2 h at room temperature in 30% normal donkey serum (Jackson ImmunoResearch, West Grove, PA), BSA fraction V (Carl Roth) dissolved in PBS or Duolink II blocking solution (1×) was used to test blocking conditions.

In situ PLA application and FRET sample preparation

The blocked cells were incubated for FRET-PLA with primary antibodies (5 µg/mL) goat anti-FLAG (NB600–344, NovusBio, Littleton, CO) and mouse anti-V5 antibody (ab7671, Abcam) and goat anti-FLAG (NB600–380, NovusBio). To verify the selectivity of the PLA experiments, we used the antibodies mouse anti-FLAG (ab18230, Abcam, Cambridge, UK) and goat anti-V5, as well as mouse anti-α1 subunit Na⁺K⁺-ATPase (ab7671, Abcam) and goat anti-V5. All cells were incubated with primary antibodies overnight at 4°C. On the following day, the cells were washed twice briefly with Duolink II buffer A and left for 2 h in Duolink II buffer A with gentle shaking. After this extended washing step, the cells were blocked again with 30% normal donkey serum for 1 h at room temperature. After the blocking, all further steps were done according to the Duolink II manual. To confirm PPI, the PLA probe anti-mouse PLUS and the PLA probe anti-goat MINUS were used; for the protein expression control, the PLA probe anti-goat PLUS and the PLA probe anti-goat MINUS were used. After incubation for 1 h at 37°C, the slides were briefly washed twice and left with gentle shaking for 2 h in Duolink II buffer A. The slides were further processed for ligation and amplification according to the Duolink II manual. All PLA cell controls transfected with empty vectors pCMV-Tag2B/pCDNA4B-V5-His were treated in the same way as cells co-transfected with the vectors pCMV-CD63-YFP-FLAG/pCDNA-gp41-CFP-V5. For normalized FRET (NFRET) measurements, cells were mounted in glycerol containing 0.1% p-phenylenediamine (Sigma Aldrich). For all other applications, Duolink II mounting medium was used. PLA values were obtained from three independent rolling circle amplification (RCA) reactions and randomly selected images. The same samples were used to obtain NFRET values in combination with indicated controls. All Duolink II reagents were obtained from Eurogentec (Angers, France). For FRET acceptor photobleaching experiments, HEK293T cells were seeded in ibiTreat 8-well slides (IBIDI, Munich, Germany) and co-transfected with FRET vectors.

Confocal laser scanning microscopy (cLSM) and FRET analysis

All images were acquired using a Zeiss LSM 780 confocal laser scanning inverted microscope (Carl Zeiss, Oberkochen, Germany) and a 63x oil immersion objective. Images for NFRET analysis were obtained using multitrack channel mode instrument settings for CFP channel at 405 nm and detected emission peak at 475 nm/27 nm bandwidth, excitation for FRET channel at 405 nm, and detected emission peak at 527 nm/48 nm bandwidth. YFP signals were additionally detected in a single track with an excitation at 514 nm and detected emission peak at 527 nm/55 nm bandwidth. Donor and acceptor bleed-through coefficients were determined by acquiring images only from a donor or an acceptor, respectively. NFRET values (9) were calculated on a pixel-by-pixel basis using a macro from the Zeiss ZEN 2010 software. The technique for obtaining NFRET
values was described in detail by others (10). Fluorescence intensity profiles were obtained from the same software. Acceptor photobleaching FRET experiments were performed using PFA-fixed HEK293T co-transfected cells as previously described in the PLA section. Within each cell, we defined regions of interest (ROIs) in the bleached and unbleached cell regions, as well as in the background region. The images were acquired in multi-track channel mode for CFP (excited at 405 nm, detected at 463–508 nm, 1.2% laser power intensity) and YFP (excited at 514 nm, detected at 515–611 nm, 1.5% laser power intensity). The 514-nm laser line was set at 100% laser power intensity at bleaching time points and calculated FRET efficiency with the Zeiss ZEN FRET implemented software was used to obtain values for each ROI. PLA signals were detected with Zeiss ZEN smart setup instrument settings for Texas Red analog dye. PLA dots were counted with the ImageJ pixel counting plugin, and nuclei were counted with the ImageJ cell counter plugin. The results were used to calculate PLA dots per cell. Indirectly immunostained Na+,K+-ATPase was detected using Zeiss ZEN smart setup settings for 594-nm dyes.

Western blot analysis
At 48 h posttransfection, cells were lysed in 100 µL 2x Laemmli buffer, chromosomal DNA was degraded with 25 U of benzonase (Novagen, San Diego, CA), proteins were reduced with 5 µL β-mercaptoethanol and separated by SDS-PAGE using TGX gels (BioRad, Munich, Germany), and then transferred onto an Immobilon-P PVDF membrane (Millipore, Schwalbach, Germany). Lysates of expressed CFP, YFP, and CFP-YFP proteins were diluted 1:10 in 2x Laemmli buffer. Proteins were probed with 0.5 µg/mL of rabbit anti-GFP (ab6556, Abcam), 1.0 µg/mL of mouse anti-α1 Na+,K+-ATPase, and 1.0 µg/mL of mouse anti-β actin (AM00194PU-N, Acris, Herford, Germany. Primary antibodies were detected with 0.125 µg/mL of HRP-conjugated goat antirabbit IgG or anti-mouse IgG (DAKO, Hamburg, Germany) and visualized with Pierce ECL Western blotting substrate (Thermo Scientific, Bonn, Germany). Western blot images were acquired...
Figure 2. Detection of PPI using the FRET-proximity ligation assay (FRET-PLA) technique. (A) Protein–protein interaction (PPI) detection by standard PLA between nonfluorescent expressed CD63-FLAG and gp41-V5 proteins. (B) Confocal images of HEK293T cells co-transfected with pCMV-CD63-YFP and pcDNA-gp41-CFP-V5 vectors. The red arrow indicates the region for which the fluorescence intensity (I) was measured. The I values for CFP, YFP, FRET, and PLA are displayed as a function of the distance. (i) PLA intensity profile within a 4-µm distance (ii) and within a 2-µm distance. Arrow length: 7.5 µm. (C) Example of acquiring images for a correlative FRET-PLA analysis. Cells with a high FRET value and the highest PLA signals are highlighted with a green arrow, cells with lower FRET values with a white arrow, and cells with the lowest FRET values with a pink arrow. (D) Displayed correlation between FRET\(--\)PLA and CFP\(--\)YFP by Pearson’s coefficient calculation of PLA signals (n = 46). (E) Quantification of PLA signals. (i) Comparison of quantified PLA signals from panel B positive for CFP (n = 60), for YFP (n = 94) from the whole cell population (n = 154), and control using the vectors pCMV-Tag2B/pcDNA4B-V5-His (mock, n = 202). (ii) NFRET values for co-expressed proteins CD63-YFP-FLAG/gp41-CFP-V5 (n = 52), for positive FRET control cells expressing YFP fused to CFP to obtain maximum NFRET values (n = 11), and for FRET negative control cells expressing CFP and YFP separately to obtain the lowest NFRET value (n = 12). *P = 0.043; †P = 0.006; ‡P = 0.00012. Scale bars, 10 µm.
using a Chemocam device (Intas, Göttingen, Germany) with implemented contrast correction.

Statistical analysis
Our results are expressed as mean ± standard deviation. We used a two-tailed, paired Student’s t-test to determine statistical significance. A P value of less than 0.05 was considered as significant between 2 groups.

Results and discussion
We identified CD63 as an interaction partner of the HIV-1 transmembrane protein gp41 in a split-ubiquitin Y2H screen (Supplementary Figure S1) and used FRET-PLA to confirm the novel interaction between these two proteins. One of the most challenging aspects in PPI studies is confirmation of Y2H results by independent techniques. PLA allows labeling of interacting proteins by primary antibodies raised in different species. The primary antibodies are detected by DNA-conjugated secondary antibodies specifically recognizing the corresponding species. The next step of PLA is based on the ligation of conjugated DNA when the epitopes are in close proximity. After successful ligation, the resulting DNA circle can

Figure 3. FRET acceptor photobleaching experiments.
(A) HEK293T cells expressing FRET pairs. Bleached (red frames), unbleached (green frames), and background bleached (white frames) areas are marked as region of interest (ROI) (B) Intensity profiles of cyan (CFP) and yellow fluorescence protein (YFP) emissions during acceptor photobleaching experiments. Acceptor photobleaching with a 514-nm laser line starts at time point 0 and is repeated at each further time point. (C) Quantification of FRET photobleaching experiments by calculating FRET efficiencies for the FRET pairs CFP-YFP (n = 12), CD63-YFP-FLAG/gp41-CFP-V5 (n = 12), and CFP/YFP (n = 11). *P = 0.28, †P < 0.0001. Scale bars: 5 µm.
Figure 4. Validation of correlative FRET-proximity ligation assay (PLA) experiments. (A) Background of nonspecific PLA signals obtained from HEK293T cells transfected with empty vectors pCMV-Tag2B/pcDNA4B-V5-His. PLA signals represent dual recognition PLA against FLAG and V5 epitope depending on blocking reagents. (B) Quantification of nonspecific PLA signals dependent on blocking reagents. The pink arrow marks the nonspecific PLA detection (0.07 ± 0.03 PLA signals per cell) using 30% normal donkey serum, which means a specificity of 93% ± 3%. (C) Protein expression control by FLAG and V5 single recognition PLA. (D) Western blot analysis of cells expressing different FRET constructs probed with anti-GFP antibody. (E) Detection of a YFP-tagged zinc finger nuclease (ZFN)-FLAG in the nucleus by single recognition PLA. (F) Detection of protein expression of CD63-YFP, gp41-CFP-V5, and co-localization with immunostained Na⁺,K⁺-ATPase. (G) Schematic illustration of experimental design. The selectivity of the PLA was validated using Na⁺,K⁺-ATPase as reference protein. Antibodies against FLAG and V5 were used in experiment PLA 1, antibodies against V5 and the α1 subunit of the Na⁺,K⁺-ATPase were used in experiment PLA 2, and all epitopes are located in the cytoplasm. (H) Western blot analysis of Na⁺,K⁺-ATPase in samples used for PLA 1 and PLA 2 (I) Quantification of PLA signals obtained for the expressed protein pair gp41-CFP-V5/CD63-YFP-FLAG (PLA 1, n = 32) and gp41-CFP-V5/Na⁺,K⁺-ATPase (PLA 2, n = 30). All scale bars are 10 µm, except in E, where it is 2 µm.

be amplified by the highly processive φ29 DNA polymerase (11,12). This RCA generates several hundred-fold more DNA circles that can be visualized by performing incubation with complementary fluorescently labeled oligonucleotide probes. When applying this method, the PPI is detected in situ with a fluorescence microscope as bright fluorescent dots (13,14) (Figure 1). In contrast to PLA, FRET analysis is based on photon-free transfer of energy from a donor fluorophore to an acceptor fluorophore and only occurs when the donor is very close (<10 nm) to the acceptor. The efficiency of this energy transfer is proportional to the inverse sixth power of the distance between the donor and acceptor (15–17).

We established the FRET-PLA technique using FLAG/V5 epitope-tagged fluorescent proteins by combining standard PLA and FRET measurements in transiently transfected cells. PLA can detect PPIs involving endogenously (18–20) or transiently (21) expressed proteins. Our PLA test results were positive for CD63 and gp41 proteins expressed without a fluorescent protein tag (Figure 2A). Introduction of fluorescent proteins in standard PLA analyses allows identification of cells expressing interactors,
and the PPI can be measured simultaneously by PLA and FRET analysis. For FRET quantification, the measurement of NFRET values was used to subtract donor or acceptor signal contaminations from the FRET image. The NFRET values are advantageous because of their independence from protein expression levels, allowing for better comparison between different samples in conjunction with whole-cell FRET measurements (9).

For the correlative FRET-PLA technique, we used the recombinant proteins gp41 and human CD63, tagged with CFP and YFP respectively. In the donor protein gp41-CFP-V5, a signal sequence from Ig kappa-chain V-J2-C (SP1) was introduced to the N terminus to increase the level of protein expression (22) in transfected HEK293T cells (Supplementary Figure S2). V5 and FLAG tags were used to detect the fluorescent hybrid proteins by PLA. Establishing correlative FRET-PLA requires that the fluorescent tags do not interact with themselves. To avoid this effect, we used modified SCFP3A and SYFP2 templates to improve the brightness and monomeric properties of the expressed fluorescent proteins (7), which for simplicity were called YFP and CFP.

We carried out the FRET-PLA technique on an HEK293T cell-based system and prepared the samples in accordance with the FRET-PLA workflow diagram (Supplementary Figure S3). The intensity plots showed that PLA signals were not produced in the absence of CFP or YFP, and the detection of positive PLA signals was possible only when CFP and YFP intensities were measured within the same distance (Figure 2B). The feasibility of correlative FRET-PLA is further supported by the observation that FRET analysis revealed high NFRET values where PLA signals occurred. Consequently, cells expressing FRET protein pairs yielded PLA signals and, most importantly, the amount of PLA signals strongly correlated with the NFRET value (Figure 2C). The lack of significant PLA signals in controls indicated a true PPI between the acceptor protein CD63-YFP-FLAG and the donor protein gp41-CFP-V5.

We characterize the correlation for each PLA intensity profile by calculating the Pearson’s correlation coefficient ($r$) between $\text{FRET} \leftarrow \leftarrow \text{PLA}$ and $\text{CFP} \leftarrow \leftarrow \text{YFP}$. The results consistently showed a strong positive correlation between PLA and FRET signals ($r = 0.62 \pm 0.29$); however, between CFP and YFP signals ($r = 0.15 \pm 0.40$) the correlation coefficient varied from no correlation to a weak–strong negative and positive correlation (Figure 2D). Furthermore, the sequential quantification of cells expressing CFP, cells expressing YFP, and the whole-cell population clearly showed that the PLA signals are not evenly distributed across all cell populations (Figure 2E i).

To classify the NFRET value as a real bimolecular FRET characteristic, FRET controls were used. The cells expressing fused CFP-YFP (ECPF-EYFP) protein showed high NFRET values and are considered positive FRET controls. In contrast, cells expressing CFP and YFP proteins
separately showed low values of NFRET and are considered negative FRET controls. When CD63-YFP-FLAG and gp41-CFP-V5 were coexpressed, high NFRET values were measured (Figure 2E ii). We can obviously exclude the possibility that fused fluorescent proteins could induce nonspecific dimerization because unfused CD63-FLAG and gp41-V5 proteins also showed PLA signals (Figure 2A).

In addition to intensity-based FRET measurement, we also obtained FRET interaction data using acceptor photobleaching. This method is based on acceptor depletion and continuous monitoring of donor and acceptor fluorescence intensities (l) during the acceptor photobleaching process (23). We examined the fluorescence intensity profiles of the FRET bleaching experiments (Figure 3A) and observed that for the FRET pair CD63-YFP-FLAG/gp41-CFP-V5, CFP intensity increased after acceptor photobleaching at several time points (Figure 3B) and was reproducible over a set of 12 cells. Photobleaching experiments were used to calculate FRET efficiencies, which also confirmed a PPI between CD63-YFP-FLAG and gp41-CFP-V5, and are clearly different from the pseudo-FRET efficiencies obtained from separately expressed CFP and YFP (Figure 3C).

A highly relevant quality criterion of PLA experiments is the ability to deliver reliable signals that are free from interference. To meet this requirement, we validated our correlative FRET-PLA and investigated the specificity and selectivity of the PLA detection. A highly sensitive method such as PLA, based on epitope recognition in combination with increased signals produced by RCA, will always be associated with nonspecific signals. We considered several factors to reduce nonspecific PLA signals. These included a shorter incubation time for antibodies and PLA probes, a shorter RCA time, increased washing stringency, and variations of blocking conditions. The only factor that remained was to find an optimized blocking condition. We performed different blocking experiments and found that an extended blocking step using 30% normal donkey serum combined with an extended washing time was the most effective strategy for reducing unspecific PLA signals (Figure 4A). Under these blocking conditions, a single detected PLA dot is more than 93% ± 3% reliable for specific epitope recognition (Figure 4B) and proves the specificity of the primary antibodies used. It was previously shown that the PLA and FRET performance can be compared in a semiquantitative manner (24). The reported saturation phenomenon in PLA applications representing nonlinearities in high protein concentrations using 1% BSA was observed by FACS analysis of PLA signals. As we showed in our specificity assay (Figure 4A), the blocking condition is a major issue in PLA experiments. We suggest that linearity can be reached by optimized blocking conditions, as we show that the blocking conditions are very important to obtain highly specific PLA results.

To evaluate expression of the acceptor and donor proteins, a single recognition PLA was performed using one polyclonal antibody to detect only FLAG or V5 tag. For single recognition PLA, we observed high numbers of positive PLA dots for FLAG or V5 recognition in contrast to the control consisting of HEK293T cells transfected with empty vectors (Figure 4C, Supplementary Figure S4). We additionally characterized the expressed proteins by Western blot analysis and showed that all fluorescent proteins are expressed (Figure 4D). To account for nonspecific antibody binding and for false-positive PLA detection on the cell surface, proteins localized in the nucleus were investigated. For this, an exogenous zinc finger nuclease (ZFN)-YFP fusion protein tagged with a FLAG epitope was used. This control experiment showed the absence of nonspecific antibody binding to the cell surface and the presence of specific binding to the target in the nucleus (Figure 4E and Supplementary Figure S5).

To provide more direct evidence that PLA is comparable with FRET analysis, we next focused on determining the selectivity of the PLA. We used the endogenous Na⁺,K⁺-ATPase as a reference because of its similarity to CD63. We showed by indirect immunostaining that these two proteins were predominantly expressed in the plasma membrane (Figure 4F) and ruled out by Western blot analysis the possibility that in the experiments PLA 1 and PLA 2, protein expression of Na⁺,K⁺-ATPase is reduced (Figure 4H). The key in our validation assays is the application of a primary antibody (clone 464.6, also known as 6H) raised against amino acids 1–21 of Na⁺,K⁺-ATPase (25,26) that detects epitopes in the intracellular part of the molecule in the PLA experiments. Our PLA results showed that gp41-CFP-V5 is not able to reach close proximity with the Na⁺,K⁺-ATPase, although we proved that the primary antibodies are able to detect Na⁺,K⁺-ATPase (Figure 4G, Figure 4I). These results suggest that PLA can be used to study specific PPIs in a pool of proteins expressed within the same cellular compartment. At this stage, we can exclude the possibility that nonphysiological protein overexpression is forcing close co-proximity between membrane proteins. CD63 and Na⁺,K⁺-ATPase are organized in...
different microdomains. Na⁺,K⁺-ATPase is Triton-insoluble, which leads to the conclusion that it is associated with lipid rafts (26–28). Membranes also contain a particular type of microdomain distinct from conventional lipid rafts called tetraspanin-enriched microdomains (TEMs). Proteins associated within TEMs can be solubilized independently from lipid microdomains, although they were found to associate with microdomains resembling lipid rafts (29). Tetraspanins such as CD63 act as molecular organizers of membrane protein complexes in the TEM. We propose that the lack of PLA signal between CD63 and Na⁺,K⁺-ATPase is due to their organization into different membrane microdomains and indicates that we only detect PPIs by PLA when interactors are in very close proximity.

In summary, our results provide evidence that PLA signals correlate strongly with FRET signals. The advantage of our method is that the combined application of FRET and PLA can be used to detect protein interactions in conditions of weak protein expression levels or low transfection efficiencies. Another benefit is that correlative FRET-PLA analysis takes much less time to obtain results by two independent methods. The difficulty of identifying and measuring direct PPIs between membrane proteins is one of the reasons why the development of various interaction techniques has remained in focus in the past decades. Considering that more than 60% of approved drugs are targeted at membrane proteins (30), it is clear that these proteins play an important role in biological processes. We believe that correlative FRET-PLA can provide a more precise characterization of PPI and will be useful in the investigation of molecular interactions between membrane proteins.

**Author contributions**

D.I. designed and performed the experiments, and analyzed the data. The manuscript was written by D.I., M.E., and J.D. All authors read and approved the manuscript.

**Acknowledgments**

This work was supported by the Peter und Traudi Engelhorn Foundation, Germany with a grant to Daniel Ivanusic and Jung-Stiftung für Wissenschaft und Forschung, Hamburg, Germany. We thank Kazimierz Madela for help with the ZEISS 780 confocal laser microscope, Theodorus W.J. Gadella for providing the plasmids pSCFP3A-C1 and pSYFP2-C1, Michael Schindler for the plasmid pECPF-EYFP, and Marwan Semaan for the plasmid pZFN-YFP.

**Competing interests**

The authors declare no competing interests.

**References**

3. Weibrecht, I., K.J. Leuchowius, C.M. Clausson, T. Conze, M. Jarvius, W.M.


Received 25 April 2014; accepted 5 September 2014.
Address correspondence to Joachim Denner, Robert Koch Institute, Nordufer 20, D-13353 Berlin, Germany. E-mail: Denner@rki.de

To purchase reprints of this article, contact: biotechniques@fosterprinting.com