A Fluorescent Two-hybrid Assay for Direct Visualization of Protein Interactions in Living Cells*§

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Genetic high throughput screens have yielded large sets of potential protein-protein interactions now to be verified and further investigated. Here we present a simple assay to directly visualize protein-protein interactions in single living cells. Using a modified lac repressor system, we tethered a fluorescent bait at a chromosomal lac operator array and assayed for co-localization of fluorescent prey fusion proteins. With this fluorescent two-hybrid assay we successfully investigated the interaction of proteins from different subcellular compartments including nucleus, cytoplasm, and mitochondria. In combination with an S phase marker we also studied the cell cycle dependence of protein-protein interactions. These results indicate that the fluorescent two-hybrid assay is a powerful tool to investigate protein-protein interactions within their cellular environment and to monitor the response to external stimuli in real time. 

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After sequencing the human genome the next challenge is now to analyze the complex protein networks underlying cellular functions. In the last decade a wide variety of methods to study protein-protein interactions ranging from biochemical to genetic or cell-based approaches have been developed. Biochemical methods like affinity purification or co-immunoprecipitation (Co-IP) allow the detection of protein complexes in vitro. Genetic methods, such as the yeast two-hybrid system (1), enable efficient high throughput screening of interactions within the cellular environment (2). However, the analysis of mammalian protein interactions in yeast may suffer from the absence or insufficient conservation of cellular factors modulating protein-protein interactions, e.g. through posttranslational modifications (3).

In the last years new fluorescence-based methods for in-cell visualization of protein-protein interactions have been introduced. Two established techniques, fluorescence resonance energy transfer (4, 5) and bimolecular fluorescence complementation (6), are based on the expression of fluorescently labeled proteins or fragments thereof. However, fluorescence resonance energy transfer requires costly instrumentation and advanced technical expertise, whereas bimolecular fluorescence complementation is based on the irreversible complementation and slow maturation of fluorophores, which does not allow real time detection of protein-protein interactions (6). Another strategy is based on the relocation of proteins to either cell membranes (7) or cytoplasmic aggregates of viral proteins (8).

All these methods have inherent shortcomings and are typically combined to obtain more reliable results. We have now developed a novel fluorescent two-hybrid (F2H) assay for the direct visualization of protein-protein interactions in living mammalian cells. The simple optical readout of this F2H assay allows observation of protein-protein interactions in real time and should also be suitable for high throughput screens.

EXPERIMENTAL PROCEDURES

Expression Constructs—The LacI-encoding sequence was PCR-amplified from the p3′SS EGFP-LacI expression vector (9) using the following primers: forward primer 5′-TCT AAG AAG CTT TCC ATG GTG AAA CCA GTA-3′ and reverse primer 5′-CCA TGC CCG GGA CAG GCT GCT TCG GGA AAC-3′ (restriction sites in italic). This PCR fragment was digested with HindIII and XmaI and cloned into the same sites of two Dnmt1-YFP expression vectors (MTNY.2 and MTB9215) generating PBD-LacI-YFP and PBD-LacI-YFP. The following primers (restriction sites in italic): PCNA forward, 5′-CTG GGC TCG GGA AAC-3′; PCNA reverse, 5′-TCT AGA AAG CTT TCC ATG GTG AAA CCA GTA-3′; and XRCC1 reverse, 5′-CTG GGC TCG GGA AAC-3′ were used for PCR amplification of the XRCC1 cDNA using the following primers (restriction sites in italic): XRCC1 forward, 5′-CTG GGC TCG GGA AAC-3′; XRCC1 reverse, 5′-TCT AGA AAG CTT TCC ATG GTG AAA CCA GTA-3′; and XRCC1 reverse, 5′-CTG GGC TCG GGA AAC-3′. The amplified PCR fragments were cloned into a LacI-RFP expression vector using the XhoI/HindIII sites for the NLS-PCNA-LacI-RFP and NLS-PCNA-LacI-RFP constructs. The amplified PCR fragments were cloned into a LacI-RFP expression vector using the XhoI/HindIII sites for the NLS-PCNA-LacI-RFP and NLS-PCNA-LacI-RFP constructs.
All other F2H constructs were generated by PCR amplification of coding cDNAs and subsequent ligation into the AsIIS and NotI sites of the bait and prey expression vectors described in Fig. 1a. The following primers were used with the restriction site indicated in italics: DDP1 forward, 5’-CCCCCGATGGATGATGAGAGGTTGG-3’; DDP1 reverse, 5’-CCCCCGATGGATGAGAGGTTGG-3’; TIMM13 forward, 5’-CCCCGGATCCAGGAGGGCGATCGTGGCTTC-3’; TIMM13 reverse, 5’-CCCCGGATCCAGGAGGGCGATCGTGGCTTC-3’; HZFH forward, 5’-GGGGGATCCAGGAGGGCGATCGTGGCTTC-3’; HZFH reverse, 5’-CCCCGGATCCAGGAGGGCGATCGTGGCTTC-3’. Deletion constructs and isolated domains of DNA Ligase II and III were described in Mortusewicz et al. (17). Deletion constructs and isolated domains of DNA Ligases I and III were described in Mortusewicz et al. (17). Immunoprecipitations were performed with a GFP-nanotrap (18) as described previously (11–16). Deletion constructs and isolated domains of DNA Ligases I and III were described in Mortusewicz et al. (17). All other F2H constructs were generated by PCR amplification of coding cDNAs and subsequent ligation into the AsIIS and NotI sites of the bait and prey expression vectors described in Fig. 1a. The following primers were used with the restriction site indicated in italics: DDP1 forward, 5’-CCCCCGATGGATGATGAGAGGTTGG-3’; DDP1 reverse, 5’-CCCCCGATGGATGAGAGGTTGG-3’; TIMM13 forward, 5’-CCCCGGATCCAGGAGGGCGATCGTGGCTTC-3’; TIMM13 reverse, 5’-CCCCGGATCCAGGAGGGCGATCGTGGCTTC-3’; HZFH forward, 5’-GGGGGATCCAGGAGGGCGATCGTGGCTTC-3’; HZFH reverse, 5’-CCCCGGATCCAGGAGGGCGATCGTGGCTTC-3’. Mammalian expression constructs encoding translational fusions of human DNM1, DNA Ligase I, DNA Ligase III, p21, FEN1, Polymerase δ p66 subunit, PARP-1, PARP-2, and PCNA were described previously (11–16). Deletion constructs and isolated domains of DNA Ligases I and III were described in Mortusewicz et al. (17). Immunoprecipitations were performed with a GFP-nanotrap (18) as described before (17). All fusion constructs were tested for correct expression and localization.

Cell Culture and Transfection—Transgenic BHK cells (clone 2) and U2OS cells (clone 2-6-3) containing lac operator repeats were cultured under selective conditions in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal calf serum and 150 μg/ml hygromycin B (PAA Laboratories) as described previously (19, 20). For microscopy cells were grown to 50–70% confluence either on 18 mm glass coverslips in μ-slides (ibidi, Munich, Germany) or in μClear 96-multwell plates (Greiner Bio-One, Frickenhausen, Germany) and then co-transfected with the indicated expression constructs using either polyethyleneimine (Sigma) or Polyplus transfection reagent jet-PEI™ (Biomol GmbH, Hamburg, Germany) according to the manufacturer’s instructions. After 6–10 h the transfection medium was changed to fresh culture medium. Cells were either observed immediately thereafter or incubated for another 12–42 h before live cell microscopy or fixation with 3.7% formaldehyde in PBS for 10 min at room temperature. Fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 3 min, counterstained with 4’,6-diamidino-2-phenylindole; a 458 nm (for red fluorescent proteins), a 488 nm (for GFP), and a 514 nm (for cyan fluorescent protein (CFP)), a 561 nm diode-pumped solid-state laser (for red fluorescent proteins). Confocal image stacks of living or fixed cells were typically recorded with a frame size of 512 × 512 pixels, a pixel size of 50–100 nm, a z-step size of 250 nm, and the pinhole opened to 1 Airy unit. A maximum intensity projection of several confocal mid-z-sections was generated with ImageJ (Version 1.38).

Multiplex analysis was performed using an INCell Analyzer 1000 (GE Healthcare) equipped with a 40×/0.6 Plan-Fluor air objective. Images of living and fixed cells were automatically acquired from several positions per well with exposure times of 200 ms for GFP fusion proteins and 600 ms for red fluorescent fusion proteins using appropriate standard filter sets.

RESULTS

To visualize protein-protein interactions in living cells in real time we developed an F2H assay. The rationale for the F2H assay is based on the fact that proteins are freely roaming the cell unless interactions with other cellular components immobilize them at specific structures (21).

We used a previously described BHK cell line and an U2OS cell line, which both harbor a stable integration of about 200–1000 copies of a plasmid carrying 256 copies of the lac operator sequence (19, 20). We generated an expression construct encoding a fluorescent bait protein consisting of a fluorescent protein (FP), the lac repressor (LacI), and the protein X to be tested for interactions (bait) resulting in the triple fusion protein FP-LacI-X (Fig. 1a) or X-LacI-FP. This fusion protein binds to the lac operator array, which then becomes visible because of the focal enrichment of the FP signal. A second, differently labeled fusion protein (FP-Y, prey) may either interact with the bait protein X leading to co-localization of the FP signals (Fig. 1b) or may not interact, resulting in a dispersed distribution of the prey fluorescence (Fig. 1c).

Visualization of Interactions between DNA Repair Proteins—To test the F2H assay, the previously described interaction between the two DNA repair proteins DNA Ligase III and XRCC1 (22, 23) was analyzed, and the results were compared with data obtained from pulldown assays. We have previously shown that this interaction is mediated by the BRCT domain of DNA Ligase III that targets it to DNA repair sites (17). We generated a bait fusion protein consisting of XRCC1 followed by the LacI and the monomeric red fluorescent protein (RFP). As expected this fusion protein localized at the lac operator array in transiently transfected BHK cells (Fig. 2a). Both the full-length GFP-tagged DNA Ligase III and the isolated GFP-labeled BRCT domain co-localized with XRCC1 at the lac operator array, whereas a fusion protein missing the BRCT domain showed a dispersed distribution. Notably the highly homologous DNA Ligase I, which catalyzes the same reaction as DNA Ligase III, did not bind to XRCC1 (Fig. 2a and supplemental Fig. 1). A direct comparison of the F2H data with data obtained from Co-IP experiments revealed that these two methods gave similar results (Fig. 2b). In addition, we could also observe the recently described interaction of XRCC1 with PCNA (24) and the two DNA damage-dependent PARPs, PARP-1 and PARP-2 (25, 26) (supplemental Fig. 2). These results demonstrate that the F2H assay is well suited to study protein-protein interactions in living cells.

Analysis of Cell Cycle Dependence of Protein-Protein Interactions—A challenge in the analysis of protein-protein interactions is to monitor transient changes caused by for example cell cycle progression or other external stimuli. We analyzed the previously described interaction between DNA methyltransferase 1 (Dnmt1) and PCNA that is mediated by the PCNA binding domain (PBD) and targets Dnmt1 to sites of DNA replication in S phase (10, 27). These findings raised the question whether this interaction occurs only in S phase at replication foci or throughout the cell cycle. We generated two bait proteins comprising parts of Dnmt1 fused to the LacI and...
YFP. One bait (PBD-LacI-YFP) comprises amino acids 118–427 of Dnmt1 including the PBD, whereas the second bait (H9004PBD-LacI-YFP) lacks the PBD and comprises amino acids 629–1089 of Dnmt1 (Fig. 3a). As a prey protein we used RFP-PCNA, which in addition marks sites of DNA replication allowing the identification of cells in S phase (11, 28). The binding possibilities of these fusion proteins at the lac operator array and the replication fork are summarized in Fig. 3b.

In non-S phase cells the LacI part of the bait proteins only bound to the chromosomally integrated lac operator array, which, dependent on the ploidy of the cell, became visible as one or two fluorescent spots in the nucleus. Interaction of RFP-PCNA with the PBD part of the bait protein resulted in co-localization of the fluorescent signals at the lac operator (visible as a yellow spot in the overlay image). c, if the prey does not interact with the bait protein it remains dispersed in the nucleus, and the lac operator array is only visualized by the bait protein (red spot). FP1 and FP2 refer to two distinguishable fluorescent proteins, e.g. GFP or YFP and mCherry or RFP. MCS, multiple cloning site; 

Fig. 1. Schematic outline of the F2H assay. a, outline of pF2H-prey and pF2H-bait expression vectors coding for fluorescently labeled prey and bait proteins used for the F2H assay. b, the LacI domain of the bait protein mediates binding to the chromosomally integrated lac operator array, which is visible as a fluorescent spot in nuclei of transfected cells. If the differentially labeled prey interacts with the bait it becomes enriched at the same spot resulting in co-localization of fluorescent signals at the lac operator (visible as a yellow spot in the overlay image). c, if the prey does not interact with the bait protein it remains dispersed in the nucleus, and the lac operator array is only visualized by the bait protein (red spot). FP1 and FP2 refer to two distinguishable fluorescent proteins, e.g. GFP or YFP and mCherry or RFP. MCS, multiple cloning site; 
P CMV IE, cytomegalovirus immediate early promoter; P SV, SV-40 promotor.
LacI-YFP), RFP-PCNA was exclusively enriched at DNA replication sites and not at the lac operator array highlighted by /H9004 PBD-LacI-YFP (Fig. 3 , lower panel). These results clearly show that the localization of RFP-PCNA (prey) at the lac operator array indicating that it does not interact with XRCC1. Scale bars, 5 μm. b, comparison of F2H results and Co-IP experiments. Co-IPs were performed with the human embryonic kidney cell line HEK 293T co-expressing RFP-XRCC1 and GFP-Ligase III or GFP-Ligase I, respectively. For interaction mapping, the GFP-tagged BRCT domain of DNA Ligase III and a deletion construct lacking the BRCT domain were used. Immunoprecipitations were performed with a GFP-nanotrap (18) (as shown before (17)). Precipitated fusion proteins were then detected with specific antibodies against RFP and GFP on Western blots. RFP-XRCC1 was co-precipitated with GFP-Ligase III but not with GFP-Ligase I. GFP-Ligase III was also co-precipitated with GFP-Ligase III BRCT but not with GFP-N-Ligase III ΔBRCT. For comparison of F2H results the input (left) and bound (right) bands from Co-IPs were aligned with corresponding signals from the F2H assay. The LacI spot of the XRCC1-LacI-RFP bait construct shown in red and the bound fraction was aligned with the respective signal of the GFP-tagged prey constructs. Whole cell images of the respective F2H experiments are shown in a and supplemental Fig. 1.

Next we analyzed the interaction of other PBD-containing proteins with PCNA. We generated a bait fusion protein comprising PCNA fused to an additional NLS followed by LacI and RFP (NLS-PCNA-LacI-RFP). When co-expressed with GFP-Ligase I, both fusion proteins localized to the lac operator array indicating interaction between PCNA and DNA Ligase I. Deletion of the PBD led to a dispersed distribution of DNA Ligase I, whereas the PBD of DNA Ligase I alone was sufficient for binding to PCNA at the lac operator array (supplementary Fig. 3). This is in agreement with previous studies showing that the PBD of DNA Ligase I is necessary and sufficient for its targeting to DNA replication and repair sites (17, 29, 30). Notably using the F2H assay we could demonstrate that DNA Ligase I and the isolated PBD are capable of binding to PCNA also outside of S phase. Likewise we could show binding of various additional replication and repair proteins like FEN1, p21, and the Polymerase δ subunit p66 to PCNA in non-S phase cells (supplemental Fig. 4). Taken together we could show that the interaction between replication proteins and PCNA is not limited to S phase but also occurs in non-S phase cells and outside the replication machinery. Furthermore we could show that interactions can be observed as early as 6 h after transfection, and even higher expression levels after 48 h still give reliable results (supplemental Fig. 5). This illustrates that the F2H assay offers the unique potential...
**Fig. 3.** F2H analysis of cell cycle-independent interaction of Dnmt1 with PCNA. 

**a.** Schematic outline of full-length mouse Dnmt1 and fusion proteins. TS, targeting sequence; ZnF, Zn$^{2+}$-binding region; BAH1 and BAH2, two bromo adjacent homology domains. 

**b.** Outline of binding possibilities of fusion proteins at the lac operator (lac op) array and at the replication fork. 

**c.** Transgenic BHK cells containing a chromosomal lac operator array were co-transfected with PBD-LacI-YFP and RFP-PCNA constructs. RFP-PCNA shows the characteristic cell cycle-dependent distribution (dispersed in non-S phase cells (top row) and focal patterns in S phase (bottom row)). The lac repressor part of the PBD-LacI-YFP fusion protein mediates binding to the lac operator array (visible as a green spot and highlighted by arrowheads), and the PBD mediates binding to PCNA at replication sites (focal pattern in S phase). Notice that RFP-PCNA is localized at the lac operator array in S and non-S phase cells indicating an interaction of the PBD of Dnmt1 with PCNA throughout the cell cycle and independent of the replication machinery. 

**d.** BHK cells were transfected with expression vectors for ΔPBD-LacI-YFP and RFP-PCNA. As above, RFP-PCNA shows a dispersed distribution in non-S phase (top row) and redistribution to replication sites in S phase (bottom row). The ΔPBD-LacI-YFP fusion protein binds to the lac operator array (green spot marked by arrowhead) but does not bind to replication sites in S phase because it lacks the PBD. Importantly in these cells RFP-PCNA (prey) is not localized at the lac operator array (marked by arrowheads) indicating that binding depends on the presence of the PBD, which is absent in ΔPBD-LacI-YFP (bait). Scale bars, 5 μm.
to quickly analyze cell cycle-specific changes in protein-protein interactions in living cells.

**Detection of Interactions between Proteins Related to Huntington Disease**—To investigate whether the F2H assay can also detect protein-protein interactions taking place in other cellular compartments, we tested the F2H assay with protein interactions identified in the context of Huntington disease by yeast two-hybrid assays (31). We analyzed the interaction of one cytoplasmic (Vimentin) and two nuclear (HZFH and SUMO3) proteins. Vimentin has been described to be a cytoskeleton component and participates in transport processes, whereas HZF and SUMO3 are involved in transcriptional regulation and DNA maintenance (31). These proteins were either fused with a red fluorescent mCherry-LacI-NLS or with NLS-GFP to generate sets of bait and prey proteins. BHK cells carrying a lac operator array were transfected with all possible combinations of expression constructs and subjected to microscopic analysis. We could detect an interaction between Vimentin and HZFH independent of whether these two proteins were used as bait or prey (Fig. 4a and data not shown). We could also detect the reported interaction between SUMO3 and HZFH, whereas Vimentin and SUMO3 did not interact as described previously (Fig. 4, b and c) (31). These results show that interactions of nuclear and cytoplasmic proteins can be studied with the F2H assay.

**Detection of Interactions between Mitochondrial Proteins**—Next we investigated whether the F2H assay is also suitable to detect protein-protein interactions occurring in other cellular organelles. To this end, we analyzed the interaction between two mitochondrial proteins, deafness dystonia peptide 1 (DDP1) and TIMM13. Both proteins are nuclearly encoded and imported into the mitochondrial intermembrane space (IMS) forming a hexameric complex (Fig. 5a). Within the IMS the DDP1-TIMM13 complex facilitates the import of hydrophobic proteins of the mitochondrial import machinery into the mitochondrial inner membrane (32). A mutation of the DDP1 gene is associated with the Mohr-Tranebjaerg syndrome, which is a progressive, neurodegenerative disorder (33). This C66W missense mutation is known to cause a full blown phenotype and affects the highly conserved Cys4 motif of DDP1. Previous studies have shown that this amino acid exchange abolishes the interaction between DDP1 and TIMM13 in the IMS (34).

Using a red fluorescent bait fusion protein comprising LacI-NLS-TIMM13 and GFP-tagged wild type (GFP-DDP1) or mutant DDP1 (GFP-DDP1C66W) prey proteins we analyzed this specific mitochondrial protein interaction with the F2H assay. We found that GFP-DDP1 co-localizes with TIMM13 at the lac operator array (Fig. 5b), whereas GFP-DDP1C66W was evenly distributed (Fig. 5c). An extended analysis of more than 30 putatively non-interacting protein pairs showed that the F2H assay is not particularly susceptible to false positive results (supplemental Fig. 6). These results demonstrate that the F2H assay is also suitable for the analysis of protein-protein interactions occurring outside the nucleus and the charac-

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**Fig. 4.** Analysis of Huntington disease-related interactions by F2H. Reported interactions between SUMO3 and HZFH (a) and HZFH and Vimentin (Vim) (b) revealed by the F2H assay are shown. c, F2H analysis shows no interaction between SUMO3 and Vimentin as described previously (31). In b the nucleus is outlined by a dashed line, and in c the lac operator array is indicated (arrowheads). Scale bars, 5 μm.
terization of disease-related point mutations disrupting these interactions.

Independent Identification of the Interaction Site for Simplified Image Analysis—Several proteins show distinct subnuclear distribution patterns, which may impair unambiguous identification of the bait structures. To distinguish the lac operator array focus from a focal distribution pattern of a bait protein we introduced a specific marker protein in the F2H assay. This marker protein comprises the CFP fused to the LacI, which exclusively binds to the lac operator array. As a proof of principle we chose the interaction between the PBD of Dnmt1 and PCNA (Fig. 3c and supplemental Fig. 5). During S phase both proteins localize at replication sites and at the lac operator array focus that are morphologically hard to distinguish. Highlighting the lac operator array with the third fluorescent fusion protein, CFP-LacI, easily circumvented this problem (Fig. 6). These results show that the CFP-LacI as a marker protein simplifies the identification of the nuclear binding site used for the F2H assay. This unambiguous identification of the interaction assay site facilitates automated image analysis with standard software tools and algorithms for high throughput screening.

DISCUSSION

Here we describe a new method to detect and visualize protein-protein interactions in living cells that we termed the F2H assay. This method is based on the immobilization of a fluorescently labeled bait protein at a distinct subcellular structure enabling the detection of protein-protein interactions as co-localization of a differently labeled prey protein at this defined structure. The F2H assay described takes advantage of cell lines with a stable integration of a lac operator array to
immobilize a lac repressor fused to fluorescently labeled proteins of interest (bait). Readily usable human, mouse, hamster, and *Drosophila* cell lines have already been described (9, 19, 20, 35–37). To be independent of specific transgenic cell lines this assay could be modified by using various cellular structures like centrosomes, the lamina, or the cytoskeleton as anchoring structures to locally immobilize bait proteins. Two recently developed methods use the cell membrane (8) or cytoplasmic aggregates of viral proteins (7) as anchors for protein association studies. However, larger anchoring structures also require higher expression levels of bait and prey proteins, which may lead to unspecific aggregations. As the F2H assay is based on lac operator arrays the number of binding sites can freely be chosen to optimize signal to noise ratios.

Like other genetic two-hybrid methods the F2H assay also may yield false positive or false negative results, which need to be controlled. Prey proteins that bind to the lac operator array in the initial screen of a bait protein can be identified by an initial screen and then be only used as baits. We analyzed more than 20 protein-protein interactions from different subcellular compartments with the F2H assay and obtained results identical to those described previously with other genetic or biochemical methods. Only one protein (SUMO3) was found to bind by itself to the lac operator array and could therefore only be used as a bait protein. An extended analysis of non-interacting proteins indicated that the F2H assay is not particularly susceptible to false positive results. Together these results show that the F2H assay is a reliable and broadly applicable method to study protein-protein interactions as early as 6 h after transfection.

In summary, this novel F2H assay allows the direct visualization of protein-protein interactions and should be ideally suited to investigate cell cycle- or differentiation-dependent changes in real time in living cells. A significant advantage of the F2H assay over other cell-based techniques is its simplicity that requires neither costly instrumentation nor advanced technical expertise. The simple optical readout of the F2H assay additionally offers the possibility to use this assay in automated high throughput screens to systematically analyze the protein interactome in living cells.

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