Visualizing protein–RNA interactions inside cells by fluorescence resonance energy transfer

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ABSTRACT

Approaches for studying protein–protein interactions in living cells have been broadly developed, but the temporal and spatial association of proteins with nucleic acids has been less explored. Here, we report a novel approach to study and visualize the association of an RNA-binding protein with its native RNA target *in situ* by fluorescence resonance energy transfer (FRET). The RNA-binding protein is tagged with a yellow variant of GFP and the RNA stained with SytoxOrange. RNA binding results in a decrease of the fluorescence lifetime of YFP due to FRET, which can be measured by fluorescence lifetime imaging microscopy (FLIM). With this method we analyzed the RNA binding of the alternative splicing repressors PTB and Raver1 and could show an RNA-specific FRET signal. Interestingly, PTB and Raver1 were bound to RNAs all over the nucleus, as expected, but additionally interacted with RNAs in the perinucleolar compartment (PNC), where only noncoding Pol III transcripts are present.

Keywords: FRET; FLIM; protein–RNA interaction; PTB; raver1

INTRODUCTION

Post-transcriptional processing and regulation of RNAs is an important mechanism to control and maintain several cellular functions and is associated with an array of protein–RNA interactions. A broad range of studies has addressed the spatial distribution of proteins and their interactions with each other. Furthermore, the distribution and movement of single mRNAs have been visualized and studied (for reviews, see Rodriguez et al. 2007; Dahm et al. 2008), and fluorescence resonance energy transfer (FRET) reporter systems were developed to detect RNA transcription (Endoh et al. 2008) or to visualize RNA self-splicing *in vivo* (Gowrishankar and Rao 2007). However, direct detection and analyses of the spatial regulation of protein–RNA interactions inside cells are still difficult. Rackham and Brown (2004) used the bacteriophage MS2–protein to mark a specific RNA template (see Bertrand et al. 1998; Rodriguez et al. 2007) and used bimolecular fluorescence complementation (BiFC) (Hu et al. 2006) to detect the interaction between the MS2-marked RNA and an RNA-binding protein. In this case the RNA must be designed and

the two binding sites (MS2 and protein) must be close to each other. Furthermore, due to the necessary formation of the fluorophore—it takes ~ 1 h to build the fluorophore—the studied RNA-binding protein is permanently cross-linked to the MS2–protein and likely to the mRNA (Hu et al. 2002). Therefore, the temporal and spatial regulation cannot be observed with this method. To overcome these limitations and to detect the interaction of a protein with its native RNA we developed a new FRET-based method to determine and to visualize RNA–protein interaction inside cells.

FRET is a photophysical effect in which energy is transferred from a fluorophore (the energy donor) to another chromophore (the energy acceptor) in close proximity (typically, 2–10 nm) (Forster 1948; Hillisch et al. 2001). Therefore, FRET microscopy is a powerful tool to quantify molecular dynamics, such as protein–protein interaction or, as shown in this paper, to visualize the association of RNA-binding proteins with their native target RNA *in situ*.

Alternative pre-mRNA splicing plays a central role to increase the number of gene products leading to tissue specific protein isoforms of the same gene. The alternative splicing repressor polypyrimidine tract binding (PTB) protein binds close to splicing sites and inhibits in this way the binding of spliceosomal proteins, resulting in a repression of splicing activity (for reviews, see Valcarcel and Gebauer 1997; Auweter and Allain 2008). Raver1 can act

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together with PTB as a co-repressor (Gromak et al. 2003; Spellman et al. 2005; Rideau et al. 2006). Raver1 has three RNA recognition motifs (RRM) and up to four binding motifs for PTB (Rideau et al. 2006) and can function as a bridge between several PTB proteins bound upstream of and downstream from the skipped exon, introducing a loop in the pre-mRNA as previously suggested (Wagner and Garcia-Blanco 2001; Rideau et al. 2006; Auweter and Allain 2008). If raver1 interacts directly with the pre-mRNA in this complex or only via PTB binding is not clear (Rideau et al. 2006).

PTB is a nucleoplasmic protein that is also highly concentrated in the perinucleolar compartment (PNC). In cell culture the appearance of PNCs is related to transformed phenotypes. Additionally, a correlation with malignancy in, e.g., breast cancer could be identified (Kamath et al. 2005; Kopp and Huang 2005). Therefore, it is important to understand the function of this structure in more detail. PNC is an irregularly shaped structure associated with the nucleolus enriched with several RNA-binding proteins, suggesting its function in RNA metabolism (Kopp and Huang 2005). Although no pre-mRNA has been found in PNCs, some noncoding RNAs transcribed by RNA Pol III are localized to and are important for the appearance of PNCs (Kopp and Huang 2005).

To validate our new approach we tagged PTB and raver1 with yellow fluorescent proteins as the FRET donor and stained the RNA with SytoxOrange as the acceptor (Cremazy et al. 2005). The occurrence of FRET indicative of RNA binding causes the fluorescence lifetime of the donor to decrease (van der Meer et al. 1994; Bastiaens and Squire 1999), which was detected by fluorescence lifetime imaging microscopy (FLIM). In both cases we could show an RNA-specific FRET signal all over the nucleus and additionally an RNA association in PNCs.

RESULTS

In this work we analyzed and imaged the association of the two well-known alternative splicing repressors PTB and Raver1 (Gromak et al. 2003; Spellman et al. 2005) with their endogenous RNA targets inside the cell by FRET-FLIM. In a previous work Cremazy et al. (2005) used SytoxOrange, a nucleic acid dye, as a FRET acceptor for GFP-tagged histone H2B to determine the association with DNA. Here we apply this technique to visualize in situ protein-RNA interactions in cells. Therefore we fused a yellow fluorescent protein (YFP or Venus) to PTB and raver1 and used SytoxOrange as the FRET acceptor dye, which stains DNA as well as RNA with high affinity (Fig. 1A; Haugland 2005). As a control and to determine whether the protein is associated with RNA or DNA, DNase or RNase treatment can be carried out, and thus only interactions with the remaining nucleic acids can be observed (see also Fig. 1A,B).

FIGURE 1. Staining nucleic acids with SytoxOrange can be used as FRET acceptor for YFP-tagged binding proteins. (A,B) Quantification of the RNase and DNase digest. HeLa cells stained with SytoxOrange, after RNase A or DNase I treatment are shown with identical normalization. Scale bar = 10 μ m (A). The staining of the nucleus is significantly reduced after DNase digest while the staining of the cytoplasmic RNA is still retained. After RNase digest the signal in the cytoplasm almost disappears while the major fluorescence, resulting from the DNA staining, remains. In B a quantification of the average fluorescence intensities of the nucleic and cytoplasmic SytoxOrange staining is shown. Error bars are SEM of $n = 36$ (nucleus) or 15 cells (cytoplasm). (C) Absorption (dashed line) and emission spectra (continuous line) of YFP (gray line) and SytoxOrange (black line). There is a considerable overlap between the YFP emission and SytoxOrange absorption, resulting in a Foerster distance R_0 of 5.6 nm. (D) Fluorescence decay of SytoxOrange (continuous line) and YFP (dashed line) in HeLa cells. YFP was best described as a single-exponential decay with a lifetime of 2.4 nsec whereas SytoxOrange showed a more complex decay curve with two components; however, the average lifetime is 3.3 nsec. The inset shows the corresponding log-plots.

SytoxOrange in combination with YFP is suitable for FRET due to a considerable overlap of the acceptor absorption and donor emission spectra (Fig. 1C). We preferred YFP instead of GFP for FRET-FLIM measurements because of (1) the pure single-exponential decay behavior of the yellow fluorescent proteins and (2) an increased spectral overlap resulting in a larger Foerster distance R_0 . The Foerster distance of YFP or Venus and SytoxOrange is 5.6 nm and is larger than for GFP as donor.

The average fluorescence lifetimes of YFP and SytoxOrange were 2.4 nsec and 3.3 nsec, respectively (Fig. 1D). The occurrence of FRET causes the lifetime of the donor YFP to decrease. If for any reason the fluorescence of the abundant acceptor is bleeding into the donor channel, e.g., due to an inappropriate filter set, an apparent increase of the donor

lifetime in the presence of SytoxOrange would be detected. Therefore, the only process decreasing the fluorescence lifetime is an energy transfer due to an RNA binding.

PTB–RNA interactions

HeLa cells were transiently transfected with Venus-tagged PTB and fixed with 4% formaldehyde. In the absence of SytoxOrange the lifetime of Venus–PTB was 2.39 ± 0.02 nsec (Fig. 2A,E, average value $\pm$ SEM of $n = 10$ cells), in agreement with previously observed values of free Venus (data not shown). In the presence of SytoxOrange, the fluorescence lifetime was decreased significantly to 2.24 ± 0.01 nsec (Fig. 2B,E, $n = 10$), indicating an energy transfer and therefore a binding of PTB to nucleic acids. While the Sytox dyes are not specific for either DNA or RNA (see Fig. 1A; Haugland 2005), the cells were treated with RNase A or DNase I before staining to digest RNA or DNA, respectively. After RNase treatment no FRET could be observed anymore and the lifetime recovered to 2.37 ± 0.02 nsec (Fig. 2C,E, $n = 10$), whereas after a DNase digest, the lifetime of Venus–PTB remained unchanged and was 2.26 ± 0.01 nsec (Fig. 2D,E, $n = 10$). In addition to an association of PTB with RNAs all over the nucleus, where splicing occurs, a binding to RNAs present in PNCs was identified (see arrows in Fig. 2 A–D). These results show that the decrease of the fluorescence lifetime of Venus–PTB in the presence of SytoxOrange was only RNA dependent.

Additionally, to exclude any nonspecific interactions of the tag with nucleic acids leading to false positive results, we performed experiments with fluorescent proteins alone or fused to the bacteriophage MS2 protein (Fig. 3). The MS2–protein is widely used to tag and visualize single RNAs and binds only with a high affinity to sequence-specific RNA–stem loops, which are not present in HeLa cells. In both cases no FRET occurs, and thus no decrease of the fluorescence lifetimes in the presence of SytoxOrange could be observed, indicating the specificity for this approach to identify and image protein–RNA interaction.

Raver1–RNA association

Raver1 as an alternative splicing corepressor is able to bind to and thus link several PTBs (Rideau et al. 2006; data not shown). To determine if Raver1 is also directly associated with the RNAs we transfected HeLa cells with YFP-tagged Raver1 and measured the fluorescence lifetime of YFP in the absence and presence of SytoxOrange. The lifetime decreased significantly from 2.37 ± 0.03 nsec (Fig. 4A,E, $n = 8$) to 2.24 ± 0.02 nsec (Fig. 4B,E, $n = 9$), respectively, indicating a Raver1–RNA association all over the nucleus, including PNC. As a control, the FRET signal was completely abolished after an RNase A treatment, and the measured lifetime was 2.36 ± 0.02 nsec (Fig. 4C,E, $n = 11$), equal to the value in the absence of staining, whereas a DNase digest, where the RNA is still intact, had no effect, and an energy transfer could be

FIGURE 2. Observing PTB binding to RNA by FRET-FLIM in situ. (A–D) Fluorescence lifetime of C-terminal Venus-tagged PTB were measured in HeLa cells in the absence (A) or presence of $2.5 \mu\text{M}$ SytoxOrange (B–D). Panels show from left to right the Venus fluorescence intensity images, the lifetime images, and the corresponding lifetime histograms of a single experiment (with mean lifetime $\pm$ SD), respectively. In the presence of the nucleic acid staining dye SytoxOrange the YFP lifetime decreased significantly due to FRET (B). The energy transfer was abolished after RNA digestion (C) but remained unchanged after a treatment with DNase I (D). PNCs are indicated by arrows. Scale bar = $5 \mu\text{m}$. (E) Statistical analysis: The bar graph shows the average lifetime of Venus–PTB under the above mentioned conditions. The lifetime was significantly lower in the presence than in the absence of SytoxOrange, while the lifetime in the absence of SytoxOrange and after an RNase A treatment or in the presence of SytoxOrange and after a DNA digestion was not significantly different ($P > 0.2$). Error bars are SEM ($n = 10$).

still observed. The determined lifetime was 2.25 ± 0.02 nsec (Fig. 4D,E, $n = 12$).

To exclude a possible indirect binding of Raver1 to mRNAs via PTB, PTB and nPTB, which can replace the

FIGURE 3. FRET-FLIM control experiments. Fluorescence lifetime of non-RNA or DNA interacting proteins (Venus [A,B] or MS2-YFP [C,D]) were measured in the absence (A,C) or presence of 2.5 μ M SytoxOrange (B,D). Left column shows the fluorescence intensity and right column shows the corresponding lifetime images, respectively. In both cases no significant differences were observed between the lifetime in absence or presence of SytoxOrange. Error bars are SEM ($n = 6-9$); scale bar = 5 μ m.

function of PTB (C. Smith, pers. comm.), were knocked down by siRNA. The protein level of PTB was analyzed ~ 48 h after siRNA transfection by Western blot and was significantly decreased (Fig. 4F). Additionally, in the majority of the HeLa cells the PNCs disappeared as previously reported, indicating the success of the siRNA (Wang et al. 2003). Only those cells were analyzed. Also without PTB an energy transfer could be observed, suggesting a direct RNA-binding capability of Raver1 (Fig. 4F).

DISCUSSION

Post-transcriptional regulation of RNA processing, stability, and localization plays a more central role in maintaining and controlling cellular processes than previously believed. These processes are mainly mediated and regulated by protein-RNA interactions. So far the spatial distribution of the proteins and/or the RNA could be studied, but their association inside cells could not be detected. Biochemical studies are limited by how well the in vitro incubation recapitulates those biological processes in

vivo and thus can identify only potential interactions but without any spatial and temporal information about where and when it occurs in the cell.

Here, we describe a novel FRET-based method to detect and image protein-RNA interactions inside cells. Using YFP-tagged proteins as the FRET donor and the nucleic acid dye SytoxOrange as the acceptor, a specific decrease of the YFP fluorescence lifetime could be observed when bound to RNA. To validate this new approach, we analyzed the RNA binding of the alternative splicing regulators PTB and Raver1. In both cases, a decrease of the donor lifetime could be detected in the presence of SytoxOrange. The FRET signal was abolished after digesting the RNA before staining but retained after a DNase I treatment. Furthermore, in control experiments with non-RNA- or DNA-binding proteins, no FRET could be observed, ruling out any unspecific interactions of SytoxOrange. This shows clearly the specificity of this technique to detect RNA interactions in situ.

As expected, PTB is bound to mRNAs all over the nucleoplasm. In previous work, we could show that PTB and Raver1 work together to efficiently promote exon 3 skipping in α -tropomyosin pre-mRNA and are associated with each other in the nucleoplasm as well as in PNCs (Rideau et al. 2006). While splicing occurs in the nucleoplasm, it is likely that PTB and/or Raver1 are associated with their target mRNAs, but the function of PNCs is still unclear. Although no mRNA was found in PNCs, non-coding Pol III transcripts could be detected. These data show for the first time that PTB is also interacting with those RNAs present in PNCs and is consistent with the finding that RNA and PTB are necessary for the appearance of this structure (Wang et al. 2003). If these RNAs act as a scaffold to preassemble, e.g., splicing complexes or if an unknown processing of these Pol III transcripts occurs has to be analyzed in the future.

Summarizing, these data show that protein-RNA interactions can be determined with a high specificity directly in cells without losing any spatial or temporal resolution. Even though this described assay is not specific for a particular RNA, it can lead to new insights of the organization and processing of RNAs through protein-RNA interactions.

MATERIALS AND METHODS

Constructs and transfection

Constructs used in this study were amplified by PCR, cloned according to standard protocols, and verified by DNA sequencing. Raver1 was cloned into the YFP-C1-plasmid (Clontech) and PTB into a Venus-C1-plasmid using the EcoRI and SalI restriction sites, respectively. HeLa cells were plated onto coverslips pre-treated with 1 μ L Cell-Tak (BD Biosciences) and transfected with 1 μ g of plasmid in 50 μ L Opti-MEM I (Invitrogen) using Fugene

FIGURE 4. Detection of Raver1-RNA interaction by FRET-FLIM in situ. (A–D) Fluorescence lifetime of N-terminal YFP-tagged Raver1 were measured in HeLa cells in the absence (A) or presence of 2.5 μ M SytoxOrange (B–D). Panels show from left to right the Raver1-YFP fluorescence intensity images, the lifetime images, and the corresponding lifetime histograms (with mean lifetime $\pm$ SD), respectively. In the presence of SytoxOrange the lifetime decreased significantly due to an energy transfer (B). FRET was abolished after RNA digestion (C) but remained unchanged after a treatment with DNase I (D). Scale bar = 5 μ m. (E) The average lifetime of Raver1-YFP was significantly lower in the presence than in the absence of SytoxOrange, while the lifetime in the absence of SytoxOrange and after an RNase A treatment or in the presence of SytoxOrange and after a DNA digestion was not significantly different ($P > 0.5$). Error bars are SEM ($n = 8$ –12). (F) Knockdown of PTB by RNAi does not abolish the FRET signal suggesting a direct RNA binding of Raver1. Error bars are SEM ($n = 11$). The inset shows the reduction of the PTB protein level in HeLa cells by Western blot analysis with actin as a loading control. (U) untreated cells; (T) siRNA treated cells.

HD (Roche Diagnostics) according to the manufacturer's protocol.

Cell fixation and nucleic acid staining

Approximately 24 h after transfection, HeLa cells were fixed for 10 min in 4% formaldehyde at 37°C. Permeabilization was per-

formed in phosphate buffered saline (PBS) containing 0.5% Triton-X100 (Sigma) and 100 mM glycine to block excess aldehyde groups for 5 min at room temperature. Nucleic acids were digested either with 0.1 mg/mL RNase A (Roche Diagnostics) in PBS or 20 U/mL DNase I (New England BioLabs) in DNase I-buffer for 30 min at 37°C, respectively. Finally, cells were labeled with 2.5 μ M SytoxOrange (Molecular Probes) for 20 min, washed several times with PBS to remove excess dye, and mounted with Pro-Long Gold antifade (Molecular Probes).

Small interfering RNA (siRNA) transfection

Preannealed siRNA duplexes against human PTB, nPTB, and a negative control were a generous gift from C. Smith (Cambridge, UK). Approximately 200,000 cells were split to a 6-well plate and at the following day, transfected with PTB-siRNA and nPTB-siRNA using FugeneHD (Roche Diagnostics). Cells were incubated 48 h before further treatment.

Western blot analysis

HeLa cells were grown in 6-well plates and were lysed in 100 μ L lysis buffer (50 mM Tris-HCl at pH 7.6, 150 mM NaCl, 0.5% NP-40) containing protease inhibitors (Roche Diagnostics) for 30 min on ice. The cell extract was denatured, separated on a 4%–12% gradient polyacrylamid gel (Invitrogen), and blotted to nitrocellulose membrane according to standard protocols. PTB was detected with a monoclonal antibody BB7 (from D. Black, University of California at Los Angeles). As a loading control actin was stained with an anti-actin antibody (Cyto-skeleton). The bands were visualized by chemiluminescence of the peroxidase activity of a peroxidase-conjugated secondary antibody by using ECL (GE Healthcare).

Fluorescence resonance energy transfer

FRET is a photophysical effect where excitation energy can be transferred by dipole–dipole interactions from one fluorophore to another in close proximity (~ 2 –10 nm).

This is in the order of the size of proteins and can be used to detect associations of biomolecules inside cells. The efficiency of this process E is strongly dependent on the distance R and/or the orientation of the two fluorophores (Equation 1):

$$E = \frac{R_0^6}{R_0^6 + R^6}, \quad (1)$$

where R_0 is the Foerster distance and represents a characteristic parameter of every dye pair defining the distance at which the efficiency is 50%. The Foerster distance for the dye pairs YFP, Venus, or GFP as the donor and the acceptor SytoxOrange was calculated according to

$$R_0 = 979(\kappa^2 Q_D J n^{-4})^{1/6} \text{ nm}, \quad (2)$$

with a donor quantum yield Q_D of 0.61 (YFP), 0.57 (Venus), or 0.6 (GFP) (Patterson et al. 2001; Nagai et al. 2002), a refraction index n of 1.33, and the orientation factor of the transition dipole moments κ^2 of $2/3$ (random orientation). The overlap integral J was determined by measuring the corrected emission spectra of YFP, Venus, and GFP in a cell extract using a FluoroMax-3 fluorescence spectrometer (Horiba Jobin Yvon) under magic angle conditions. The absorption spectrum of SytoxOrange was obtained from Invitrogen with an excitation coefficient of $79,000 \text{ M}^{-1} \text{ cm}^{-1}$ (Haugland 2005).

If FRET takes place, next to the fluorescence intensity of the donor also the fluorescence lifetime of the donor τ will decrease, which can be measured by FLIM:

$$E = 1 - \frac{\tau_{\text{FRET}}}{\tau_{\text{free dye}}}. \quad (3)$$

Fluorescence lifetime measurements

Fluorescence lifetime measurements were performed by wide-field time-domain FLIM. As an excitation light source, a Chameleon XR Ti:Sapphire Laser (Coherent) with 200 fsec pulses and a repetition rate of 90 MHz was tuned to 980 nm and frequency doubled to 490 nm using a second-harmonic generator (SHG) (APE). The SHG was equipped with an autotracker device to correct the crystal position for different wavelengths and to keep the final beam at a constant position. Finally, the laser beam was coupled into a short graded index fiber, expanded with a collimator, and coupled into the microscope. Images were recorded on a Zeiss Axiovert S100TV with a 63×1.4 NA oil immersion objective and an optovar lens of 1.6 or 2.5 through a 495LP dichroic mirror (Chroma) and a BL HC514/30 emission band-pass filter (Semrock) for YFP fluorescence or BL HC536/40 emission band-pass filter (Semrock) for SytoxOrange emission. The images were acquired at the microscope's bottom port with an intensified PicoStar HR 12 CCD camera system (LaVision). The time-gated image intensifier was triggered directly by the laser trigger through a programmable picosecond-trigger-delay unit (LaVision). The microscope was fully automated and controlled with self-written software (LabView 8.2, National Instruments). Images were acquired and analyzed with the same software.

The fluorescence decay curves were recorded by 16 images with increasing delay times (step 500 psec) after the excitation laser pulse with a camera binning of 2×2 and an exposure time of 100–200 msec for each image. The laser power was reduced by ND filters to avoid photobleaching during the acquisition. Images were background corrected by subtracting the mean value of a region without any fluorescence signal, and the fluorescence lifetime was calculated for each pixel according to

$$I = A \cdot e^{-t/\tau}, \quad (4)$$

with I being the measured intensity in each pixel at a timepoint t , the preexponential factor A , and fluorescence lifetime τ .

Data analysis

Significance of the fluorescence lifetime shifts was analyzed using unpaired two-tailed Student's t -test; P -values < 0.01 were considered as significant.

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