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Research Paper

Antisense Molecular Beacon Strategy for In Situ Visualization of snRNA and Fibrillarin Protein Interaction in *Giardia lamblia*

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KEY WORDS

FRET, FCET, antisense molecular-beacon, *Giardia lamblia*, snRNA, fibrillarin

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ABSTRACT

Use of confocal microscopy has provided many recent developments in the study of functional aspects, especially localization and distribution of proteins, DNA and RNA within the cells. In the present investigation, we have applied for the first time, antisense molecular beacon based Fluorescence Resonance Energy Transfer (FRET) and Flow Cytometric Energy Transfer (FCET) techniques to demonstrate binding and co-localization of fibrillarin protein with small nuclear RNA (snRNA) to form ribonucleoprotein particle (RNPP) complex in *Giardia lamblia*. It has been observed by FRET and FCET that energy transfer occurs from fluorescence tagged fibrillarin to snRNA antisense molecular beacon confirming the clear physical interaction between them during RNPP complex formation. This is the first demonstration of in situ detection of RNA-protein complex formation by antisense molecular beacon based FRET and FCET in *Giardia lamblia*.

INTRODUCTION

Macromolecular interactions are gaining their importance in today's biological research. RNA-protein interactions play very vital role in regulating the different activities within a cell. Although RNA-protein complexes are very common for macromolecular processing and biogenesis and there are several techniques like gel elctrophoretic mobility shift assay (GEMSA) and north western blotting which can be successfully implemented for detection of such interactions in vitro, but, very few methods are available for the detection of RNA-protein interactions in situ.

In eukaryotes, rRNA processing was found to involve small ribonucleoprotein particles (RNPP) consisting of fibrillarin protein and small nucleolar RNAs (snoRNAs) in the nucleolus. *Giardia lamblia* contains no nucleolus[1] but fibrillarin[23] and small nuclear RNAs (snRNAs)[24] have been identified in the parasite.

Fluorescence resonance energy transfer (FRET) is a technique used for quantifying the distance between two molecules conjugated to different fluorophores.[12] By combining optical microscopy with FRET it is possible to obtain quantitative temporal and spatial information about the binding and interaction of proteins and RNA in vivo. Although FRET is a very sensitive and specific technique, but, sometimes a cross talk between FRET donor/acceptor pair is observed. This type of error in FRET has not been accounted in flow cytometric energy transfer (FCET) technique[32] which is similar to that of FRET. Here change in the mean fluorescence emission is the marker of energy transfer and interaction between the two different biomolecules.

In the present investigation, an attempt has been made to understand the snRNA-fibrillarin interaction in situ by FRET and FCET even in absence of any suitable snRNA specific dye by using an antisense molecular beacon.[16,17,19]

MATERIALS AND METHODS

Localization of Fibrillarin in Nuclei of *G. Lamblia*. To study the localization of fibrillarin protein in *Giardia lamblia*, the nucleus was double stained with Propidium Iodide (PI) and anti-fibrillarin antibody [10]-anti-rabbit conjugate tagged with FITC. Briefly, 48 hrs old 10^6 axenically grown trophozoites of *G. lamblia* (strain Portland I) were washed with PBS, fixed in cold methanol and 0.5% Tween 20 for 30 min and stained with PI with a final concentration of 1μg/ml. Two times washed cells were incubated with 1 ml of 1:200 diluted (optimal during standardization) anti-fibrillarin antibody, for 1 hr at 4°C, washed and probed with 1ml of 1:50 diluted anti-rabbit FITC conjugate (Jackson Immunochemicals, USA). Washed cell pellet was dissolved in minimum volume of PBS and examined under confocal microscope (Zeiss LSM 510) in a slide at 488 and 543 nm

excitations and 530 and 630 nm emissions respectively for PI and FITC. Co-localization of fibrillarin within the nuclei was studied by LSM 510 software (Zeiss).

Determination of Fibrillarin-snRNA Interaction In Situ—Designing of Antisense Molecular Beacon Against snRNA. *G. lamblia* snRNA D[10] was used for designing antisense molecular beacon (IDT DNA). The detailed strategy for designing the molecular beacon is shown in Figure 3. The antisense sequence was designed against a 28 bp conserved 3' terminal end sequence of snRNA D tagged with rhodamine as fluorophore and blackhole quencher as the fluorophore quencher.

Localization of snRNA in *G. Lamblia*. Specificity of the antisense molecular beacon designed against snRNA D was demonstrated by a localization study within the nuclei of *G. lamblia* trophozoites. *G. lamblia* trophozoites were fixed in cold methanol and 0.5% Tween 20 as described earlier and incubated for 2 hrs at 4°C with 1:50 diluted antisense molecular beacon (final concentration 0.05 mM) in PBS. Washed pellet was dissolved in minimum volume of PBS and examined under confocal microscope (Zeiss LSM 510) at 543 nm excitation and 610 nm emission.

Fluorescence Resonance Energy Transfer (FRET). Axenic *G. lamblia* (strain P-I) cells were fixed as earlier and incubated with 1:50 PBS diluted antisense molecular beacon (final concentration 0.05 mM) for 2 hrs at 4°C. Cells were washed with PBS and again incubated in 1 ml of 1:200 diluted anti fibrillarin antibody for 1 hr, then washed and conjugated with anti-rabbit FITC tagged antibody for 1 hr. Washed pellet was dissolved in minimum volume of PBS and one drop was used to make a thin film on a glass slide. The slides were examined under confocal microscope (Zeiss LSM 510) at 488 and 543 nm excitation and 530 and 610 nm emission for FITC and Rhodamine respectively. The physical interaction between snRNA and fibrillarin was demonstrated by FRET. Slides were exposed to laser excitation of 488 nm (for FITC), while excitation laser for Rhodamine (i.e., 543 nm) was switched off, but, emission spectra for Rhodamine was measured at 610 nm.

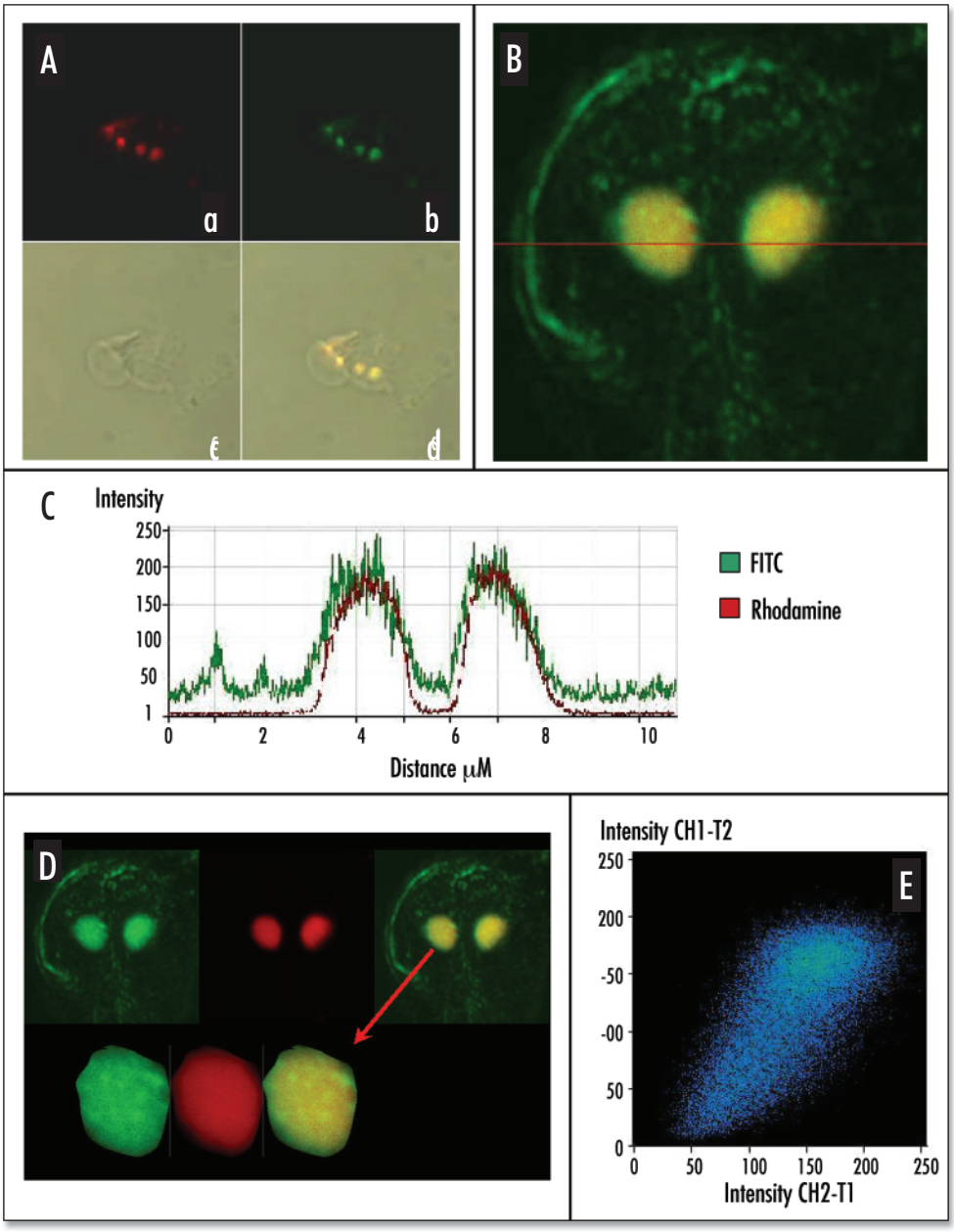

Figure 1. Co-localization of fibrillarin protein inside *Giardia lamblia* nuclei. (A) Fibrillarin is stained with FITC and nucleus with PI etailed procedure is given in text). a, PI stained nuclei; b, FITC stained fibrillarin protein; c, phase contrast picture of *Giardia* whole cell; d, co-localization of fibrillarin in nuclei. (B) Enlarged picture of co-localization of fibrillarin in nuclei. (C) Co-localization profiles of the two dye in nuclei, the two picks corresponding to the two nuclei showed complete co-localization. (D) Out of two nuclei, one is enlarged and further analyzed by LSM 510 software in graphical mode to show extent of co-localization. (E) Presence of 45° angle of scattered graph clearly demonstrates the complete co-localization of fibrillarin protein inside the nucleus.

A control molecular beacon of equal size and Tm but with non specific sequence has been used for a control reaction (not shown in results).

The FRET signal was further confirmed by a "Fluorescence Recovery After Photobleaching" (FRAP) test. After detection of FRET signal by exciting the double stained cells with excitation laser for FITC, the Rhodamine emission of the cells has been bleached out by flushing the cells with a long wavelength laser emission for 1 min. This bleaching do not hamper the intensity of FITC emission. Then, the intensity of FITC and Rhodamine emission have been measured in that double stained cells.

Flow Cytometric Energy Transfer (FCET). A Fluorescence Activated Cell Sorter (FACS: BD Biosciences) has been used to measure the energy transferred (FCET) between fibrillarin and snRNA during complex (RNPP) formation. The procedure was essentially the same as discussed earlier in FRET by confocal microscope. To demonstrate if the energy was transferred from fibrillarin to snRNA, laser excitation for both the lasers, i.e., FL1 for FITC tagged fibrillarin and FL2 for Rhodamine tagged snRNA, were kept open. Emission spectra for both FITC and Rhodamine were measured at 530 and 610 nm respectively.

Figure 2 (Above). Demonstration of snRNA D inside *Giardia lamblia* nucleus using antisense molecular beacon tagged with rhodamine. (A) presence of snRNA D stained with rhodamine tagged molecular beacon within the nucleus. (B) Phase contrast picture of whole cell. (C) Figure generated after superimposition of (A) and (B).

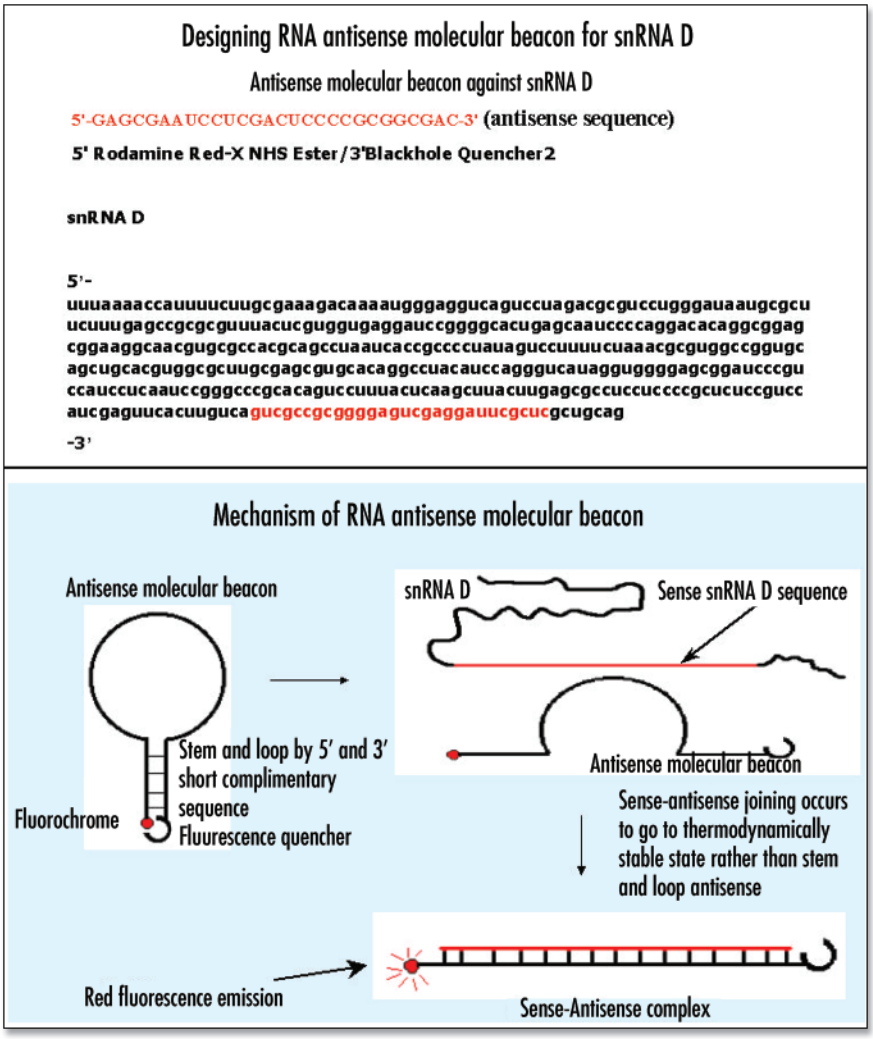

Figure 3. Detailed description of designing and mechanism of antisense molecular beacon for FRET and FCET. 3′ and 5′ end tag of complementary aaaa-uuuu has not been shown in the picture.

RESULTS AND DISCUSSIONS

The evolutionary position and atypical nature of rRNA has largely been exploited to study the factors involved in its biogenesis, but there are still many unanswered mysteries about them like: how does the post transcriptional processing of the native rRNA occur? Although previous studies have confirmed that different snRNAs, fibrillarin and other post-transcriptional factors are involved in this type of modification.[9,20,31] The complex formation (RNPP) ability of snoRNAs with fibrillarin[10,13,27] and its role in rRNA processing has also been shown in other organisms.[2,6,8] We have mentioned earlier that *G. lamblia*, the ancient protozoan parasite has the potential for being an useful model for studies of rRNA processing and both fibrillarin and different snRNAs play very crucial role during post transcriptional modification of rRNA. But, till now, there are no reports regarding the direct confirmation of fibrillarin-snRNA complex formation in situ within *G. lamblia* cells. In this present study efforts have been made to show the direct interaction between fibrillarin and snRNA using a very new strategy namely FRET and FCET using antisense molecular beacon. This strategy can not only be used in *G. lamblia* but also in other systems, where due to unavailability of small RNA or DNA specific dyes, co-localization and interaction studies between different proteins and RNA/DNA is not possible using FRET.

The localization study of fibrillarin and snRNA clearly demonstrates that both the protein (fibrillarin) (Fig. 1) and the snRNA (snRNA D) (Fig. 2) are present within the nucleus of the cell. snRNA D has been chosen randomly for the complex formation study from a large database of different Giardia snRNAs, previously found to be involved in complex formation with fibrillarin in vitro.[10] The control molecular beacon has not hybridized with snRNA or with the sense strand of DNA giving rise to the specificity of binding of antisense molecular beacon against snRNA D and its suitability for future FRET and FCET studies. The mere co-localization does not signify the interaction between fibrillarin and snRNA to form RNPP, to prove the firm interaction, FRET and FCET studies have been done. There is an increasing interest in the detection of interactions between intracellular molecular species and FRET provides a powerful technique for achieving this goal.[7] To carryout FRET, the most important objective was to choose a dye pair specific for snRNA and fibrillarin. As, till date, no specific dye is available that binds with snRNA, so a unique approach has been tried, viz. antisense molecular beacon[11] against the snRNA D. The approach worked well because the beacon has been designed from a partially conserved region of snRNA D at the 3' end for binding properly and specifically only to the sense snRNA inside the cell and to avoid steric effect at the protein (fibrillarin) binding site of the snRNA (Fig. 3). The binding site of fibrillarin on snRNA D is not yet known, but, it is already known from the previous observations[15,25] that small RNA-protein binding mainly occurs

using specific secondary structures in the small RNA and not at the linear ends. Keeping in mind this fact, we have designed the beacon from linear 3' end of snRNA D, not to hamper its secondary structure for taking part in complex formation. Thus it has produced a FRET signal. Confocal FRET imaging usually starts with the visualization of FITC and the energy transfer signal. Due to their physical properties, the spectra of the FITC partly overlap. Their simultaneous excitation and detection in a coexpressing cell can lead to significant cross-talk. With newly introduced multitracking technology the cross-talk problem has effectively been tackled.[3] In a multitrack configuration for FRET, first FITC is excited and detected, then finally a emission signal for rhodamine is recorded under the excitation conditions for FITC. If the fluorophores are more than 10 nm distant from each other only FITC signals should be detectable but the FRET channel (rhodamine emission in this case) should not show any signal. If, however, both fluorophores are interacting with each other and located within a few nanometers, the FITC signal should decrease since energy is transferred to Rhodamine leading to a signal in the FRET channel. The later indicates that the labeled RNA partners are potentially interacting.[21] As the excitation laser for rhodamine has been turned off during the whole procedure, there were no chance of any red emission due to its normal excitation. Thus, the emission spectra for rhodamine in FRET is truly detected as energy was transferred from emission spectra of FITC to excitation spectra of rhodamine. Thus, interaction of snRNA and fibrillarin has been confirmed to form the RNPP in situ (Fig. 4A). Fluorescence recovery after photobleaching, again confirms that this FRET signal is not merely an artefact.[36] In Figure 4B a visual comparison between panel b and d clearly suggests the emission intensity of panel d is much higher than panel b. This is due to gain in emission intensity of FITC by loss of FRET signal transfer after photobleaching of rhodamine emission in panel d.

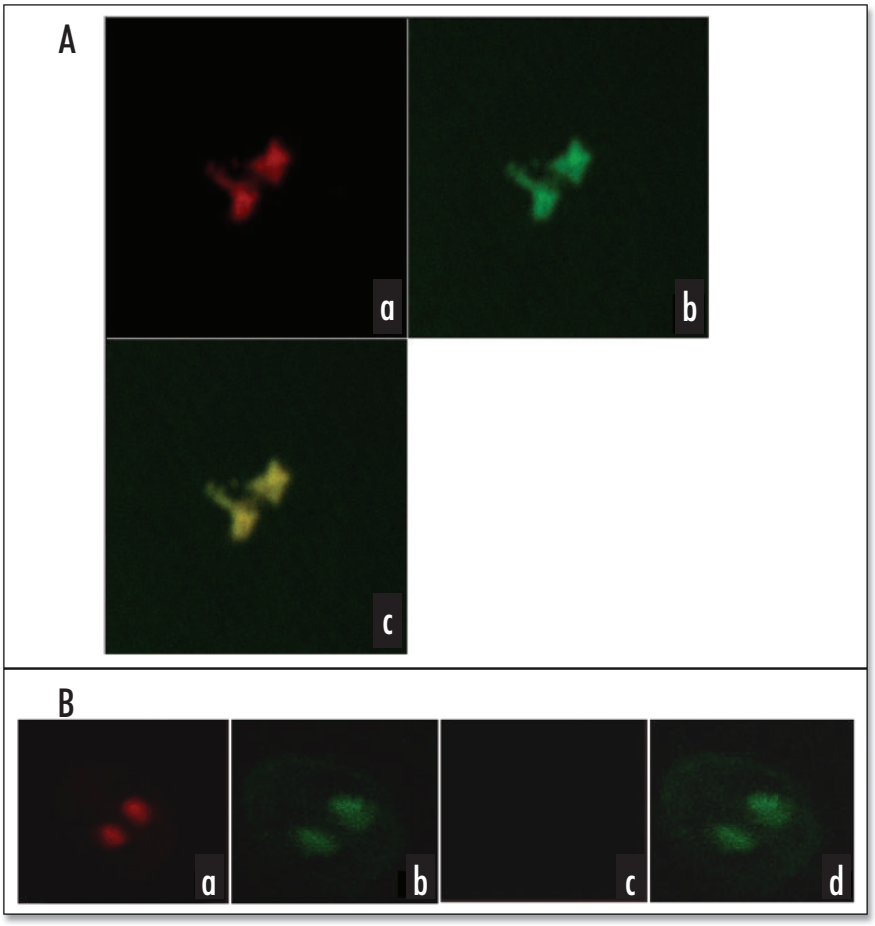

Figure 4. Fluorescence Resonance Energy Transfer by confocal microscope. Here Rhodamine tagged molecular beacon and anti fibrillarin antibody-FITC conjugate have been used for double staining *G. lamblia* trophozoites. Images have been taken in absence of excitation spectra for Rhodamine (A). FRET signal is further confirmed by increase in FITC green emission after photobleaching the Rhodamine red emission (B). (A) Confocal microscopic demonstration of FRET. a, Rhodamine emission channel detected at 610 nm with 488 nm excitation for FITC but in absence of Rhodamine excitation at 534 nm; b, FITC Emission channel at 530 nm with excitation at 488 nm; c, Figure generated after superimposition of (a) and (b). (B) Change in fluorescence emission for FRET signal after photobleaching . a, Rhodamine emission channel detected at 610 nm with 488 nm excitation for FITC but in absence of Rhodamine excitation at 534 nm; b, FITC Emission channel at 530 nm with excitation at 488 nm; c, Rhodamine emission channel bleached by specific laser line that does not hamper FITC emission; d, FITC emission increased in green channel at 530 nm with excitation at 488 nm after laser bleaching of rhodamine emission by specific laser line that does not hamper FITC emission.

In steady-state FRET can be detected by exciting the labeled specimen with light of wavelengths corresponding to the excitation spectrum of the donor and detecting light emitted at the wavelengths corresponding to the emission spectrum of either the donor and/or the acceptor. When FRET occurs, the donor emission is decreased and the acceptor emission is increased (sensitized emission). Proper use of FRET measurements to characterize molecular interactions requires that corrections be made for cross talk (the detection of donor fluorescence through the acceptor emission filter and the detection of acceptor fluorescence through the donor emission filter). A number of donor/acceptor pairs suffer significant cross talk. This type of error in FRET measurement has not been accounted for in the flow cytometry energy transfer (FCET) method.[32] It is also an efficient method to map associations between biomolecules because of its high sensitivity to changes in molecular distances in the range of 1–10 nm.[28] During FCET mean fluorescence intensity change also suggests the energy transfer in the overlapping region of the two dyes. However, in FCET increase in red fluorescence of rhodamine and decrease in green fluorescence of FITC in presence of both FL1 and FL2 excitation confirms the energy transfer as well as physical interaction between snRNA and fibrillarin. FCET is used to give more support to FRET findings by confocal microscope and our results clearly showed that there is a positive energy shift from fibrillarin to snRNA because the mean fluorescence intensity of FITC has decreased and that of Rhodamine has increased. The change in mean fluorescence intensity is a marker of energy transfer from overlapping electronically excited state of FITC to rhodamine (Fig. 5). As energy is transferred from overlapping emission-excitation region of FITC

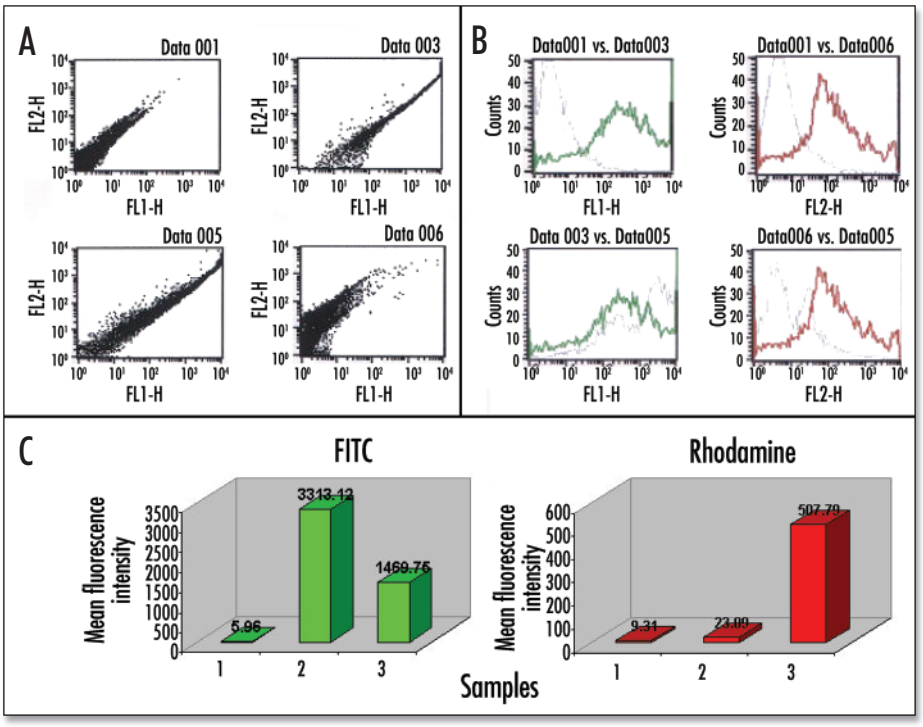

Figure 5. FACS is used to analyse the FCET between fibrillarin to snRNA D. (A) Scattered plot of different sets of *Giardia lamblia* cells in flow cytometer. Data 001, Control; Data 003, *Giardia lamblia* cells stained with anti fibrillarin antibody/FITC conjugate; Data 005, *Giardia lamblia* cells double stained with anti fibrillarin antibody/FITC conjugate and rhodamine tagged antisense molecular beacon; Data 006, *Giardia lamblia* cells stained with rhodamine tagged antisense molecular beacon. (B) FCET—Data 001 vs. 003 shows positive staining by anti-fibrillarin antibody/FITC conjugate; Data 001 vs. 006 shows positive staining by rhodamine tagged antisense molecular beacon; Data 003 vs. 005 shows decrease in FITC green fluorescence emission during FCET in double stained *G. lamblia* cells and Data 006 vs. 005 shows increase in rhodamine red fluorescence emission during FCET in double stained *G. lamblia* cells. Gray line denotes emission intensity of single stained cells and green/red line denotes emission intensity of double stained cells. (C) Change in mean fluorescence intensity. Several sets of experiments were performed using the same parameters and mean of all has been plotted in the graph. 1, Control; 2, FITC or rhodamine single stained cells; 3, FITC and rhodamine double stained cells.

and rhodamine, the mean fluorescence intensity of FITC has been dropped down compared to single FITC staining. Simultaneously, the mean fluorescence intensity for Rhodamine has increased compared to single rhodamine stained cells. Different sets of *G. lamblia* cells have been used in FCET. Although similar conditions have been properly maintained throughout, but there might be a chance that differences in emissions observed in single and double stained cells, i.e., FCET signal, may be due to difference in staining efficiencies and not a proper FCET signal. To avoid this discrepancy we have taken several sets of single and double stained cells and measured their emission spectra and finally a statistical mean is used to show FCET signal.

It is clearly evident from the above results that snRNA-fibrillarin interacts to form complex during preRNA processing required in the formation of ribosomal RNA. This is the first report where FRET and FCET using antisense molecular beacon has been used in *G. lamblia* to demonstrate RNA protein interaction inside cells (in situ). The technique will be highly useful for in situ analysis of RNA–protein interaction in other systems, because till date RNA specific dyes are very meager.

Reference

1. Adams RD. Biology of *giardia* spp. Microbiol Rev 1991; 55:706-32
2. Amiri KA. Fibrillarin like proteins occur in the domain Archaea. J Bact 1994; 176:2124-7.
3. Ankerhold R. Fluorescence resonance energy transfer (FRET) analysis with the ZEISS LSM 510. GIT lab j 2001; 2:13-5
4. Bonnet G, Tyagi S, Libchaber A, Kramer FR. Thermodynamic basis of the enhanced specificity of structured DNA probes. Proc Natl Acad Sci USA 1999; 96:6171-6.
5. Caffarelli E, Losito M, Giorgi C, Fatica A, Bozzoni I. In vivo identification of nuclear factors interacting with the conserved elements of box C/D small nucleolar RNAs. Mol Cell Biol 1998; 18:1023-8.
6. Cappai R, Osborn AH, Handman E. Cloning and sequence of a Leishmania major homologue to the fibrillarin gene. Mol Biochem Parasitol 1994; 64:353-5.
7. Dirks RW, Molenaar C, Tanke HJ. Methods for visualizing RNA processing and transport pathways in living cells. Histochem Cell Biol 2001; 115:3-11.
8. Dunbar DA, Wormsley S, Lowe TM, Baserga SJ. Fibrillarin associated box C/D small nucleolar RNAs in trypanosoma brucei. J Biol Chem 2000; 275:14767-76.
9. Filipowicz W, Pelczar P, Pogacic V, Dragon F. Structure and biogenesis of small nucleolar RNAs acting as guides for ribosomal RNA modification. Acta Biochim Pol 1999; 46:377-89.
10. Ghosh S, Ghosh R, Das P, Chattopadhyay DJ. Expression and purification of recombinant Giardia fibrillarin and its interaction with small nuclear RNAs. Pro Express Purific 2001; 21:40-8.
11. Gionata Leone, Harm van Schijndel, Bob van Gemen, Fred Russel Kramer, Cornelis Dirk Schoen. Molecular beacon probes combined with amplification by NASBA enable homogeneous, real-time detection of RNA. Nucl Aci Res 1998; 26:2150-5.
12. Gordon GW, Berry G, Liang XH, Levine B, Herman B. Quantitative fluorescence resonance energy transfer measurements using fluorescence microscopy. Biophys J 1998; 74:2702-13.
13. Henriquez R, Blobel G, Aris JP. Isolation and sequencing of NOPI. A yeast gene encoding a nucleolar protein homologues to a human autoimmune antigen. J Biol Chem 1990; 265:2209-15.
14. Heyduk T, Heyduk E. Molecular beacons for detecting DNA binding proteins. Nat Biotech 2002; 20:171-6.
15. Jian-Ying Zhang, Edward KL, Chan Xuan-Xian Peng, Eng M Tan. A novel cytoplasmic protein with RNA-binding motifs is an autoantigen in human hepatocellular carcinoma. J Exp Med 1999; 189:1101-10.
16. Leone G, van Schijndel H, van Gemen B, Kramer FR, Schoen CD. Molecular beacon probes combined with amplification by NASBA enable homogeneous, real-time detection of RNA. Nucl Aci Res 1998; 26:2150-5.
17. Liu J, Feldman P, d Chung TD. Real-time monitoring in vitro transcription using molecular beacons. Anal Biochem 2002; 300:40-5.
18. Liu J, Feldman PA, Lippy JS, Bobkova E, Kurilla MG, Chung TDY. A scintillation proximity assay for RNA detection. Anal Biochem 2001; 289:239-45.
19. Marras SA, Kramer FR, Tyagi S. Multiplex detection of single-nucleotide variations using molecular beacons. Genet Anal 1999; 14:151-6.
20. Maxwell ES, Fournier MJ. The nucleolar small RNAs. Annu Rev Biochem 1995; 35:897-934.
21. Miyawaki A, Llopis J, Heim R, McCaffery JM, Adams JA, Ikura M, et al. Fluorescent indicators for Ca^{2+} based on green fluorescent proteins and calmodulin. Nature 1997; 388:882-7.
22. Molenaar SA, Marras JCM, Slats JC, Truffert M, Lemaitre AK, Raap RW, et al. Linear 2' O-methyl RNA probes for the visualization of RNA in living cells. Nucl aci res 2001; 29:E89-9.
23. Narcisi EM, Glover CV, Fechheimer M. Fibrillarin, a conserved preribosomal RNA processing protein of Giardia. J Euk Microbiol 1998; 45:105-11.
24. Niu XH, Hartshorne T, He XY, Agabian N. Characterization of putative small nuclear RNAs from *Giardia lamblia*. Mol Biochem Parasitol 1994; 66:49-57.
25. Phinikoula S, Katsamba David, Myszka G, Ite A. Laird-Offringa. Two Functionally Distinct Steps Mediate High Affinity Binding of U1A Protein to U1 Hairpin II RNA. J Biol Chem 2001; 276:21476–81.
26. Prasher DC, Eckenrode VK, Ward WW, Prendergast FG, Cormier MJ. Primary structure of the *Aequorea victoria* green-fluorescent protein. Gene 1992; 111:229-33.
27. Schimmang T, Tollervey D, Kern H, Frank R, Hurt EC. A yeast nucleolar protein related to mammalian fibrillarin is associated with small nucleolar RNA and is essential for viability. EMBO J 1989; 8:4015-24.

28. Sebestyen Z, Nagy P, Horvath G, Vamosi G, Debets R, Gratama JW, et al. Long wavelength fluorophores and cell-by-cell correction for autofluorescence significantly improves the accuracy of flow cytometric energy transfer measurements on a dual-laser benchtop flow cytometer. Cytometry 2002; 48:124-35.
29. Sokol DL, Zhang X, Lu P, Gewirtz AM. Real time detection of DNA RNA hybridization in living cells. Proc Natl Acad Sci USA 1998; 95:11538-43.
30. Tollervey D, Lehtonen H, Camro-Fonseca M, Hurt EC. Temperature sensitive mutations demonstrate role of yeast fibrillarin in pre rRNA processing, pre rRNA methylation and ribosome assembly. Cell 1993; 72:443-57.
31. Tollervey D, Lehtonen H, Camro-Fonseca M, Hurt EC. The small nucleolar RNP protein NOPI (fibrillarin) is required for prerRNA processing in yeast. EMBO J 1991; 10:573-83.
32. Tron L, Szollosi J, Damjanovich S, Helliwell SH, Arndt-Jovin DJ, Jovin TM. Flow cytometric measurement of fluorescence resonance energy transfer on cell surfaces. Quantitative evaluation of the transfer efficiency on a cell-by-cell basis. Biophys J 1984; 45:93.
33. Tyagi S, Kramer R. Molecular beacons: Probes that fluoresce upon hybridization. Nat Biotech 1996; 14:303-8.
34. Willhoeft U, Tannich E. 2000 Fluorescence microscopy and fluorescence in situ hybridization of *Entamoeba histolytica* nuclei to analyse mitosis and the localization of repetitive DNA. Mol Biochem Parasito 2000; 105:291-6.
35. Wouters FS, Verveer PJ, Bastiaens PI. Imaging biochemistry inside cells. Trends Cell Biol 2001; 11:203-11.
36. Zimmermann B, Ankerhold R. FRET and FRAP using Emission Fingerprinting with the LSM 510 META. G.I.T.lab J 2002; 4:3.