

Fluorescence Resonance Energy Transfer using molecular beacon as a probe, a new approach for in vivo macromolecular interaction study

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Introduction

Macromolecular interactions are gaining their importance in today's biological research. RNA-protein interactions play very vital role in regulating different activities within a cell. Although RNA-Protein complexes are very common for macromolecular processing and biogenesis and there are several techniques like gel electrophoresis 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 vivo.

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.

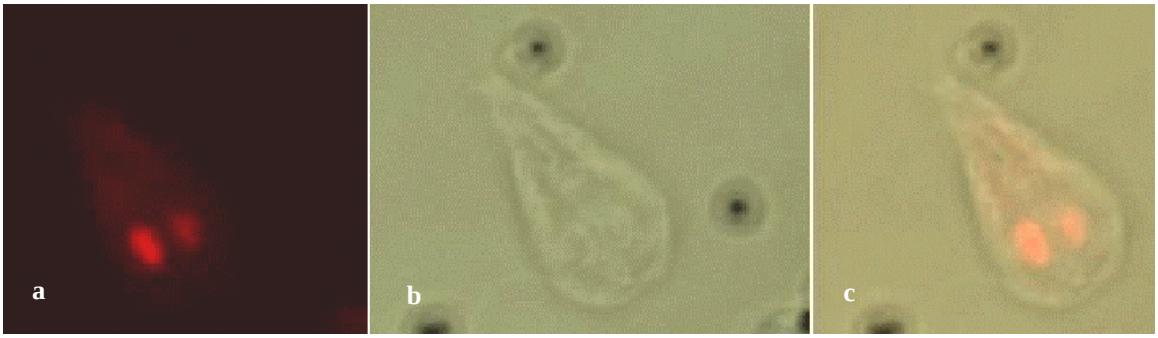

Fig. 1 : Demonstration of snRNA D inside *Giardia lamblia* nucleus using antisense molecular beacon tagged with rhodamine. Panel a) presence of snRNA D stained with rhodamine tagged molecular beacon within the nucleus, Panel b) Phase contrast picture of whole cell. Panel c) Figure generated after superimposition of panel a and panel b

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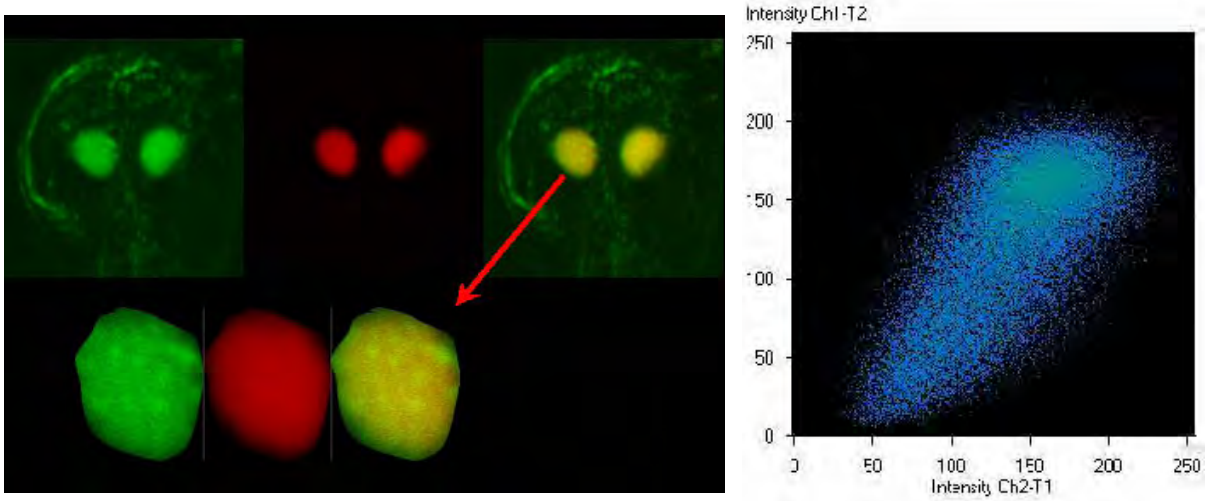


Fig. 2 : Co-localization of fibrillarin protein and snRNA in *Giardia lamblia* nuclei. Fibrillarin is stained with FITC and snRNA with antisense molecular beacon shows extent of co-localization. Presence of 45 degree angle of scattered graph during analysis of colocalization by LSM 510 software clearly demonstrates the complete co-localization of fibrillarin protein inside the nucleus.

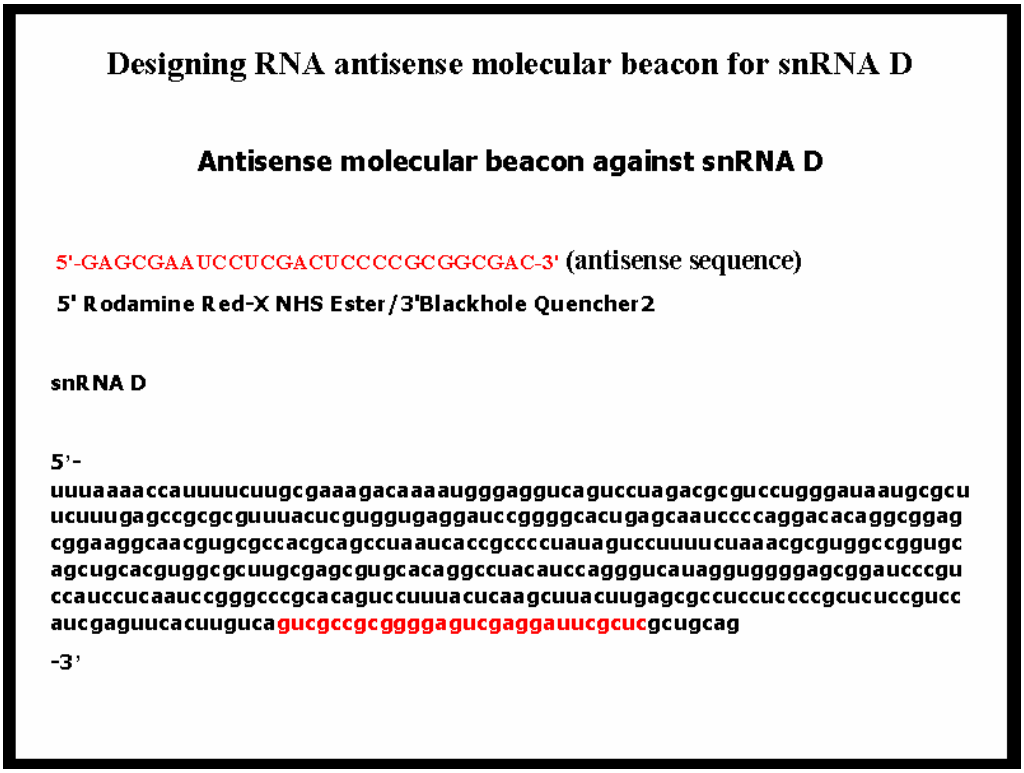
Methods

A Zeiss LSM 510 Meta system with LSM 510 software was used throughout the study. It was previously observed that snRNA and fibrillarin is a nucleolar protein present in Giardia lamblia nuclei although the organism does not contain any defined nucleolus. Fibrillarin was found to be involved in formation of RNPP complex with different snRNAs. In *Giardia lamblia*, the primitive eukaryotic enteric parasite, it has been found to interact with snRNAs as suggested by molecular biological techniques like GEMSA, north western blotting etc. But, all these techniques are confirming the interaction *in vitro*. To study this interaction *in vivo* a new technique is designed here. Fluorescence resonance energy transfer (FRET) is a technique used for quantifying the distance between two molecules conjugated to different fluorophores (Gordon *et al*, 1998). 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*. Here change in the mean fluorescence emission is the marker of energy transfer and interaction between the two different biomolecules.

G. lamblia snRNA D (Ghosh *et al.* 2001) was used for designing antisense molecular beacon (IDT DNA). The detailed strategy for designing the molecular beacon is shown in Fig. 3. The antisense sequence was designed against a 28bp conserved 3' terminal end sequence of snRNA D tagged with rhodamine as fluorophore and blackhole quencher as the fluorophore quencher.

This antisense molecular beacon designed against snRNA D was used for a localization study within the nuclei of *G. lamblia* trophozoites. *G. lamblia* trophozoites were fixed in cold methanol and 0.5% Tween 20 and incubated for 2 hrs at 4^{o}C with 1:50 diluted antisense molecular beacon (final concentration 0.05mM) in PBS. Washed pellet was dissolved in minimum volume of PBS and examined under confocal microscope (Zeiss LSM 510) at 543nm excitation and 610nm emission. snRNA was identified in red color within the nucleus.

After localization of SNRNAs FRET was performed. Axenic *G.* cells were fixed as earlier and incubated with 1:50 PBS diluted antisense molecular beacon (final concentration 0.05mM) 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



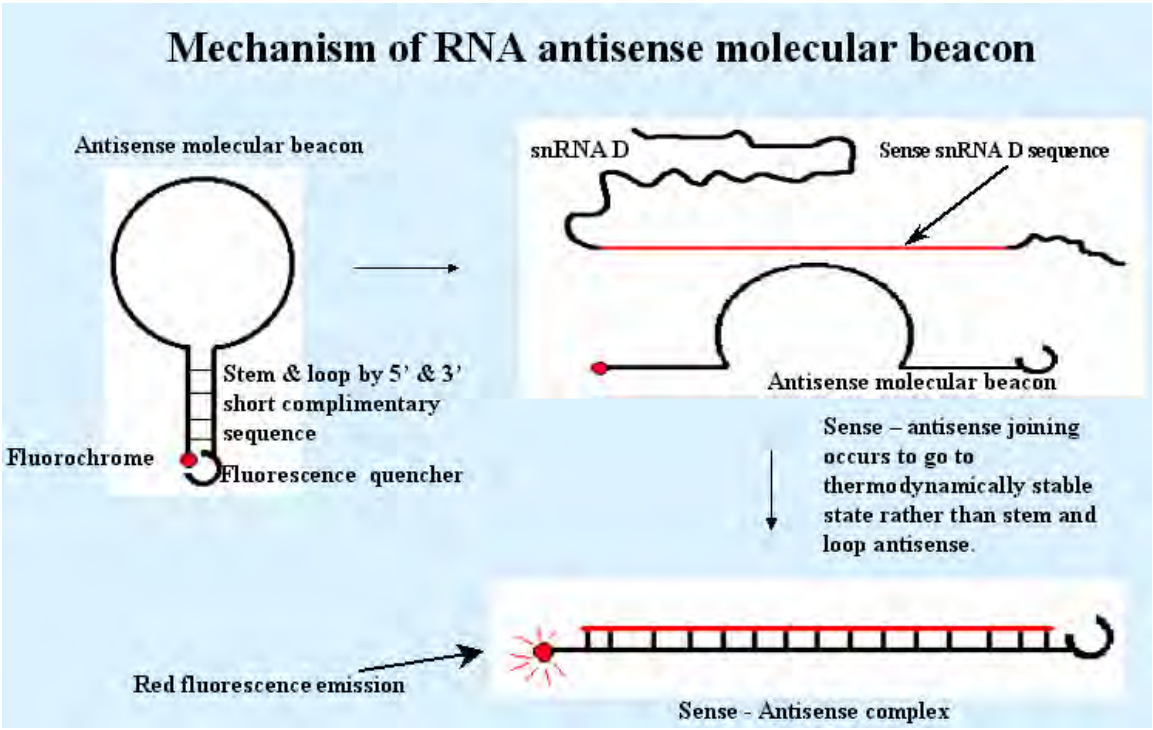


Fig. 3 : Detailed description of designing and mechanism of antisense molecular beacon for FRET and FCET. 3' and 5' end tag of complementary aaaa-uuuu has not been shown in the picture.

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 & 543 nm excitation and 530 & 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.

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).

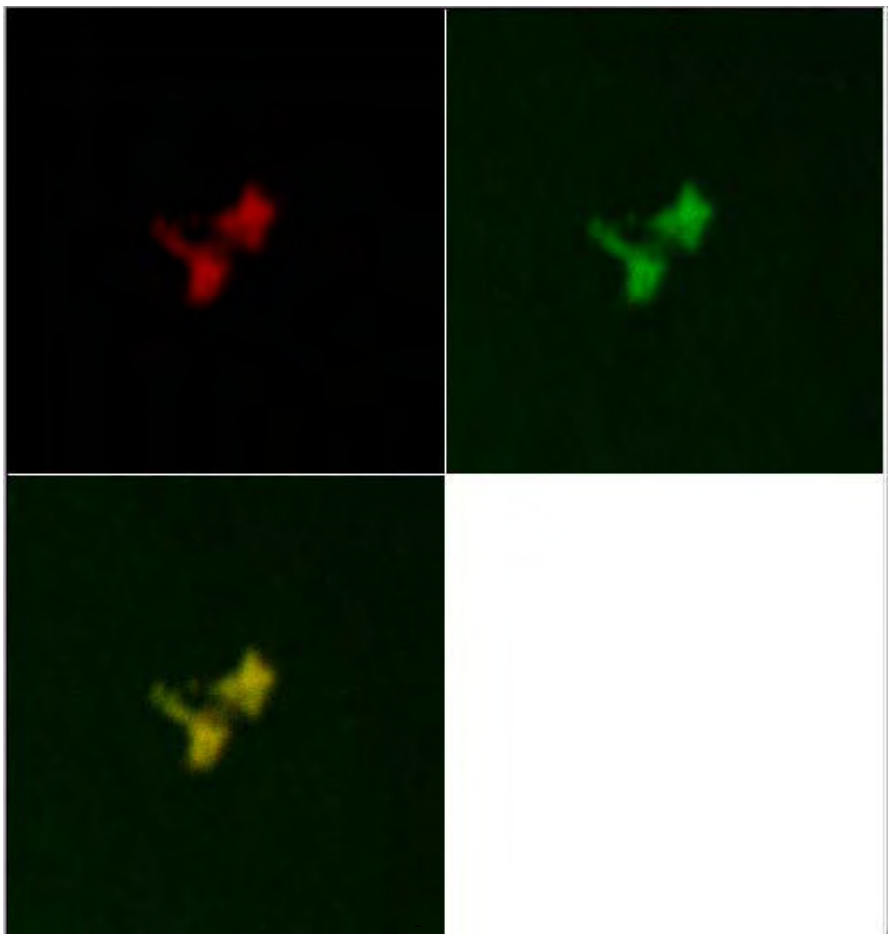
The FRET signal was further confirmed by a "Fluorescence Recovery After Photobleaching" (FRAP) test. After detection of FRET signal by exciting the double stained cells with excitation laser for FITC, the Rhodamine emission of the cells has been bleached out by flushing the cells with a long wavelength 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.

Results and Discussion

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 snoRNAs, fibrillarin and other post-transcriptional factors are involved in this type of modification (Maxwell *et al.* 1995, Tollervy *et al.* 1991, Filipowicz *et al.* 1999). The complex formation (RNPP) ability of snoRNAs with fibrillarin (Schimmang *et al.* 1989, Henriquez *et al.* 1990, Ghosh *et al.* 2001, Ganguly et. al. 2004) and its role in rRNA processing has also been shown in other organisms (Amiri *et al.* 1994, Cappai *et al*. 1994, Dunbar *et al.* 2000). 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 FRET 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 snRNA clearly demonstrates that snRNA (snRNA D) is present within the nucleus of the cell and the designed molecular beacon can specifically be used for staining the snRNA.. 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 studies. Although antisense molecular beacon stained snRNA and FITC stained fibrillarin was found to be co-localized in the nucleus, but, the mere co-localization does not signify the interaction between fibrillarin and snRNA to form RNPP. To prove the firm interaction, FRET study has 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 (Dirks *et al*. 2001). 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 (Gionata Leone *et al.* 1998) 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. The binding site of fibrillarin on snRNA D is not yet known, but, it is already known from the previous observations (Jian-Ying Zhang *et al*. 1999, Phinikoula S. Katsamba *et al*. 2001) 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 co-expressing cell can lead to significant cross-talk. With newly introduced multitracking technology the cross-talk problem has effectively been tackled (Ankerhold, 2001). 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 latter indicates that the labeled RNA partners are potentially interacting (Miyawaki *et al*. 1997). 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*.



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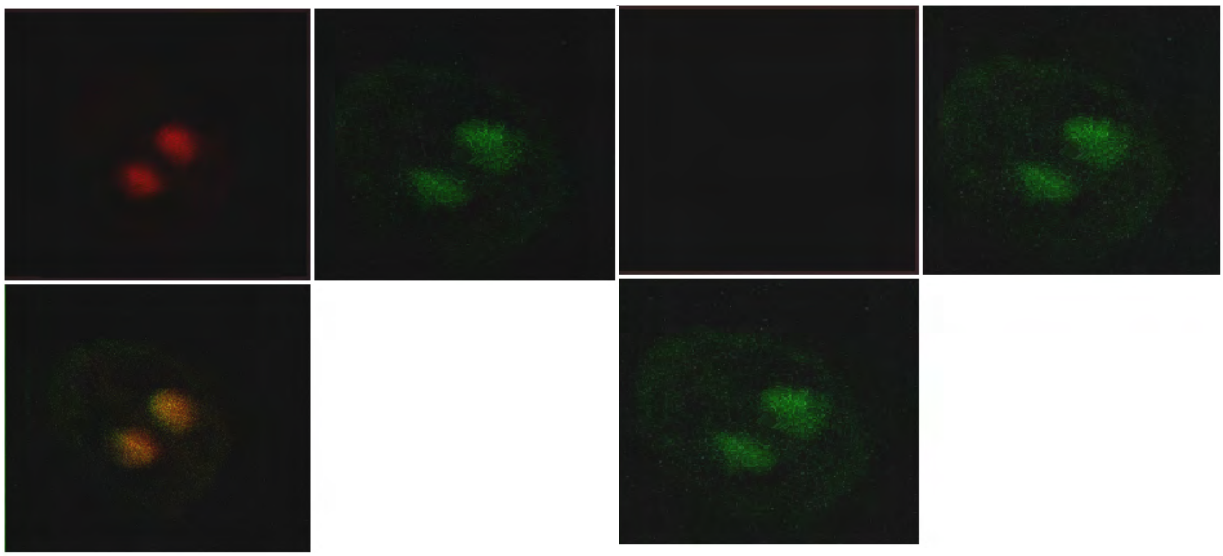

II

Fig. 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 (IA). FRET signal is further confirmed by increase in FITC green emission after photobleaching the Rhodamine red emission (II).

I : Confocal microscopic demonstration of FRET. Panel 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.

II : Change in fluorescence emission for FRET signal after photobleaching . Panel 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.

Fluorescence recovery after photobleaching, again confirms that this FRET signal is not merely an artefact (Zimmermann *et al.* 2002). In Fig. 4-II 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.

It is clearly evident from the above results that snRNA-fibrillarin interacts to form complex during pre-RNA processing required in the formation of ribosomal RNA. This is the first report where FRET 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.

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