Electroporation of snoRNA in *Giardia lmblia*

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Giardia lamblia is a gastrointestinal flagellated protozoan parasite causing giardiasis. It is estimated that about 300 million people worldwide are affected by this disease. The organism not only infects humans but also infects other species of mammals, birds and reptiles. *Giardia* is placed in the phylum Sarcomastigophora under the class Zoomastigophora that includes other protozoan parasites like *Trypanosoma*, *Leishmania*, *Trichomonas* and *Dientamoeba*. Though placed in this class, *Giardia* is different from other protozoans in many respects. The sequence of small subunit rRNA, lack of mitochondria and some other eukaryotic organelles make this organism an interesting/intriguing subject to study various molecular and biochemical mechanisms. Electroporation is an important tool used in molecular biology to introduce various micromolecular substances into a cell. The technique involves a significant increase in the permeablity of the cell membrane caused by an externally applied electrical field. The process can be utilized in *Giardia* to introduce various micromolecules into the organism and visualise them using a suitable microscope. In this chapter we describe the process of electroporating *invitro* transcribed snoRNA into *Giardia lamblia* and its visualization using laser scanning confocal microscopy.

1. Introduction

Giardia lamblia is a binucleate flagellated protozoan parasite that causes giardiasis in a variety of animals that includes members of the class amphibian to the mammals (Adams, 1991). Although the organism is a major cause of diarrhea worldwide (Young, 2005), it was not accepted as a human pathogen until recently, because in is non-invasive and the infection it causes is frequently asymptomatic (Adams, 1991). *Giardia* is placed in the phylum Sarcomastigophora under the class Zoomastigophora that includes other protozoan parasites like *Trypanosoma, Leishmania, Trichomonas* and *Dientamoeba* (Adams, 1991). Phylogenetic analyses have consistently placed Giardia near the root of the eukaryotic branch of evolution (Sogin, 1991) and the organism also exhibits certain features that are rather prokaryotic in nature (p2). The lack of cellular organelles like mitochondria, peroxisomes and smooth endoplasmic reticulum, or the sequence of its small subunit rRNA are features that are very much unlike of other eukaryotes (Adams, 1991). *Giardia* can be regarded as an ancient eukaryote (Ghosh, 2001) and a missing link between eukaryotes and prokaryotes (Young, 2005). It is this feature of the organism that makes it an interesting model to study the various aspects of molecular and biochemical innovations that led to the formation of eukaryotic cells (Young, 2005).

Different biochemical methods have been developed for transferring genes into the cells. Incubation of cells with co-precipitates of DNA and Calcium Phosphate, micro injection of genes in the nucleus of the recipient cell, use of viral vectors and liposomes as vehicle to transfer genes into recipient cells are some of them (Graham and van der Eb, 1973; Diacumakos, 1973; Hamer and Leder, 1979; Mulligan, 1979; Fraley et al., 1980; Wong et al., 1980; Schafer-Ridder et al., 1982). Apart from these, electroporation is often used for transformation of cells (Karmakar, 2013) as it is much more effective than other processes of chemical transformation (Neumann, 1982). Biological membranes are co-operatively stabilized organization of lipids and proteins containing locally limited structural defects. Under the effect of externally applied electric field, the membranes, transiently increases their permeability when certain threshold value of the field strength is exceeded. This increase in permeability leads to a transient exchange of matter across the membrane.

Small nucleolar RNAs (snoRNAs) are a group of small RNAs that are involved in maturation of rRNA (Smith, 2005). Categorized into 2 classes, Box C/D (have conserved sequence elements known as box C characterized by the sequence RUGAUGA and box D having the CUGA characterized sequence) and Box H/ACA (the conserved sequence in box H is ANANNA and the ACA sequence) the snoRNAs modifies the pre rRNA by attaching a methyl group to the 2'-Oxygen of the ribose sugar and converting the uridine residue to pseudo uridine respectively (Smith, 2005). *Giardia* is reported to have 20 different snoRNA like RNAs out of which 16 belongs to Box C/D class and the rest are H/ACA type (Young, 2005). snoRNAs can be an important tool to study several molecular biological mechanisms and in *Giardia lamblia* it can be quite interesting.

In this chapter we will describe the process of electroporating a Fluorescein-12-UTP labeled *in vitro* transcribed snoRNA of *Giardia* into the organism and visualized using a laser scanning confocal microscope.

2.1 *Giardia lamblia*

Giardia was one of the first protozoans to be described when Anton von Leeuwenhoek discovered the trophozoites of the organism in 1681 in his own diarrheal stool (Dobell, 1920). In 1859, Lambl described the genus *Giardia* in greater

details (Lambl, 1859) and hence, the human species was named after him (Adams, 1991). The organism has a simple life cycle consisting of an infective cyst and vegetative trophozoites. The cyst is relatively resistant to harsher environmental conditions such as desiccation and is also resistive to gastric acid in the host stomach. After a cyst is ingested, it excysts in the small intestine and here it forms two trophozoites. These trophozoites then divide by binary fission and are responsible for the symptoms of giardiasis. Later, some of these trophozoites are induced to encyst and are egested out through feces which are again ingested by another host, thus completing the life cycle (Adams, 1991).

2.2 Trophozoites of *Giardia lamblia*

The trophozoites measure approximately 10-12 μm in length and 5-7 μm in width. It consists of a funis (body), median body, a ventral disk formed by a concave surface on the anterior two thirds of the ventral surface and four pairs of flagella (Adams, 1991). The trophozoites have cellular organelles like the two nuclei, lysosomal vacuoles and also Golgi apparatus that has been reported in encysting trophozoites. Glycogen and ribosomal granules are present in the cytoplasm. But the organism lacks many of the usual eukaryotic organelles like the mitochondria, peroxisomes and smooth endoplasmic reticulum.

2.3 Cysts of *Giardia lamblia*

Cyst is the infective stage of *Giardia* and as mentioned earlier, is relatively resistant to external environment as well as to gastric acid in the stomach of the infected host. The cyst is approximately 5-8 μm and is surrounded by a wall that is about 0.3 μm in thickness (Sheffield, 1977). The outer surface of the wall consist a structure formed by 7 to 20 filaments (Erladsen, 1989) and the inner part contains an outer and inner cyst wall membrane separated by a thin layer of cytoplasm. Inside the *Giardia lamblia* cyst wall, flagellar axonemes, vacuoles, ribosomes and fragments of the ventral disk are found (Sheffield, 1977; Erladsen, 1984; Erladsen, 1989). Normally, the cyst contains four nuclei, whereas those that have not undergone nuclear division contain two.

2.4 Ribosomal RNA (rRNA) of *Giardia lamblia*

Sequence analysis of the gene encoding the small subunit RNA has been used to classify *Giardia lamblia* as the most primitive eukaryote (Sogin, 1989). The rRNAs of *Giardia lamblia* are unique in that they are smaller than those of other eukaryotes and in fact are smaller than those of the eubacteria (Boothroyd, 1987; Edlind, 1987). The rDNA gene is only 5566 bp (Healey, 1990) and is tandemly repeated in the genome. The large subunit (28S), small subunit (16S) and 5.8S of the rRNA molecule is encoded by this tandem repeat and are much smaller than their other eukaryotic counterparts. The sequence of *Giardia lamblia* small-subunit rRNA shows greater similarity to the archaebacteria sequence than do the sequences from other eukaryotes (Adams, 1991). It is this uniqueness of the rRNAs of *Giardia* that makes it particularly intriguing to study its various aspects and thus the study of snoRNAs becomes important as they are involved in the processing of pre-rRNA to form the mature rRNA (Maden, 1997)

2.5 Importance of studying *Giardia lamblia*

Apart from its evolutionary importance, *Giardia* has certain clinical correlations which make it particularly important to study. *Giardia* causes giardiasis, the clinical manifestations of which vary from asymptomatic infections to chronic diarrhea with loose, foul smelling stools that are greasy, frothy or bulky. Other symptoms like abdominal cramps and bloating, nausea and decreased appetite, malaise and weight loss are seen in majority of patients. Fever is occasionally present, especially in early part of the infection. *Giardia* is the most common protozoan isolated from human stool specimens (Flores, 1983) and is a common cause of diarrhea in humans as well as in dogs and cats (Kirkpatrick, 1984).

2.6 Small nucleolar RNA (snoRNA)

snoRNAs are a class of small RNA molecules that primarily guide chemical modifications of other RNAs, mainly ribosomal RNAs. They are categorized into 2 families, box C/D and box H/ACA snoRNAs based on their common sequence motifs and structural features (Bachellerie, 2000). The box C/D snoRNA carries out 2'O-ribose-methylation of pre rRNA (where a methyl group is attached to the ribose group) and box H/ACA is responsible for pseudouridylation (conversion of uridine residue to pseudouridine) of rRNA (Maden, 1997). *Giardia lamblia* is reported to have 20 snoRNA like RNAs (Yang, 2005) and in this chapter we will describe the process of electroporating *in vitro* transcribed RNA J, a C/D box snoRNA (Ghosh, 2001) of *Giardia* into the organism.

2.7 Electroporation of snoRNA into *Giardia lamblia*

Electroporation is an important tool for molecular biology and use of this technique to introduce foreign molecules in *Giardia* could help us understand the cell biology of this organism in a better way. To study the process of electroporation in *Giardia*, RNA J which is a U14 class snoRNA of *Giardia lamblia* and characterized by the presence

of A and B conserved domains, along with the C and D conserved sequence (Ghosh et al., 2013) was electroporated into the organism. The RNA cloned in pGEM 4z vector as described previously (Ghosh et al., 2013) was single digested and linearized with EcoR I restriction enzyme (NEB) and used for *in vitro* transcription (IVT) by using T7 Maxiscript kit (Ambion), following the manufacturer's protocol. The RNA was tagged with Fluorescein 12 UTP (Roche) for easy detection inside the cell by the help of a confocal microscope (LSM 510 Meta, Zeiss). Fluorescein is a common flurophore that emits green light when stimulated with blue excitation light (Semwogerere, 2005). The transcribed RNA was purified by using mini Quick Spin Columns (Roche) according to manufacturer's protocol for removing the excess and unincorporated Fluorescein 12 UTP. Electroporation was carried out in Electro Cell Manipulator ECM 2001 (BTX) electroporator. Giardia trophozoites grown axenically in modified TYIS 33 medium were subjected to cold shock by keeping the culture tubes in ice for 10 mins and the cells were collected by centrifugation at 1600 rpm for 10 mins. Cells were then washed with sterile PBS (NaCl 137mM, KCl 2.7 mM, Na2HPO4 10mM, KH2PO4 2.0 mM) for 3 times and resuspended in it at a concentration of 107 cells/ml. 0.8 ml of this cell suspension was taken in a 2 mm gap cuvette, *in vitro* transcribed RNA J was added, and incubated on ice for 10 mins. This was then electroporated at 2.5 kV for a pulse length of 20 ms. Pulses of 8, 10 and 12 were applied to determine their optimum number for maximum electroporation. Electroporated cells were then incubated on ice for 15 mins, washed with PBS to remove excess RNA and observed under a confocal microscope. It was observed that at a potential difference of 2.5 kV across a distance of 2mm, snoRNA incorporation in *Giardia lamblia* depended on and is directly proportional to the number of pulses (Fig. 1). As is evident from the figure, with 8 pulses almost negligible amount of RNA could be imported into the cells. When the number of pulses was increased to 10, a higher amount of RNA is electroporated, but many cells are visible which has no electroporated RNA inside them. When the pulse number was increased to 12, it was evident that the proportion of cells containing the electroporated RNA was the highest compared to others. Almost all the cells contain the electroporated RNA. It is always desirable to use a minimum electrical field for electroporation as an unnecessary increase in the electric field compromises the viability of the cells. For a potential difference of 2.5kV across a distance of 2mm, 12 pulses of 20msec each is the minimum number required to electroporate sufficient snoRNA into *Giardia lamblia*. A lower number of pulses lead to insufficient incorporation of snoRNA into the cell.

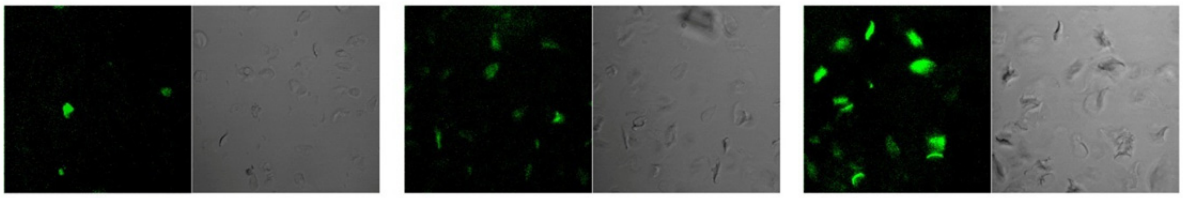

Fig. 1 showing electroporation of Fluorescein labelled snoRNA into *Giardia lamblia*: Panel A shows *Giardia lamblia* cells after 8 pluses, Panel B shows cells after 10 pulses and Panel C shows cells after 12 numbers of pulses.

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