SNAP-tag mediated live cell labeling as an alternative to GFP in anaerobic organisms

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Keywords:
SNAP-tag, AGT, GFP, Giardia, microaerotolerant, parasite, fluorescence labeling

The protozoan human parasite Giardia intestinalis represents one of the earliest known branches of the eukaryotic lineage. Therefore its simple compartmental organization may hold answers as to how the complex eukaryotic endomembrane system has evolved and what early eukaryotes may have looked like. Using fluorescent protein technology to investigate compartmental trafficking and dynamics in this organism has been only partially successful, as GFP and its autofluorescent protein relatives need oxygenation in order to mature and become fluorescent. Giardia is a microaerotolerant parasite and tolerates only very low oxygen levels, which makes the use of autofluorescent proteins at the least very problematic. As an alternative to such reporters, SNAP-tag labeling is used here to label and visualize proteins in live Giardia parasites.

The microaerotolerant protozoan parasite Giardia intestinalis belongs to one of the earliest known branches of the eukaryote lineage (1) and this proposed basic phylogenetic position is also reflected on an ultrastructural level. This has made Giardia an interesting model for the investigation of eukaryotic evolution from both the molecular and subcellular perspectives (2). Indirect immunofluorescence microscopy on fixed cells has proved invaluable as a tool to identify and study the endomembrane compartments of Giardia (3). Time-lapse microscopy studies of parasites expressing autofluorescent proteins such as green fluorescent protein (GFP) would help to answer important questions on protein trafficking and organelle dynamics. However, GFP needs to be oxygenated in order to mature and become fluorescent (4). It is possible to expose Giardia to oxygen levels sufficient for GFP visualization after harvesting the cells from culture, although they sustain damage to internal compartments, and following the fate of GFP in the cell over a longer period of time seems impossible under these conditions. Recently, Keppler et al. have described an alternative method to covalently label fusion proteins in vivo (5) using modified human O\(^6\) alkyl guanine DNA alkyl transferase (AGTm) or SNAP-tag which is not oxygen dependent. The SNAP-tag becomes covalently labeled when exposed to fluorophores presented in the form of a suitable benzyl guanine substrate. In their work, Keppler et al. used the SNAP-tag to specifically detect fusion proteins in vivo in mammalian AGT-
deficient cells. Since endogenous AGT is not present in unicellular organisms, including yeast and important pathogens such as *Plasmodium falciparum*, this method may be a useful tool to study a wide range of organisms under either aerobic or anaerobic conditions. Here, we show labelling of fusion proteins in live *Giardia* with fluorescent dyes using the SNAP-tag.

To target a reporter to the nucleoplasm of the two *Giardia* nuclei we expressed a fusion protein containing a conserved N-terminal SV40 nuclear localization signal (NLS), which is also functional in *Giardia* (6), followed by a 182 amino acid SNAP-tag sequence (snap22). Expression cassettes for stable transformation of *Giardia* trophozoites were constructed as described previously (4,7). Complementary oligonucleotides (Microsynth AG, Balgach, Switzerland) encoding the simian virus 40 (SV40) NLS and the *Haemophilus influenza* hemagglutinin (HA) tag containing restriction site overhangs indicated in italic letters were annealed by mixing them in an equal molar ratio, incubating them in PCR buffer at 94°C for 2 minutes and cooling slowly to room temperature. These mixes were used directly for ligation reactions. The SNAP-tag gene (snap22) was amplified from a plasmid based on the vector pUC 18 (kind gift of Covalys Biosciences AG, Witterswil, Switzerland) by PCR. The snap22 gene preceded by the short sequence coding for the SV40 NLS was inserted into the giardial expression cassette in a plasmid based on a PBS KS- (Stratagene, La Jolla, CA, USA) backbone containing a constitutively expressed bacterial neomycin resistance gene for selection of transgenic parasites (4). Expression of the NLS-snap22 hybrid gene was under the control of the inducible cyst wall protein 1 (CWP1) promoter (Figure 1).

Transcription from this promoter is activated by placing the trophozoites in conditions that favor encystation. Briefly, more than hundred fold induction is achieved by growing trophozoites without cholesterol for two days followed by seven hours incubation of cells at pH to 7.85 and the addition of porcine bile and lactic acid to the growth medium (3,4). The rate of induction is normally between 70 and 85%.

Trophozoites of the *Giardia intestinalis* strain WB (ATCC Nr. 50803) clone C6 were grown vegetatively in TYI-S-33 medium as described previously (4). Parasites were harvested after induction by chilling the cells on ice for 30 minutes followed by centrifugation at 800 x g, then washed with ice-cold PBS. For SNAP-tag labeling, the cells were incubated in 5 µM O6- benzyl guanine diacetyl fluorescein (BGAF) (kind gift of Kai Johnsson, Swiss Federal Institute of Technology Lausanne, EPFL) in growth medium at 37°C for 30 minutes, then washed three times with medium warmed to 37°C and resuspended in ice cold PBS for observation on a Leica SP2 AOBS confocal laser scanning microscope (CLSM, Leica Microsystems, Wetzlar, Germany) using the appropriate settings. Parasites expressing the NLS-snap22 protein labeled with BGAF showed brightly fluorescent nuclei (Figure 2, A), whereas wild type cells stained with BGAF showed no signal (Figure 2, B) demonstrating specific labeling and the expected nuclear localization of the fusion protein. The viability and morphology of cells treated with BGAF was not changed compared to wild type cells in any observable way. To directly compare the localization of a SNAP-tag fusion protein in *Giardia* with a conventional HA-tagged reporter we expressed a fusion protein containing either the HA tag or the SNAP-tag fused to the N- terminus of giardial RabA (GiRabA). Overexpressed HA tagged GiRabA specifically targets the membranes of the nuclear envelopes in *Giardia*, which results in a fluorescent signal at the nuclear envelopes but not in the nucleoplasm when immunostained with anti-HA-FITC conjugate (our unpublished results and Figure 2, C). The open reading frame of GiRabA was amplified by genomic PCR and cloned in frame with the SNAP-tag sequence as described above. For detection of the HA epitope, parasites were harvested for indirect immunofluorescence microscopy as above and fixed with 3% formaldehyde for 40 minutes at room temperature, followed by a
5 minute incubation with 0.1 M glycine in PBS. Fixed cells were permeabilized with 0.2% Triton X-100 in PBS for 20 minutes and blocked >2 hours in 2% BSA in PBS. Anti-HA antibody conjugated with FITC (Roche Applied Science, Rotkreuz, Switzerland) was diluted 1: 20 in 2% BSA / 0.2% Triton X-100 in PBS to stain fixed cells expressing HA-RabA. After washing the cells with PBS, they were embedded with Vectashield containing DAPI (Vector Labs, Emeryville, USA). For detection of SNAP-tagged GiRabA we used the protocol described above. Both the HA-tagged and the SNAP-tag GiRabA localized to the nuclear envelope (Figure 2 C, D). This confirmed the correct targeting of the SNAP-tag labeled GiRabA fusion protein in living cells. Living cells expressing NLS-snap22 and snap22RabA and labeled with BGAF showed a slight fluorescent background (Fig 2, A and D). This was probably due to the presence of reporter protein in the cytoplasm of living cells, since wild type cells exposed to BGAF showed no fluorescent background after washing (Fig 2, B). Moreover, in fixed and detergent-permeabilized cells used for conventional IFA with the anti-HA antibody, much of this cytoplasmic signal is lost because no cross-linking agent was used in the fixation protocol and the background therefore appears lower (compare Fig. 2A, D with 2C).

The fluorescence emitted by live cells expressing either the NLS-snap22 or snap22-RabA proteins labeled with BGAF disappeared 5-6 seconds after the start of observation because of photobleaching. This was insufficient for time-lapse analysis or FRET experiments. In the meantime, however, significantly more photostable membrane-permeable fluorophores have become commercially available as SNAP-tag labels (BG-505 and BG-TMR-star, Covalys, Witterswil, Switzerland, http://www.covalys.com/?menu=products&sub=snapcell#SNAPcell505). To test the photostability of these improved fluorophores and their suitability for extended live-cell analysis, we labeled Giardia expressing the snap22-RabA reporter with BG-505 or BG-TMR-star for time-lapse CLSM analysis as described above. With both fluorophores we observed a lower overall labeling intensity compared to BGAF, possibly due to decreased membrane permeability. Labeled and washed cells were continuously scanned at a rate of 6 images per minute (including 2 x line-averaging) for 25 minutes. Only a very minor loss of signal was observed for both fluorophores during the procedure. The representative images in Fig. 2E show a cell labeled with BG-TMR-star (scanned with a 543 nm laser line at 45% power), demonstrating the vastly superior photostability of the new fluorophores.

Taken together, the experiments described above show that, unlike oxygen-dependent autofluorescent proteins such as GFPs, the SNAP-tag system can be used to investigate organisms grown under anaerobic culture conditions and to detect and precisely localized reporter proteins in living Giardia parasites. This approach has a wide range of applications and should not only be practical in other important anaerobic or microaerotolerant pathogens such as Entamoeba or Trichomonas that live in oxygen-deprived environments, but generally in all organisms lacking endogenous AGT.

Acknowledgments
We would like to thank Kai Johnsson from the University of Lausanne for his scientific advice and for providing BGAF, BG-505 and BG-TMR. The authors would like to acknowledge Tom Gibbs for critical reading of the manuscript. This work was supported by a grant from the Swiss National Science Foundation (3100A0-100270) to A. B. H.

Competing interests statement
The authors state that they have received materials free of charge, as specified above, from Covalys Biosciences AG, and that the authors’ interest in this work is purely scientific. The authors do not own any stocks, nor are they in paid employment or board members of Covalys Biosciences AG, and no patent applications on the authors’
side (pending and actual) exist for any of the results obtained in this work. Further the authors state that they have received no financial support whatsoever from Covalys Biosciences AG. The work presented here is eligible for the SNAP-tag award 2005, under the conditions stated by the organizers (http://www.covalys.com/downloads_cms/snap-tag_award_2005.pdf).

References


Figure Legends

Figure 1: Graphical depiction of the expression vector constructs (not to scale). Expression of target genes was based on a pBluescript vector containing the bacterial neomycin resistance gene (NEO8) under the control of a constitutive promoter in a head to head conformation to the inducible CWP1 promoter, which controlled the transcription of inserts of interest. For the experiments described, giardial RabA preceded by an HA tag or the snap22 gene, or snap22 gene preceded by the SV40 NLS, respectively, were cloned in the inducible expression site. Flanking regions included the CWP1 3’UTR at the 3’ end of the cassette.

Figure 2: Confocal microscopy of fluorescently labeled Giardia. A. Live cells expressing NLS-snap22 stained with BGAF show a staining typical for the nucleoplasm of the two nuclei of Giardia. B. Wild type cells processed identically show no fluorescence. C. Cells expressing the HA-tagged RabA construct were fixed and labeled with a monoclonal anti HA antibody conjugated to FITC and nuclear DNA was stained with DAPI. Image shows signal from HA (green) surrounding the two nuclei (blue, see inset on upper right, HA + DAPI). A merged fluorescent signal and differential interference contrast image is shown in the lower left inset (DIC). D. Live cells expressing the snap22-RabA construct labeled with BGAF show a fluorescent signal similar to C, confirming correct targeting of the snap22-RabA protein to the nuclear envelope. Note that not
all cells are in the same focal plane and thus do not display the same signal intensity. E. Continuous time-lapse analysis of a representative cell expressing the snap22-RabA construct labeled with BG-TMR-star. Four of 150 images taken at regular intervals during 25 minutes demonstrate excellent photostability of the fluorophore. Scale bars: A, C and insets in C: 5 µm. B, D and inset in D: 10 µm.

Table 1. Primers and oligonucleotides used to create expression constructs

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