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

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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 labeled fusion proteins in vivo (5) using modified human O⁶ alkyl guanine DNA alkyl transferase (AGTm) or SNAP-tagTM (Covalys Biosciences AG, Witterswil, Switzerland), 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 labeling 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 simian virus 40 (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 SV40 NLS and the *Hemophilus influenza* hemagglutinin (HA) tag containing restriction

site overhangs (see Table 1) were annealed by mixing them in an equal molar ratio, incubating them in PCR buffer at 94°C for 2 min, 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) 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 pBlueScript[®] KS⁻ vector (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 was activated by placing the trophozoites in conditions that favor encystation. More than 100-fold induction was achieved by growing trophozoites in growth medium without bile for 2 days, followed by a 7-h incubation of cells at pH to 7.85, and the addition of porcine bile and lactic acid (3,4). The rate of induction is normally between 70% and 85%.

Trophozoites of the *G. intestinalis* strain WB (ATCC no. 50803; ATCC, Manassas, VA, USA) clone C6 were grown vegetatively in TYI-S-33 medium as described previously

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 (neo^R) under the control of a constitutive promoter in the opposite orientation to the inducible cyst wall protein 1 (CWP1) promoter, which controlled the transcription of inserts of interest. For the experiments described, giardial RabA preceded by a *Hemophilus influenza* hemagglutinin (HA) tag or the snap22 gene, or snap22 gene preceded by the simian virus 40 (SV40) nuclear localization signal (NLS), respectively, were cloned in the inducible expression site. Flanking regions included the CWP1 3' untranslated region (UTR) at the 3' end of the cassette.

(4). Parasites were harvested after induction by chilling the cells on ice for 30 min, followed by centrifugation at $800\times g$, and then washed with ice-cold phosphate-buffered saline (PBS). For SNAP-tag labeling, the cells were incubated in $5\text{ }\mu\text{M}$ O^6 -benzyl guanine diacetyl fluorescein (BGAF; kind gift of Kai Johnsson, Swiss Federal Institute of Technology Lausanne, EPFL, Lausanne, Switzerland) in growth medium at 37°C for 30 min,

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 2A), whereas wild-type cells stained with BGAF showed no signal (Figure

2B), demonstrating specific labeling and the expected nuclear localization of the reporter protein. The viability and morphology of cells treated with BGAF was not changed compared to wild-type cells in any observable way. To 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 resulted in a fluorescent signal at the nuclear envelopes but not in the nucleoplasm when immunostained with anti-HA-fluorescein isothiocyanate (FITC) conjugate (our unpublished results and Figure 2C). 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 min at room temperature, followed by a 5-min incubation with 0.1 M glycine in PBS. Fixed cells were permeabilized with 0.2% Triton[®] X-100 in PBS for 20 min and blocked for $>2\text{ h}$ in 2% bovine serum albumin (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[®] (Vector Laboratories, Burlingame, CA, USA) containing 4',6-diamidino-2-phenylindole (DAPI). 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 and 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 (Figure 2, A and D). This was probably due to the presence of reporter protein in the cytoplasm of living cells, since wild-

Figure 2. Confocal microscopy of fluorescently labeled *Giardia*. (A) Live cells expressing nuclear localization signal (NLS)-snap22 stained with O^6 -benzyl guanine diacetyl fluorescein (BGAF) show a staining typical for the nucleoplasm of the two nuclei of *Giardia*. (B) Wild-type cells processed identically show no fluorescence. Inset, a differential interference contrast (DIC) image showing the cells present in the field of view. (C) Cells expressing the *Hemophilus influenza* hemagglutinin (HA)-tagged RabA construct were fixed and labeled with a monoclonal anti-HA antibody conjugated to fluorescein isothiocyanate (FITC), and nuclear DNA was stained with 4',6-diamidino-2-phenylindole (DAPI). The image shows signal from HA (green) surrounding the two nuclei (blue, see inset on upper right, HA + DAPI). A merged fluorescent signal and DIC image are shown in the lower left inset. (D) Live cells expressing the snap22-RabA construct labeled with BGAF show a fluorescent signal similar to panel 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. Inset, DIC image. (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 min demonstrate excellent photostability of the fluorophore. Scale bars for panels A, C, and insets in C, 5 μm . Scale bars for panels B, D, and inset in D, 10 μm .

Table 1. Primers and Oligonucleotides Used To Create Expression Constructs

Name	Sequence
tandemHA-s	5'-TGGCTACCCATACGATGTCCCAGACTACGCCGGCTA CCCATACGATGTCCCAGACTACGCCGGCA-3'
tandemHA-as	5'- GATCT GCCGGCGTAGTCTGGGACATCGTATGGGTAGC CGGCGTAGTCTGGGACATCGTATGGGTAGCCAT GCA -3'
SV40NLS-Nsi-s	5'-TAAGAAGAAGCGCAAGGTGA-3'
SV40NLS-Bgl-as	5'- GATCT CACCTTGCCTTCTTCTT GCA -3'
snap22-Bgl-s	5'-GAAGATCTAAGGATTGTGAAATGAAAC-3'
snap22-Bgl-as	5'-CATAGATCTTCCCAAGCCTGGCTTCCCCAAC-3'
RabA-s	5'-CATAGATCTATGCGGTACAAAGTCAGTG-3'
RabA-as	5'-CCTTAATTAACAGTAGCACCCTGAAG-3'

Overhangs appropriate for ligation with restriction enzyme-digested DNA are bolded.

type cells exposed to BGAF showed no fluorescent background after washing (Figure 2B). Moreover, in the fixed and detergent-permeabilized cells used for conventional immunofluorescence assay (IFA) with the anti-HA antibody, much of this cytoplasmic signal was lost because no cross-linking agent was used in the fixation protocol, and the background therefore appears lower (Figure 2, compare A and D with C)

The fluorescence emitted by live cells expressing either the NLS-snap22 or snap22-RabA proteins labeled with BGAF disappeared 5–6 s after the start of observation because of photobleaching. This was insufficient for time-lapse analysis or fluorescence resonance energy transfer (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 Biosciences AG; 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. Although we observed a lower overall labeling intensity compared to BGAF, possibly due to decreased membrane permeability, both fluorophores interacted specifically with the reporter and tolerated extended excitation without bleaching (Figure 2E). Labeled and washed cells were continuously scanned at a rate

of 6 images/min (including $2 \times$ line-averaging) for 25 min. Only a very minor loss of signal was observed for both fluorophores during the procedure. The representative images in Figure 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 localize 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.

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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 have no financial interest and are in no way affiliated with Covalys Biosciences AG, and no patent applications on the authors' side (pending and actual) exist for any of the results obtained in this work. The work presented here is eligible for the SNAP-tag award 2005, under the conditions stated by the organizers (www.covalys.com/downloads/cms/snap-tag_award_2005.pdf).

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