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New fluorescence markers to distinguish co-infecting *Trypanosoma brucei* strains in experimental multiple infections

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Abstract

Multiple-genotype infections are increasingly recognized as important factors in disease evolution, parasite transmission dynamics, and the evolution of drug resistance. However, the distinction of co-infecting parasite genotypes and the tracking of their dynamics have been difficult with traditional methods based on various genotyping techniques, leaving most questions unaddressed. Here we report new fluorescence markers of various colours that are inserted into the genome of *Trypanosoma brucei* to phenotypically label live parasites of all life cycle stages. If different parasite strains are labelled with different colours they can be easily distinguished from each other in experimental studies. A total of 10 *T. brucei* strains were successfully transfected with different fluorescence markers and were monitored in culture, tsetse flies and mice, to demonstrate stability of marker expression. The use of fluorescence activated cell sorting (FACS) allowed rapid and accurate identification of parasite strains labelled with different markers. Cell counts by FACS were virtually identical to counts by traditional microscopy ($n = 75$, Spearman's ρ: 0.91, $p < 0.0001$) but were considerably faster and had a significantly lower sampling error (66% lower, d.f. = 73, $t = -17.1$, $p < 0.0001$). Co-infecting strains transfected with fluorescence genes of different colour were easily distinguished by eye and their relative and absolute densities were reliably counted by FACS in experimental multiple infections in mice. Since the FACS can simultaneously determine the population sizes of differently labelled *T. brucei* strains or subspecies it allows detailed and efficient tracking of multiple-genotype infections within a single host or vector individual, enabling more powerful studies on parasite dynamics. In addition, it also provides a simple way to separate genotypes after experimental mixed infections, to measure responses of the single strains to an applied treatment, thus eliminating the need for laborious cloning steps. The markers presented broaden the spectrum of tools available for experimental studies on multiple-genotype infections. They are fundamentally different from isoenzyme analysis and other genotyping approaches in that they allow the distinction of parasite genotypes based on an easily recognizable phenotypic trait. They will be of specific interest to researches addressing ecological, evolutionary and epidemiological questions using trypanosomes as an experimental system.

Keywords: *Trypanosoma brucei*; Strain distinction; Multiple genotype infection; Co-infection; Fluorescence; FACS

1. Introduction

Trypanosoma brucei, a protozoan parasite transmitted by the bite of the tsetse fly (*Glossina* spp.), is of great medical and economic interest in Africa. It causes

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human sleeping sickness, which has seen a dramatic comeback over the last decade (Van Nieuwenhove et al., 2001) and Nagana, a cattle disease that prevents cattle farming and the use of work animals over huge areas of sub-Saharan Africa (Mulligan and Potts, 1970), thus profoundly affecting the economics of the entire continent. To understand the transmission and spread of the disease, the evolution of its virulence, and its dynamics within individual hosts, we need to be able to distinguish genetic strains of the parasite and accurately estimate their densities in living hosts in the laboratory. Distinguishing multiple genotypes in mixed infections has become particularly important, as multiple-genotype infections are increasingly recognized as important factors in disease evolution (Ebert and Mangin, 1997; Frank, 1996; Read and Taylor, 2001), the evolution of drug resistance (de Roode et al., 2004) and transmission dynamics (Gupta et al., 1994; Hudson et al., 2002; Plebanski et al., 1999). In *T. brucei*, multiple-genotype infections are especially relevant in the tsetse fly, for parasite recombination can occur only in tsetse salivary glands (Gibson and Bailey, 1994; Gibson et al., 1997; Jenni et al., 1986). Multiple-strain infections of *T. brucei* are reported to be common in tsetse flies (MacLeod et al., 1999) and may be a factor in the spread of the *SRA* gene responsible for human infectivity of East African *T. brucei rhodesiense* (Xong et al., 1998).

So far, mainly two approaches have been used to make inferences about relative frequencies of genotypes in mixed trypanosome infections: in most cases parasitaemias were recorded only as a total number for all genotypes, and then a PCR on a variable region, isoenzyme analysis or an immuno-fluorescence assay was performed to determine what genotypes were still present (e.g. Butikofer et al., 2002; Turner et al., 1991). Alternatively, to further determine the relative proportions of genotypes, for each investigated sample many clonal populations had to be grown up from isolated single parasites and their genotypes analyzed by PCR. However, this approach is extremely tedious and thus limits either the number of host individuals that can be followed in an experiment – limiting statistical power – or the frequency of replication per host—limiting the accuracy of measurement. Both approaches require some sort of genotyping of the parasites. Immunofluorescence is another method that could be applied to distinguish different strains but it has the disadvantage that it is not stable, i.e. strains have to be labelled repeatedly. Furthermore, to study multiple infections, it would need to be applied to parasites that are already mixed and we are not aware of a broad range of immuno-fluorescence markers that would allow the strain-specific marking of different parasites in a mix of strains.

To improve on these methodological limitations, our aim was to construct markers that would allow us to visually, i.e. phenotypically, distinguish and quantify multiple co-infecting *T. brucei* strains both in the mammalian host and in the insect vector. We therefore constructed genetic markers that are stably inserted into the parasite genome to label live trypanosomes in all life-stages with fluorescence of four different colours. Such markers enable us to distinguish different strains or subspecies (e.g. *T. b. brucei* versus *T. b. rhodesiense*) in multiple infections if the co-infecting genotypes are labelled with different colours. Live fluorescence markers are fundamentally different from isoenzyme analysis and other genotyping approaches in that they allow parasite distinction based on phenotype rather than genotype and thus allow rapid and accurate determination of relative frequencies of co-infecting strains. Similar markers have been used before to address cell biological questions in *T. brucei* (e.g. Biebinger et al., 1996; Bingle et al., 2001; Sheader et al., 2004) or to investigate procyclic parasites in the tsetse fly (Gibson and Bailey, 2003) but they have not been applied for population biological studies of multiple infections. Traditional genotyping approaches remain important because unlike fluorescence markers inserted into the parasite genome they can be applied to unknown field samples. We tested if our markers could be integrated into several different *T. brucei* strains and if fluorescence expression was stable in all life-stages. We also verified that a fluorescence activated cell sorter (FACS) can be used to automate parasitaemia counts and that the FACS accurately distinguishes between different co-infecting strains if they have strain-specific labelling. This would allow us to efficiently follow mixed populations of several parasite strains through all life-stages and to physically separate live parasites of different strains from mixed infections.

2. Materials and methods

2.1. Fluorescent protein fusion constructs

We made genetic constructs that allow the insertion of fluorescent protein genes of several colours into the genome of *T. brucei* to express fluorescence in the parasite's cytoplasm. The coding sequences of EGFP, ECFP, EYFP (green, blue and yellow fluorescent protein genes, respectively, BD Biosciences Clontech) and mRFP1 (red, Campbell et al., 2002) were cloned into the *Hind*III/*Bam*HI sites in the expression vector pHD1034, which contains a puromycin resistance gene

and a ribosomal RNA promoter for constitutive expression during all parasite life-stages (Quijada et al., 2002). The pHD1034 construct is inserted into the ribosomal spacer of *T. brucei*. To increase the labelling variety, we also made recombinant-fusions that target to individual organelles. Specifically, DsRed.T3 (Bevis and Glick, 2002) was fused to the N-terminus of the paraflagellar rod protein A in a pXS2 vector (Bangs et al., 1996). The resulting fluorescence is therefore restricted to the trypanosome's flagellum. Gene expression in this construct is under control of the PARP promoter and therefore it is expressed only in the procyclic (tsetse fly midgut) life-stage of *T. brucei*. The pXS2 construct is inserted into the tubulin intragenic region of *T. brucei*. We thus have four constructs (green, red, blue, yellow) that label the cytoplasm of *T. brucei* in all live-stages and one construct that labels only the flagellum of procyclic parasites red. All constructs are available from the authors.

For stable transfection (i.e. insertion of the construct into the *T. brucei* genome), constructs were linearized using restriction enzymes *Not*1 or *Mlu*1 (New England Biolabs) for the pHD1034 or the pXS2 vectors, respectively. Parasites were transfected using standard protocols (Beverley and Clayton, 1993). Eight hours after transfection, the selecting drug (1 μg/ml Puromycin (Sigma) for pHD1034, and 2.5 μg/ml Geneticin G418 (Neomycin analogue, Invitrogen) for pXS2) was added to select parasites that had incorporated the construct into their genome. Parasites were selected for 10 days, at which point no more non-fluorescent parasites were detectable. Bloodstream form parasites were maintained at 37 °C and 5% CO_2 in minimal essential medium (MEM, Gibco) supplemented with 15% inactivated horse serum, MEM non-essential amino acids (Gibco), 21 mM HEPES, 4.6 mM D-(+)-glucose, 22 mM $NaHCO_3$, 0.014% 2-mercaptoethanol, 0.1 mM hypoxanthine, 2 mM sodium pyruvate, and 16 μM thymidine, pH 7.4. Procyclic parasites were maintained at 26 °C and ambient CO_2 in Beck's medium (Hyclone) with 15% fetal bovine serum and 1× PenStrep (Sigma). Both media were supplemented with the respective selecting drug.

2.2. Stability of fluorescence and automated trypanosome counts

We attempted transfection in bloodstream and procyclic forms of various *T. b. brucei*, *T. b. rhodesiense*, and *T. b. gambiense* strains. The stability of expression of fluorescent protein genes successfully inserted into the *T. brucei* genome was assessed in parasites in culture, in the tsetse fly vector, and in mice (pHD1034-based constructs only) using standard fluorescence microscopy. To test the stability of fluorescence expression in culture, drug (puromycin/neomycin) addition was stopped and parasites maintained in culture medium without selecting drugs. Stability of expression in tsetse and mice was assessed by infecting teneral tsetse (*Glossina m. morsitans*, S. Aksoy laboratory colony, Yale University, USA) and 3–4-week-old female NMRI mice (*Mus musculus*, RCC Ltd., Itingen, Switzerland) and checking for non-fluorescent parasites for up to 8 weeks (tsetse midgut and salivary gland infections) and 10 weeks (mice), respectively. Twenty-six flies were infected with *T. b. brucei* strain RUMP503 transfected with construct pHD1034-R1 and 40 flies were infected with both RUMP501 transfected with construct pHD1034-G1 and RUMP503 transfected with pXS2-RF1. To infect tsetse flies, we washed procyclic parasites in PBS and added 10^6 parasites/ml to 37 °C defibrinated cow blood (Rockland Immunochemicals Inc., Gilbertsville, PA, USA) that was then fed to the tsetse. Care was taken to keep the time between parasite addition and blood meal as short as possible to minimize parasite mortality. To infect mice, we washed and resuspended bloodstream form parasites in PBS and injected between 10^3 and 10^7 parasites in 250 μl total volume i.p. While establishing the growth characteristics of the newly transfected strains, 36 mice were singly infected with all fluorescent strains available in bloodstream form (Table 1). The constructs containing ECFP and EYFP were only tested in culture using transient transfection. Except for the colour of their fluorescence they are identical to the other pHD1034-based constructs and should behave the same when integrated into chromosomes for stable expression.

To efficiently count parasites from mouse blood we used a Becton Dickinson FACScan Flow Cytometer (FACS). The standard for counting trypanosome parasitaemias is microscopic counting in Neubauer-improved counting chambers. We therefore tested the reliability of the FACS counts by comparing them to counts of the same samples with a Neubauer-improved counting chamber (Paul Marienfeld GmbH & Co. KG). To obtain the parasitaemia of one sample with the FACS, we diluted 5 μl of sample in 0.995 ml of Becton Dickinson FACS Flow (sheath fluid) and measured 10 times for 30 s each. A fixed volume of fluid (6 μl) is analyzed by the FACS in 30 s under the machine setting used ('low pressure'). Therefore the number of parasite cells per ml in the original sample can be calculated from the FACS cell count. To increase accuracy, counts are calibrated by adding a known quantity of fluorescent beads to the samples. The average of the 10 consecutive counts was taken as the FACS parasitaemia. To obtain the parasitaemia of

Table 1
Trypanosoma brucei strains successfully transfected with pHD1034-based constructs for cytoplasmic fluorescence expression in four colours in all life stages, and with pXS2-RF1 for procyclic fluorescence expression in the paraflagellar rod

Species	Strain[a]	Fluorescence construct				
		pHD1034-G1	...-R1	...-C1	...-Y1	pXS2-RF1
T. b. brucei	STIB246BA[b,c]	Yes	Yes	–	–	–
T. b. brucei	STIB247MCM[b]	Failed	Yes	–	–	–
T. b. brucei	STIB345AA[b]	Yes	Failed	–	–	–
T. b. brucei	STIB763B[b]	Yes	Yes	–	–	–
T. b. brucei	STIB777AE[b]	Yes	Yes	–	–	–
T. b. brucei	STIB920[b,c]	Yes	Yes	–	–	–
T. b. rhodesiense	STIB900[b]	Yes	Yes	–	–	–
T. b. brucei	RUMP501[d]	Yes	–	Yes[e]	–	Yes
T. b. brucei	RUMP503[d]	Yes	Yes[e]	–	Yes[e]	–
T. b. rhodesiense	Ytat1.1[d]	Yes	–	Yes	Yes[e]	Yes

'Yes' indicates successful transfection, 'failed' indicates unsuccessful attempts, and '–' indicates not attempted. Transfections of three *T. b. gambiense* strains were attempted but failed. Constructs are described in the text.
[a] STIB strains from R. Brun, Swiss Tropical Institute, others from S. Aksoy, Yale University, USA.
[b] Bloodstream form parasites.
[c] STIB246BA and STIB920 derive from the same original field isolate.
[d] Procyclic parasites.
[e] Transient transfection only (insertion of plasmid, no integration into trypanosome chromosomes).

the same sample with the Neubauer-improved counting chamber, 10 subsamples were counted. For each subsample two opposite corner quadrants were counted out and their average calculated up to the parasitaemia per milliliter mouse blood. The average of the 10 subsamples was taken as the Neubauer parasitaemia. We compared the variance around the mean of the two methods as a measure of their respective sampling error and tested if they were significantly different by performing a *t*-test on the null hypothesis that the ratio of their standard deviations is 1. To measure how well the results of the two methods correspond we calculated the correlation between the two methods. Statistical analyses were performed in R version 2.01 (R Development Core Team, 2005).

2.3. Application to multiple infection experiments

The FACS distinguishes trypanosomes from blood cells based both on their fluorescence and on their size and shape. It can distinguish between different trypanosome strains or subspecies if they contain different-colour fluorescence. To test the applicability of our markers to distinguishing and following strains in multi-strain infections we infected 20 mice with different combinations of one green and a different red labelled *T. brucei* strain and analyzed their blood in the FACS. As a specific example demonstrating the applicability of the method to experimental studies we co-infected an additional group of four female NMRI mice with the green-labelled strain STIB777AE-G1 (inoculum 1.5×10^6 parasites) and the red-labelled strain STIB246BA-R1 (4.5×10^6 parasites) of *T. b. brucei*. Relative and absolute densities of the two strains were determined daily by FACS. For this trial, blood samples were diluted 1/1000 in PBS buffer and measured for 3 min in the low-pressure setting. The detection limit was thus 2.8×10^4 parasites/ml. If no parasites could be detected the parasitaemia was set at a nominal value of 5×10^3 cells/ml for graphing purposes.

3. Results

3.1. Stability of fluorescence expression

All constructs were successfully transfected into procyclic *T. brucei* and expressed the expected fluorescence. Constructs pHD1034-G1 and pHD1034-R1 were additionally transfected into bloodstream form parasites of several *T. brucei* strains. In total, we successfully transfected eight *T. b. brucei* and two *T. b. rhodesiense* strains in different life-stages (Table 1). Transfection failed in three *T. b. gambiense* strains. No transfection was attempted in other *Trypanosoma* species.

The strength of fluorescence varied between strains and was more variable in some strains than in others. We therefore cloned single parasite cells of the most variable strain (*T. b. brucei* STIB763B-G1) to see if the variability was due to a mixture of several populations with different fluorescence characteristics. The variability of fluorescence strength decreased only slightly in some cloned

lines and remained unchanged in the others. Because we were interested in maintaining maximal genetic diversity within strains we did not clone the other strains.

Once properly selected, transfected trypanosomes maintained fluorescence expression in culture, even in the absence of continued antibiotic selection. A loss of fluorescence was never observed. Parasites in tsetse flies were first checked 15 days post-infection and then at irregular intervals. Of the 66 infected tsetse flies, 21 developed midgut infections and 4 salivary gland infections. Strong fluorescence was observed in these parasites more than 8 weeks post-infection. Parasites containing construct pHD1034-G1 (green) or pHD1034-R1 (red) expressed fluorescence both in midguts and salivary glands. Parasites containing construct pXS2-RF1 expressed red fluorescence in midguts but never in salivary glands as expected because the pXS2 vector was constructed for expression in only the procyclic life-stage (Bangs et al., 1996). Infections in mice were checked for up to 10 weeks and passaged into new mice by syringe up to five times. Fluorescence expression remained stable throughout in all strains.

3.2. Automated trypanosome counts

The FACS was able to recognize and accurately count fluorescent trypanosomes in blood from infected mice. Repeated counts on the same samples revealed a significantly smaller measurement error around the mean (d.f. = 73, $t = -17.1$, $p < 0.0001$) for the FACS than for microscopic counting in a Neubauer-improved counting chamber for the counting methods described (ratio of $S.D._{FACS}/S.D._{Neubauer} = 0.34 \pm 0.33$ (mean $\pm$ 1 S.D.), median = 0.25, $n = 74$).

T. brucei parasitaemias determined by FACS matched parasitaemias determined by the Neubauer-improved counting chamber closely across the entire range of naturally occurring parasitaemias (correlation of FACS and Neubauer counts of samples containing green or red fluorescent trypanosomes: FACS parasitaemia = 1.01 × Neubauer parasitaemia + 37,1341, Spearman's ρ: 0.91, $p < 0.0001$, $n = 75$). The lowest parasitaemia detectable by FACS depends on the dilution of the original sample and the length of sampling. With a 1/200 dilution measured for one minute it is around 7.5×10^3 parasites/ml.

3.3. Application to multiple infection experiments

The FACS reliably distinguished between green and red fluorescent trypanosomes in mixed blood samples containing two parasite strains expressing fluorescence

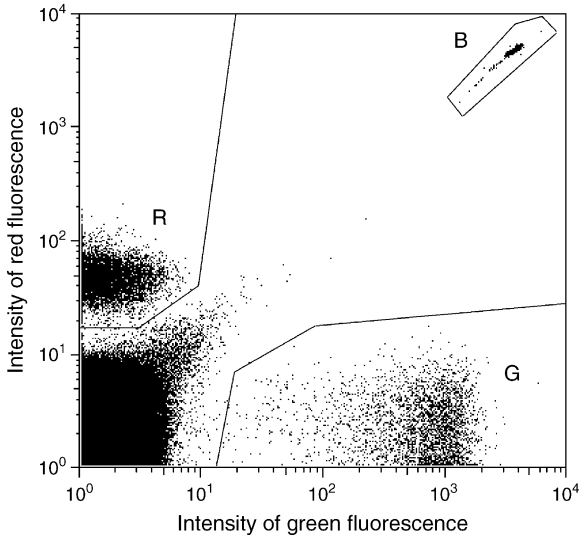

Fig. 1. Distinction of two co-infecting *T. brucei* strains in the fluorescence activated cell sorter (FACS). The plot depicts the intensity of green (*x*-axis) vs. red fluorescence (*y*-axis) of all particles measured by the FACS in a blood sample from a mouse infected with *Trypanosoma b. brucei* strains STIB777AE-G1 (green) and STIB246BA-R1 (red). Each dot represents one particle. Clearly recognizable are the well-separated populations of the two co-infecting parasite strains ('G' and 'R'), the fluorescent beads to calibrate the counts ('B') and all remaining non-fluorescent particles such as blood cells or medium particles (lower left corner). The polygons around the parasite and bead populations can be adjusted depending on the characteristics of the trypanosome strains and calibration beads used and define which particles the FACS counts as green or red trypanosomes or beads, respectively.

of different colour (Fig. 1). Detected green fluorescence was stronger than the red one because the FACS used had a 488 nm laser which is closer to the excitation optimum of the green fluorescent protein used (488 nm) than the red one (584 nm). The cells counted by the FACS could thus be unambiguously attributed to one of the two co-infecting strains based on the colour of their fluorescence. Daily absolute and relative parasitaemias could be tracked well as the example of four mice with a mixed infection of *T. b. brucei* strains STIB777AE-G1 and STIB246BA-R1 demonstrates (Fig. 2). Whereas traditional methods could only have established that the total parasitaemia was increasing throughout and that both strains were present until at least day 5 post-infection, the FACS measurements revealed a dramatic decline of strain STIB777AE-G1 after day 3 in all four mice.

4. Discussion

Fluorescence genes integrated into the parasite genome are commonly used to visualize protein

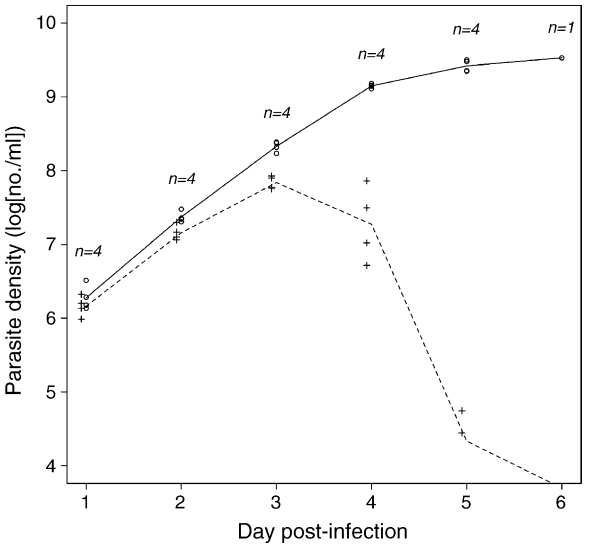

Fig. 2. Parasitaemia of two co-infecting *Trypanosoma b. brucei* strains STIB777AE-G1 ('+') and STIB246BA-R1 ('o') in four mice measured in a fluorescence activated cell sorter (FACS). There is a pair of '+' and 'o' for every mouse on each day. The lines represent the mean parasitaemia per strain. The density of STIB777AE-G1 fell below the detection threshold in one mouse on days 5 and 6 and was set to a nominal value of 5×10^3 cells/ml.

expression in live trypanosomes. They have been used for cell biological studies in *T. brucei* (e.g. Biebinger et al., 1996; Sheader et al., 2004) and have also been applied to document recombination events in *T. brucei* (Bingle et al., 2001). Their use for ecological and evolutionary studies of *T. brucei* populations has been very limited. The markers we present in this paper express fluorescence in all stages of the trypanosome life-cycle and are thus a powerful tool to study multiple parasite strains in culture, in the insect vector and in the mammal host. Once the constructs have been incorporated and all non-fluorescent parasites have been cleared, fluorescence markers are stably expressed and passed on to all following parasite generation. High-level expression of GFP has been shown to be non-toxic in many cell lines (Pines, 1995) and has no apparent detrimental fitness effects for the parasite (Bingle et al., 2001). The transfection itself can alter cell growth characteristics (own observations), probably because of the selection of the subpopulation of parasites that has incorporated the gene construct. However, the changes observed were much smaller than differences between different untransfected strains of *T. brucei* and are certainly smaller than between wild strains and the culture-adapted strains in most laboratories. Also, the direction of change varied, with some strains growing more vigorously, some less, and some remaining unchanged, indicating that the effect was probably not due to the introduced genes per se. But most importantly it has to be stressed that for many experimental questions (e.g. on the interaction of two transfected strains within the host) altered growth characteristics are unproblematic as long as the newly transfected strains are not directly compared to their untransfected progenitors. Of course, if the results from experiments with labelled parasites are to be compared to previous results with untransfected parasites of the same strain then care has to be taken that parasites still have the same growth characteristics.

A major benefit of the described markers is that they enable us to study multiple-genotype infections in hosts in much greater detail by allowing us to distinguish living parasites of different genotypes. This is significant because mixed infections are claimed to be important for the ecology and evolution of parasites on theoretical grounds (e.g. Frank, 1996; Hudson et al., 2002; Read and Taylor, 2001), yet little empirical support exists for this claim. This lack of data is largely due to the lack in most parasite systems of markers to distinguish different genotypes efficiently, i.e. based on their phenotype. Genotyping approaches such as isoenzyme analysis can also distinguish different strains but being based on PCR they are not able to attribute a genotype to single live parasites in samples and are thus unable to establish relative frequencies of different strains without recourse to tedious cloning steps.

A further significant aspect of the described live fluorescent markers is that they enable us to use fluorescence activated cell sorters (FACS) with two main benefits. First, they greatly increase the efficiency of tracking multiple strains because they automate counting and distinction of multiple trypanosome strains in samples. Our results show that compared to the traditional method using a counting chamber, the FACS can distinguish and count more accurately and has a 66% lower sampling error. It also reduces observer effects and is considerably faster, allowing larger sample sizes and increasing the statistical power of experiments. The second main benefit of the FACS is that it can physically separate live parasite cells of different strains or subspecies based on their fluorescence colour. This is a significant step forward compared with the parasite cloning that was previously necessary to reliably separate parasite genotypes. It yields much greater parasite numbers in much shorter time, which also reduces the danger of introducing selection bias.

The described fluorescent markers are also useful if only a fluorescent microscope but no FACS is available. They enable the researcher to determine relative frequencies of strains under the microscope very efficiently

because fluorescent parasites are very clearly visible even in very thick blood. It is in this context that markers labelling specific organelles, such as the described pXS2-RF1 construct, are interesting. Most simple fluorescent microscopes require to switch between two filters to see two different colours. If two markers of the same colour but expressed in different cell compartments as used, however, different strains can be distinguished with only one filter. So for studies in tsetse midguts and not taking advantage of the FACS, the pXSRF1 construct offers an alternative to the multi-colour labelling.

Fluorescent markers enable parasitologists to address novel ecological questions about trypanosomes in hosts and facilitate the use of trypanosomes as a model system for ecologists and evolutionary biologist interested in parasite population biology. They enable studies on strain-specific growth and on interactions between multiple trypanosome strains or between *T. b. brucei* and *T. b. rhodesiense* within a single host animal. While the presented markers also improve the counting of parasites in single infections, their real power lies in distinguishing different genotypes in experimental multiple infections. Here, they offer a vast improvement over traditional methods both in terms of efficiency and accuracy. In addition, the FACS opens the way for studies on single trypanosome strain evolution that require the physical separation of different parasite strains after experimental mixed infection treatments.

One hurdle still remains, though. Gene exchange between *T. brucei* strains occurs in the tsetse fly salivary glands (Jenni et al., 1986). Therefore the markers are not stable if multiple recombining strains are passed through a tsetse fly. This is, however, a potential problem for any marker based on single genes, and we see no easy solution for it.

In conclusion, the distinction of live parasites based on an easily recognized phenotypic trait and the physical separation of genotypes from mixed infections made possible by live fluorescence markers make trypanosomes an attractive system for experimental studies on parasite population dynamics. The marker described here will be especially important by opening new avenues of research on the ecology and evolution of mixed trypanosome infections and their implications for disease dynamics and control.

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