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Stable transformation of pleomorphic bloodstream form *Trypanosoma brucei*

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Abstract

African trypanosomes differentiate between various developmental stages both in mammalian hosts and their tsetse vector to adapt to and survive in the different environments they encounter. In the bloodstream, trypanosomes naturally exist as either proliferative slender-forms or non-proliferative stumpy-forms, the latter being responsible for both prolonged infection and transmission. However, most trypanosome studies are carried out on laboratory-adapted monomorphic cell lines, incapable of differentiating to stumpy-forms or completing the life cycle through the tsetse fly. Partly, this has been due to the inefficiency of transfection of pleomorphic strains which have retained the ability to generate stumpy-forms. Recently, Amaxa Nucleofector® technology was shown to increase transfection efficiency for monomorphic bloodstream forms. Using this technology we have optimised a similar method for pleomorphic bloodstream form transfection, generating transfection efficiencies of 10^{-7}–10^{-6}. This permits routine genetic manipulation of pleomorphic lines, which have the most biological relevance for trypanosomes in the field.

Keywords

Trypanosome; Transfection; Pleomorphic; Stumpy; *Trypanosoma brucei*

A wide range of genetic manipulations, including gene knock-outs, endogenous-tagging, inducible RNA interference and over-expression are all commonly used in the study of monomorphic bloodstream form and procyclic form *Trypanosoma brucei*, made possible by the ability to efficiently and reliably transfect these cells. Transfection of procyclic form trypanosomes has been carried out with high efficiency for many years [1], but, historically, bloodstream form trypanosomes have been more difficult to transfect. Recent advances have allowed a marked increase in transfection efficiency for monomorphic bloodstream forms, particularly through the use of the Amaxa Nucleofector® system which increases transfection efficiency 1000-fold compared to traditional electroporation methods [2]. The introduction of site-specific double strand breaks at targeted sites for vector integration also

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increases transfection efficiency a further 250-fold [3]. These developments have allowed the production of monomorphic bloodstream form RNAi libraries, which have been successfully utilised for whole-genome RNAi screens [4–6].

Laboratory-adapted monomorphic bloodstream forms are proliferative cells which grow uncontrolled *in vivo*, quickly overwhelming the host. Naturally occurring populations of bloodstream form trypanosomes, in contrast, are pleomorphic; that is they differentiate in a density-dependent manner from the proliferative slender form, through an intermediate stage, to the non-proliferative, transmission-competent stumpy form, limiting parasitaemia and prolonging infection [7,8]. Hence, the stumpy form is a key developmental life stage that plays an important role in disease transmission and within-host infection dynamics [9]. Although monomorphic cell lines are a vital resource for trypanosome research, their inability to generate stumpy forms *in vivo* limits their utility for the study of genes or pathways involved in infection dynamics, bloodstream form differentiation, or analyses where progression through the complete life cycle is desired. Conversely, pleomorphic cell lines have retained the ability to generate stumpy forms and so can be used for such analyses, yet these lines are less amenable to genetic manipulation. Indeed, the generation of transgenic pleomorphic bloodstream forms, although possible [1], is still considered problematic. In consequence, even common genetic approaches carried out in monomorphic cell lines, such as RNAi, are rarely carried out in pleomorphic cell lines. Based on the success of the Amaxa Nucleofector® system for the transfection of monomorphic bloodstream forms [2] we report here an optimised method for the transfection of pleomorphic cell lines. This method is used routinely in our laboratory for the stable transfection of pleomorphic bloodstream form trypanosomes.

The pleomorphic cell line *T. brucei brucei* AnTat1.1 90:13 [10], (containing pLew90 and pLew13 [10,11]) has proven particularly amenable to transfection, likely due to a degree of culture adaptation in this cell line. Nonetheless, these cells differentiate to stumpy forms *in vivo*: stumpy forms are visible from day 4–6 post infection and the parasitaemia plateaus around day 5–7 post infection whereupon the population comprises >80% morphological stumpy forms. The AnTat1.1 90:13 cell line also grows well *in vitro* when maintained at low parasite density (below 10^6 cells/ml) and passaged regularly (at least every 2 days) in HMI-9 [12] supplemented with 20% FCS. Although it is possible to transfect such pleomorphic slender grown *in vitro* that maintain pleomorphism, we found that it is preferable to harvest slender form cells from an *in vivo* infection, this providing the most healthy starting population for transfection and thereby increasing the likelihood of successfully isolating transfectants. Their limited passage history *in vitro* could also reduce the risk of a selection for monomorphism. Importantly, the cells were harvested while the population was overwhelmingly slender in morphology (<1×10^8 parasites/ml of blood) since intermediate and stumpy cells are committed to irreversible cell cycle arrest and therefore would not proliferate *in vitro*. Approximately 1–1.5 ml of blood was harvested using 200 μl of 2% citrate as an anticoagulant from an infected mouse by cardiac puncture. The blood was maintained at 37 °C throughout isolation and was then added as promptly as possible to 25 ml HMI-9 (20% FCS; 100 U/ml penicillin and streptomycin) in vented tissue culture flasks which had been pre-warmed and pre-equilibrated at 37 °C with 5% CO_2. To separate the

trypanosomes from the mouse blood cells, the flask was positioned upright in the incubator allowing the blood cells to settle to the bottom of the flask. After 2–3 h the supernatant was carefully pipetted (avoiding clumps of red blood cells) into a fresh flask containing pre-warmed and pre-equilibrated HMI-9 to produce a final volume of 60 ml. Further settling of the remaining blood cells was then allowed to occur overnight and the following day the trypanosome containing medium was again transferred to a fresh flask avoiding settled blood cells. The resulting medium was enriched for trypanosome cells but not completely free of blood cells such that the trypanosome density needed to be determined *via* a haemocytometer rather than, for example, a particle counter. The quantity of trypanosomes generated by this method varied due to variation in the initial parasitaemia and the volume of blood isolated but was typically in the order of $2–4 \times 10^7$ cells per mouse.

For the transfection (Fig. 1A), cells were pelleted at 800 g for 8 min in a clinical centrifuge and the supernatant removed. The cell pellet was then resuspended in 100 μl Amaxa transfection buffer (basic parasite nucleofector solution 2) with 10 μg linearised DNA in 5 μl 1 mM Tris-HCl, pH8, 0.1 mM EDTA. The trypanosomes were then transferred to an Amaxa cuvette and transformed in an Amaxa Nucleofector® II using the CD4+ T cells X-001 program. Following transfection, the cells were immediately transferred into 25 ml of pre-warmed and pre-equilibrated HMI-9 and incubated for 6 h. After 6 h, selective drug (0.5 μg/ml puromycin; 10 μg/ml blasticidin; 1.5–3 μg/ml phleomycin, where effective doses required titration) was added and cells were transferred into 24 well plates, diluted 1:2, 1:5, 1:25 and 1:125 using HMI9. Approximately 5–8 days post-transfection resistant clones became detectable and were transferred to fresh selective media with care being taken to avoid cell density from exceeding 10^6 cells/ml. After expansion *in vitro*, transfectant clones were cryopreserved in HMI-9 with 7–10% glycerol and genomic DNA harvested, if required. Additionally, to generate blood stocks of the resulting transfectants, cells were infected into a mouse and blood stabilates prepared after harvest when the parasitaemia was slender in morphology.

To quantify the transfection efficiency obtained using this method, replicate stable transfections of AnTat1.1 90:13 pleomorph cells [10] were carried out using the ‘empty’ pALC14 RNAi vector (a modified version of pZJM, created from pLew100, which is targeted to insert between ribosomal RNA genes [11,13,14]). Using cells harvested from three independent *in vivo* infections (as described above) or from three independent cultures (after 7 days growth *in vitro*) we obtained transfection efficiencies with 10 μg of DNA of $1.5 \times 10^{-7}–1.4 \times 10^{-6}$ and $1.7 \times 10^{-7}–2.7 \times 10^{-6}$, respectively, such that multiple independent transfectant lines were isolated for each transfection (Fig. 1B). In our laboratory over 30 independently transfected lines have been generated by this approach and in no case has a loss of pleomorphism been observed. Moreover, *T. brucei brucei* AnTat.1.1 cells as well as *T. brucei brucei* AnTat1.1 90:13 cells have been successfully transfected.

This method enables the routine stable transformation of pleomorphic bloodstream form *T. brucei* at efficiencies in the order of $\sim 10^{-7}–10^{-6}$. This makes the genetic manipulation of differentiation competent pleomorphic trypanosome lines readily achievable using simple methodology and materials and equipment available in most trypanosome research laboratories. This facilitates medium-throughput gene function analyses for trypanosome

lines most relevant to parasites in the field, enabling phenotypic analysis throughout the life cycle of the parasite.

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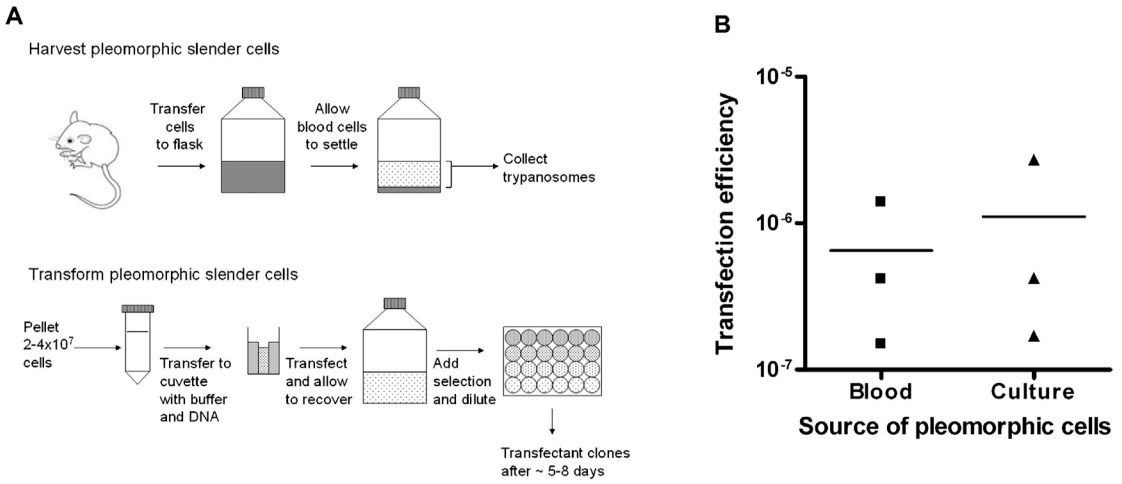

Fig. 1.

Transfection of pleomorphic transfections from cells harvested from blood or from culture. (A) Diagram outlining the method for the harvest and transfection of pleomorphic slender forms (details described in main text). (B) Pleomorphic bloodstream form AnTat1.1 90:13 cells were harvested from mouse blood 3 days post infection or after 7 days *in vitro*. Between 2.8 and 4.06 × 10^7 cells were transfected with 10 μg of pALC14 vector in the "Amaxa basic parasite nucleofector solution 2" transfection buffer and selected in 24 well plates at 1:2, 1:5, 1:25 and 1:125 dilutions with 0.5 μg/ml puromycin. Transfection efficiencies were calculated from the number of positive wells per dilution (excluding any dilution where >50% of wells were positive) and extrapolating for the total number of cells transfected. Replicates were carried out from three independent mouse infections and three independent *in vitro* cultures.

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