Cell-cycle synchronisation of bloodstream forms of Trypanosoma brucei using Vybrant DyeCycle Violet-based sorting

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Cell-cycle synchronisation of bloodstream forms of *Trypanosoma brucei* using Vybrant DyeCycle Violet-based sorting

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Abstract

Studies on the cell-cycle of *Trypanosoma brucei* have revealed several unusual characteristics that differ from the model eukaryotic organisms. However, the inability to isolate homogenous populations of parasites in distinct cell-cycle stages has limited the analysis of trypanosome cell division and complicated the understanding of mutant phenotypes with possible impact on cell-cycle related events. Although hydroxyurea-induced cell-cycle arrest in procyclic and bloodstream forms has been applied recently with success, such block-release protocols can complicate the analysis of cell-cycle regulated events and have the potential to disrupt important cell-cycle checkpoints. An alternative approach based on flow cytometry of parasites stained with Vybrant DyeCycle Orange circumvents this problem, but is restricted to procyclic form parasites. Here, we apply Vybrant Dyecycle Violet staining coupled with flow cytometry to effectively select different cell-cycle stages of bloodstream form trypanosomes. Moreover, the sorted parasites remain viable, although synchrony is rapidly lost. This method enables cell-cycle enrichment of populations of trypanosomes in their mammal infective stage, particularly at the G1 phase.

Keywords

Trypanosoma brucei; Cell-cycle; Vybrant DyeCycle Violet; Flow cytometry; Synchronisation

1. Introduction

Isolating cells within a particular stage of the cell-cycle offers opportunities for the analysis of periodic cytological and molecular events during cell division. In kinetoplastids, cell-cycle control is atypical with respect to the model eukaryotic organisms used for studying cell-cycle events [1]. In particular, replication and segregation of the genome of the single mitochondrion, the kinetoplast, is co-ordinated with replication and segregation of the nuclear genome [2,3]. Further, cytokinesis of this group of organisms can be uncoupled from successful mitosis, such that anucleate cytoplasts (zoids) or cells with an atypical number or configuration of nuclei and kinetoplasts can be generated under particular drug regimens [4,5] or upon the perturbed expression of a variety of genes directly or indirectly linked to cell-cycle control (e.g. [6-8]). Although several methods for induction of cell-cycle synchronisation in kinetoplastids have been reported [9-12], historically attempts to

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synchronise *Trypanosoma brucei* have usually been unsuccessful or difficult to reproduce. This has changed recently, however, with the development of methods to either induce reversible cell-cycle arrest by hydroxyurea treatment [13,14], or to selectively enrich different cell-cycle stages utilising vital DNA dyes, such as Vybrant DyeCycle Orange in conjunction with flow cytometry [3]. Each of these approaches, however, has some limitations. For example, hydroxyurea-induced synchronisation (and other chemically induced arrest protocols) have the potential to perturb underlying cell-cycle controls through the inappropriate activation of cell-cycle checkpoints or via block-release effects on cell growth and cell age [15,16]. In contrast, selective sorting protocols, which are less likely to be subject to these issues, have been confined to procyclic forms and do not allow the efficient selection of distinct bloodstream form cell-cycle stages [3,17]. The method reported here allows the effective isolation of enriched G1 and post-DNA-replication cell-cycle stages of bloodstream forms of *Trypanosoma brucei* using Vybrant DyeCycle Violet staining and flow cytometry, and the subsequent recovery of viable sorted cells that are able to progress back into a proliferative cell-cycle.

Bloodstream form Lister 427 *T. brucei* were grown in 5% CO_2 at 37 °C in HMI-9 medium [18] containing 20% foetal calf serum (FCS) at 37 °C and harvested during exponential growth by centrifugation for 10 min in a clinical centrifuge (930 × g) at room temperature. The parasite pellet was then resuspended at a concentration of 1×10^6 cells/ml in HMI9 medium supplemented with 2% FCS and 10 μg/ml penicillin/streptomycin. Vybrant DyeCycle Violet (Molecular Probes, Invitrogen V35003) was added to a final concentration of 1 μg/ml and the cell suspension incubated for 30 min at 37 °C, the tube being protected from light by wrapping in aluminium foil. The samples were then centrifuged and resuspended back into 3 ml of staining media prior to sorting on a FACSAria (BD Biosciences). PSG (488.8 mg/l NaH_2PO4, 2.55 g/l NaCl, 8.08 g/l Na_2HPO_4, 15 g/l D-glucose, pH 7.8) was used as the sheath fluid in order to minimise cell stress, and the FACS machine cooled to 4 °C, such that when the stained sample and collection tubes were placed into the machine, the samples would have been cooled to below ~20 °C to limit cell metabolic activity. This cooling also served to limit the post-sort effects of Vybrant DyeCycle Violet on cell proliferation (our unpublished observations). Intact cells were gated based on FSC/SSC profiles, combined with pulse processing for doublet discrimination, in order to select single cells (Fig. 1A). The dye was excited using a 407 nm Violet laser and emission detected via a 450/40 bandpass filter. Gates were set up to collect only the 2C fraction (G0/G1 cells) and 4C fraction (G2, mitotic and post-mitotic cells), with the 2C gate set to include 1 quartile either side of the G0/G1 peak and the 4C gate set to exclude the first quartile of the G2/M peak, to ensure efficient discrimination and selection of these cell-cycle stages. In all cases, cells were sorted under conditions of minimal ambient light to prevent photoinactivation of the Vybrant DyeCycle Violet. During sorting, cells were collected into 100% FCS and maintained below 20 °C until the overall sort for all cells was completed (this taking approximately 60–90 min). Analysis of sorted cell numbers revealed that around half of the initial cell population was recovered (the typical recovery was 20 million cells), with approximately 70% of these comprising the 2C (G0/G1) gated fraction and 30% comprising cells with 4C DNA content (G2, mitotic and post-mitotic cells). These sorted populations were concentrated by centrifugation at 930 × g in a clinical centrifuge, PSG removed and the cells resuspended in HMI9 media supplemented with 40% FCS and 10 μg/ml penicillin/streptomycin. Post-sort analysis demonstrated the existence of distinct peaks corresponding to the DNA content of the selected cell sub-populations, although there was a significant reduction in overall fluorescence when compared to the pre-sorted population probably due to dye leaching or photobleaching (Fig. 1B). The pre-sorted and post-sorted populations were also assayed microscopically by preparing air-dried smears of concentrated cell samples, fixing these in methanol at −20 °C and analysing the configuration of kinetoplasts and nuclei after staining with 4′, 6-diamidino-2-phenylindole

(DAPI). The sorted populations were scored for the proportion of cells in the configurations 1 kinetoplast 1 nucleus (1K1N), 2K1N and 2K2N. The G0/G1 sort population consisted of 99% (range 98–100%) of cells with 1K1N configuration, whereas the G2/M population contained an enrichment of 2K 1N cells (χ = 36%; range = 18–48%) and 2K2N cells (χ = 13%; range 4–26%) (Fig. 1C and D). As the majority (~80%) of cells in an asynchronous culture were 1K1N it was possible to be quite discerning when establishing the gates for isolating the G0/G1 fraction and thus recoup a very pure population at reasonable yield. The G2/M peak, in contrast was somewhat broader generating a distinct emphasis toward 2K1N or 2K2N populations in different experiments depending on the gates applied.

Vybrant DyeCycle Violet staining and sorting of mammalian cells can result in delayed re-entry into a proliferative cell-cycle, whereas the post-sort viability of Vybrant DyeCycle Orange stained procyclic trypanosomes has not been reported [3,14]. Therefore, the progression of Vybrant DyeCycle Violet stained trypanosome populations was followed post-sorting for 7–17 h, the cells being assayed for the proportion of distinct cell-cycle stages by DAPI staining of methanol fixed cell samples. Analysis of the G1 population post-sort revealed a reappearance of 2K1N and 2K2N cells after 5h (Fig. 2A) whereas the G2/M sort contained 36% 2K1N cells with only 9% showing 2K2N immediately post-sorting (Fig. 1C), yet after 2 h the proportion of cells with a 2K2N configuration had risen to over 20%, there being a concomitant drop in the proportion of 1K1N cells from 55% to 38% of the total population (Fig. 2B). This suggests that many of the 1K1N cells detected in the G2/M sort are in late S-phase (but before kinetoplast segregation) and that the sorted cells progress from 1K1N and 2K1N configurations to generate an enriched post-mitotic population after 2 h and without significant post-sort delay. This observation was consistent between several independent assays, such that 2 h post-sort of G2/M samples represents the best time to isolated enriched 2K2N populations.

The sorted cell populations remained motile and viable and returned eventually to a proliferative cell-cycle. Hence, the G2/M sorted population returned to pre-sort ratios of different cell-cycle stages within 5 h of sorting, whereas the G1 sort returned to pre-sort ratios within 7 h, this apparent delay likely being due to the enhanced ability to capture 2C cells early in the cell-cycle and a possible delayed progression into S-phase induced by Vybrant Dye-Cycle Violet. Nonetheless, the synchrony of cells during post-sort recovery was less than with block-release protocols because sorting on the basis of DNA content selects populations from quite broad ranges of the cell-cycle (G0/G1, approximately 0.4 of one cell-cycle; G2/M, approximately 0.4 of one cell-cycle [2]). Moreover, although most cells appeared motile post-sort, the overall population growth over 24 h was significantly suppressed with respect to unsorted, unstained, populations, this being predominantly caused by exposure to the Vybrant DyeCycle Violet rather than low temperature during sorting (our unpublished observations). Hence, this protocol is well suited to generating enriched populations of distinct cell-cycle phases, but may have limitations in analysing post-sort cell-cycle events.

The method described uses Vybrant DyeCycle Violet to select enriched cell-cycle phases of bloodstream forms of *T. brucei* which remain motile and viable. The improved selection of bloodstream cells over previously published protocols used in procyclic forms is dependent on the use of Vybrant DyeCycle Violet rather than other Vybrant DyeCycle dyes (Prof. Michael Boshart and Markus Kador, LMU, Munich, Germany; personal communication). By selecting trypanosome populations enriched in distinct cell-cycle stages by selectional rather than chemically or genetically induced arrest, cell-cycle regulated events in trypanosomes can be studied under conditions where its underlying controls are not perturbed by block-release effects on cell-cycle control. Moreover, this methodology complements hydroxyurea-based protocols which, although able to select greater cell

numbers, are unable to select enriched G1 populations. Hence, processes that are cell-cycle regulated, or apparently linked to cell-cycle control such as differentiation processes, can be meaningfully dissected and interpreted.

Acknowledgments

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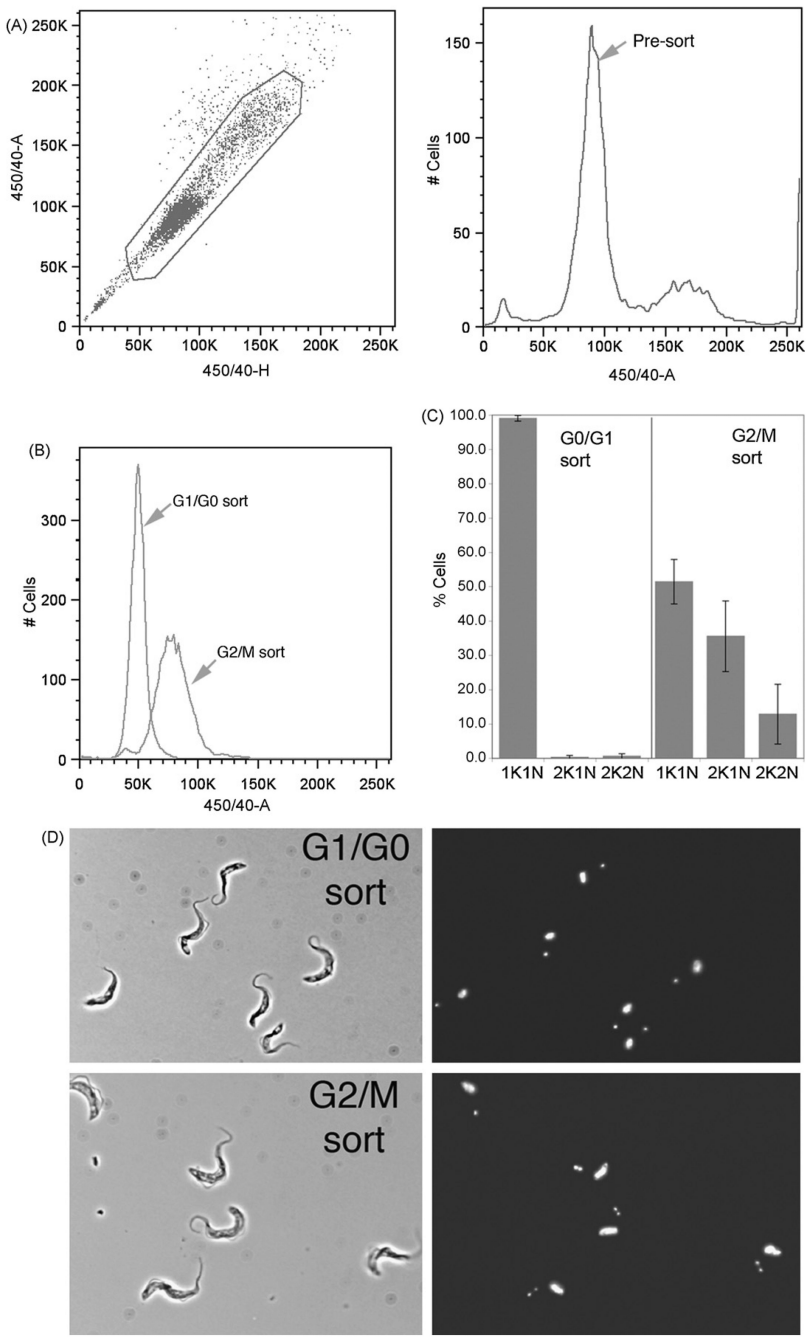

Fig. 1.

(A) Flow cytometry profile of Vybrant DyeCycle Violet stained bloodstream form *T. brucei*, the selected area showing those cells gated for sorting. The DNA staining profile of the population of Vybrant DyeCycle Violet stained bloodstream form *T. brucei* prior to sorting is shown in the right hand panel. (B) Post-sort flow cytometry profile of Vybrant DyeCycle Violet stained bloodstream form *T. brucei*. The G1/G0 and G2/M sorted cell populations are indicated. Note that the overall fluorescence intensity is less than in Panel A due to dye leaching post-sort. (C) The proportion of 1K1N, 2K1N and 2K2N cells in the G1/G0 post-sort population (left panel) and the G2/M post-sort population (right panel) determined by fluorescence microscopy of DAPI stained cells. The data represent analyses of 7

independent sort experiments, with >250 cells being analysed in each population. Error bars represent the standard deviation. (D) Cell images of the G1/G0 and G2/M post-sort populations. The left hand panels show phase contrast images of the sorted populations indicating their morphology and integrity. The right hand panel shows the same cell samples stained with DAPI to reveal the cell nucleus and kinetoplast(s) in each parasite. In both cases cells were air dried and fixed in methanol at −20 ° for at least 30 min, then rehydrated in PBS and stained for 1 min with DAPI, before washing in PBS and mounting in MOWIOL. All cells in the G1/G0 sort were 1K1N, 4 of the 5 cells in the G2/M sort exhibited a 2K1N configuration.

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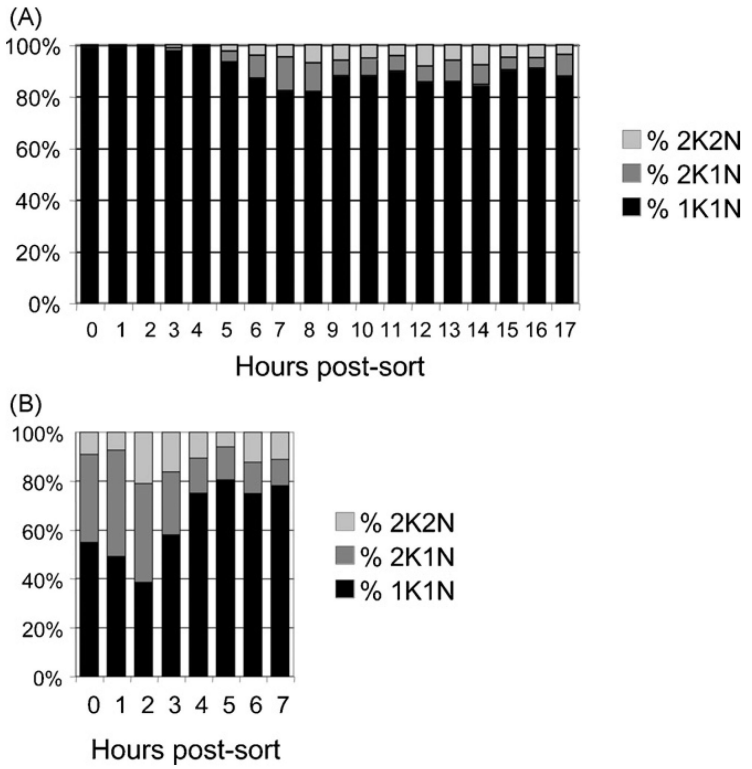

Fig. 2.

The proportion of 1K1N, 2K1N and 2K2N cells in the G1/G0 (A) and G2/M (B) populations at time points after sorting and incubation in HMI-9 medium containing 40% FCS. Each population remained viable post-sort and proliferated for at least 48 h. The G1/G0 sorted population was tracked over 17 h, the G2/M population was tracked over 7 h, this being governed by the number of cells available post-sort for analysis. All graphs are based on an analysis of >250 cells at each time point.