Producing cellular transport proteins without cells: a key to study novel molecular targets of malaria parasites

Malaria tropica, the most severe and fatal form of malaria, is caused by mosquito-transmitted, single-celled *Plasmodium falciparum* parasites. More than 3 billion people in 100 countries live in malaria-endangered areas. Every year, > 200 million new infections cause 400,000 deaths mostly of young children. Despite strong efforts, a vaccine is missing and drugs are becoming unusable because of increasing resistance. Therefore, it is urgent to replenish the therapeutic arsenal by identifying novel antimalarial targets and drugs.

We are aiming at inhibiting transport proteins of the parasite cell membrane. This will for instance shut off nutrient supply from the host blood, or block release of toxic metabolites, such as lactic acid. We have just discovered that the parasite's energy generation fully depends on a single lactic acid transporter, which we termed PfFNT. We also identified drug-like PfFNT inhibitors that potently kill the parasites. For further development and basic understanding of the transporter properties, we require sufficient protein and a suitable assay system.

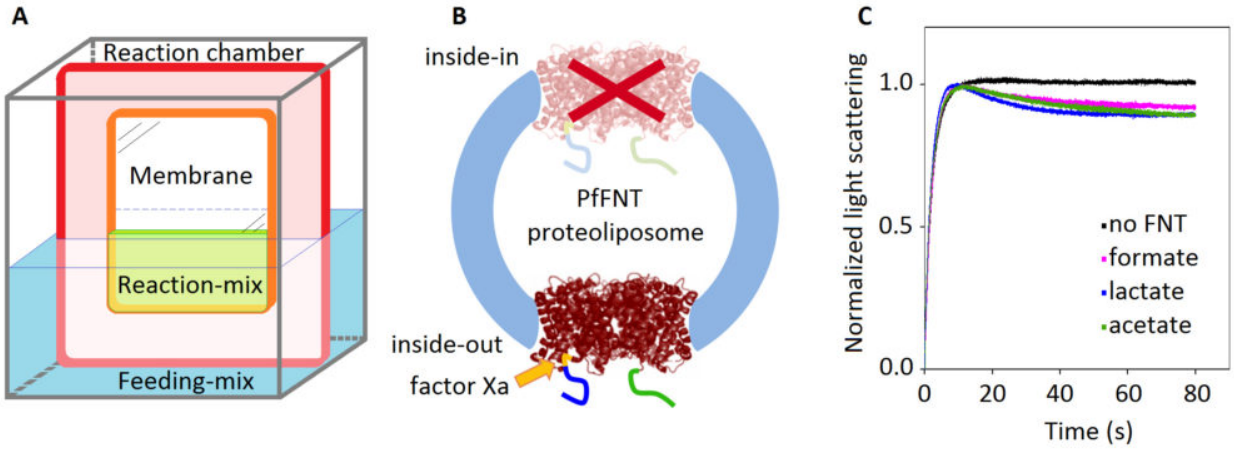

Fig. 1. Cell-free process line for PfFNT production (A), orientational integration into proteoliposomes (B), and transport assays by following changes in light scattering (C).

Typically, a protein of interest is produced by introducing the genetic information into a laboratory cell strain, e.g. baker's yeast. This way, PfFNT from malaria parasites facilitated lactic acid uptake by yeast cells in which the endogenous lactic acid transporters had been deleted. The strain is excellently suited for inhibitor screenings from compound libraries. However, the system has limitations regarding transport studies in the export direction. Further, for crystallization and structure elucidation, milligram quantities of the protein are needed, which would require several

liters of yeast culture.

To overcome these shortcomings, we employed a cell-free system for PfFNT production (Fig. 1A). It comprises two compartments separated by a membrane with permeability for small molecules: 1. a reaction chamber harboring the complete machinery for gene transcription and protein translation including enzymes, ribosomes from *Escherichia coli*, and an energy generation system; 2. a chamber-surrounding feeding mix as a supply of nucleotide and amino acid building blocks, and to dilute out waste products that would impede the reaction. Since PfFNT is an insoluble membrane protein, a detergent must be added, which we chose from 50 µl screening reactions. A suitable detergent holds the protein in solution and stabilizes its correct fold. To indicate correct folding, we generated a PfFNT fusion with green fluorescent protein, GFP. The fluorophore of GFP forms by a secondary chemical reaction of three amino acid residues that require optimal positioning, i.e. correct folding, and several minutes time, i.e. stable solubilisation. The optimized conditions were then used to produce PfFNT without GFP but with an N-terminal histidine affinity tag and a factor Xa protease cleavage site in 1 ml reaction chambers. This yielded milligram quantities of PfFNT permitting crystallization trials.

To assay transport, we reconstituted the cell-free produced PfFNT into proteoliposomes of about 200 nm diameter (Fig. 1B). We were quite surprised when analyzing the PfFNT orientation in the proteoliposome bilayer by cleaving off the histidine-tag. Factor Xa can cleave proteins only if the histidine-tag is accessible. Instead of an expected random orientation, we found 100% cleavage meaning that all PfFNT proteins were evenly integrated. The termini, i.e. the actual intracellular protein face, was exposed to the outside. This eventually allowed us to investigate the physiological transport direction by simply adding lactic acid to the proteoliposome suspension. We monitored resulting proteoliposome volume changes due to initial osmotic water efflux and subsequent lactic acid influx by particle size-dependent light scattering (Fig. 1C).

Together, the fully cell-free process line is highly suitable for producing PfFNT and assaying directional transport allowing for in-depth studies on this novel antimalarial target.

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