Fluorometric assay of ion-coupled amino acids transport mediated by SLC transporters for large screening

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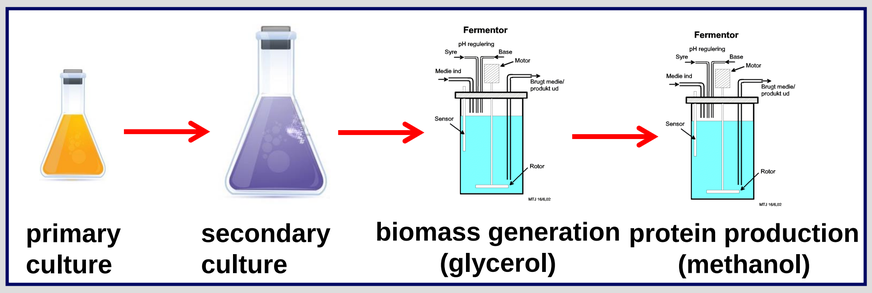
Overview:
The measurement of ion coupled flux of metabolites through cell membranes is historically performed using electrophysiological techniques based on electrical current measurements in cell systems. However, this methodology is not suitable for measuring transport with single protein experimental systems, which are mandatory for understanding transport mechanisms and describing interactions of transporters with xenobiotics. To address this technical issue, we set up a fluorometric assay for measuring H^+ or sodium Na^+ fluxes coupled to amino acid transport in the *in vitro* experimental model of proteoliposomes. This model is made up by inserting a purified recombinant human transporter in artificial phospholipid membrane vesicles, namely proteoliposomes. This experimental tool allows for studying transport properties of a single protein without interferences deriving from other molecular systems normally present in cell (*ex vivo*) models. A plasma membrane transporter for neutral amino acids, namely ASCT2 (SLC1A5) was chosen as benchmark, moving from the deep knowledge about this SLC and from the great interest of the international scientific community due to the key role played by ASCT2 both in human physiology and pathology. We have exploited the glutamine-coupled Na^+ transport or the glutamate-coupled H^+ transport, to point out a fluorometric method for assaying transport as an alternative to the radioisotope assay, so far adopted for ASCT2. Using the Na^+ sensitive probe, Sodium GreenTM (SGI), we revealed the transport of 2 Na^+ ions coupled to glutamine thus establishing a 2:1 stoichiometry Na^+/glutamine with an overall transport reaction $2Na^+$-aa_{ex}/aa_{in}. With the same experimental setup, we showed that ASCT2 is also able to mediate the transport of the negatively charged amino acid glutamate, coupled to the flux of a proton. This was demonstrated using the H^+ sensitive probe, Pyranine. These technical advancements described novel information on ASCT2 mechanisms and function. The fluorometric assay could then be standardized and applied to large screening of small molecules and chemicals on ASCT2, as well as other ion coupled transporters, substituting the radiolabelled assays for decreasing the costs and more importantly for the "do-not-harm" policy.

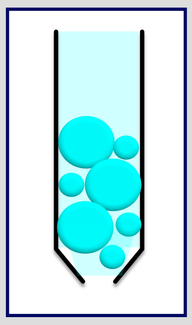
Introduction:
ASCT2 is a neutral amino acid transporter also accepting glutamate and exhibiting a double transport mode. Indeed, Gln is exchanged with other neutral AA in a Na^+-dependent manner, whereas Glu is exchanged with other neutral AA in a Na^+ and H^+ dependent manner. This transporter is crucial for highly proliferating cells, whose need for glutamine is much increased. Accordingly, ASCT2 is over-expressed in most human cancers thus representing a hot pharmacological target. The recombinant human ASCT2 was produced in *P. pastoris* and purified by Ni-chelating Hi-TRAP FPLC chromatography and then reconstituted in active form in proteoliposomes. The 3D structure of ASCT2 was solved using the *P. pastoris* recombinant protein.
The objective of this work is to present an up-to-date tool for large scale screening of membrane transporter-drug interactions helping prediction of efficacy and/or toxicity of hypothetical drugs before animal experimentation.

1. BIOTECHNOLOGY PRODUCTION AND PROTEOLIPOSOME RECONSTITUTION OF THE HUMAN TRANSPORTER

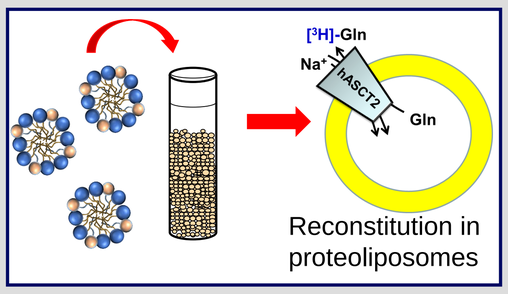
Transformation of *P.pastoris* with hASCT2 construct



primary culture → secondary culture → biomass generation (glycerol) → protein production (methanol)



Ni^{2+} chromatography for hASCT2 purification

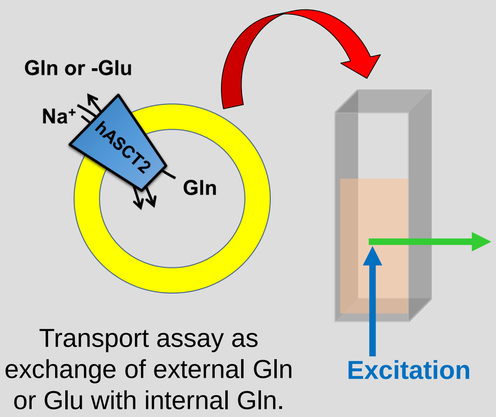


Reconstitution in proteoliposomes

Detergent removal in a batch-wise procedure with Amberlite XAD-4 resin

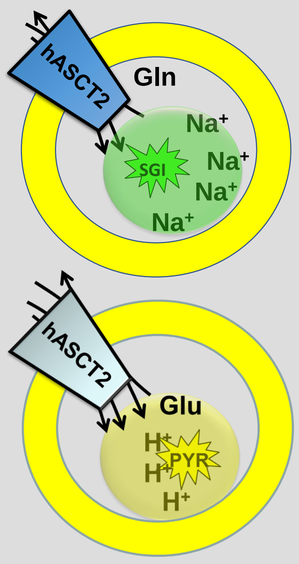
Material and methods

2. FLUOROMETRIC ASSAY IN PROTEOLIPOSOMES FOR ION FLUX MEASUREMENTS



Transport assay as exchange of external Gln or Glu with internal Gln.

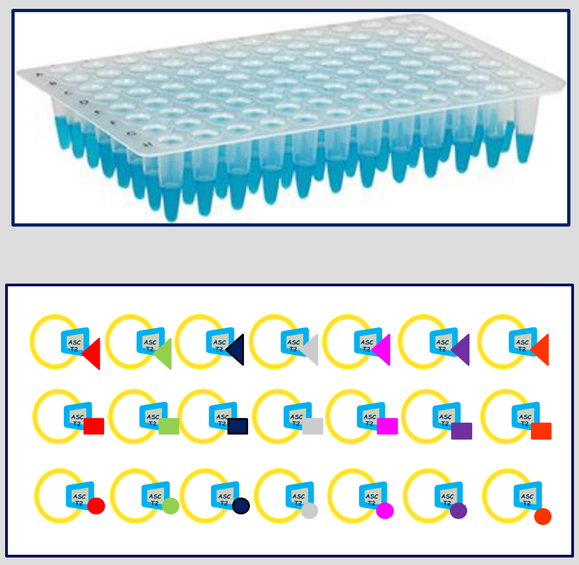
Fluorescence measurement for evaluation of Na^+ or H^+ fluxes coupled to amino acid exchange



Na^+-flux measurement with **SodiumGreen dye**

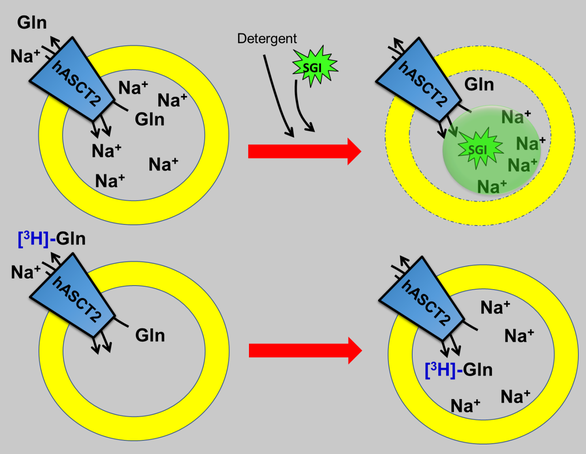
H^+-flux measurement with **Pyranine dye**

Technique scalable for **automation** and **drug screening**

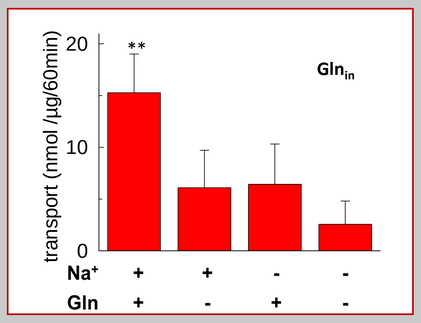


Material and methods

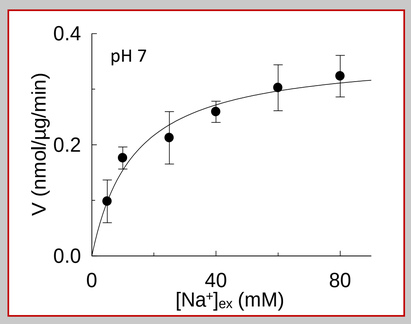
3. Na^+ UPTAKE IN PROTEOLIPOSOMES



Radiometric and **fluorometric** assays in parallel

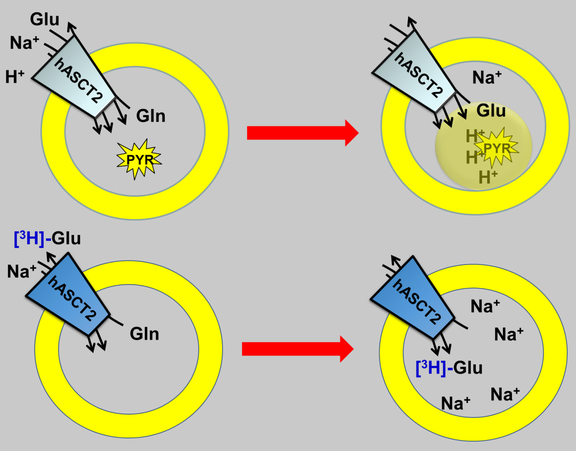


Transport was started by adding glutamine together (+) or not (-) with Na-gluconate. From parallel experiments with radiolabeled Gln a stoichiometry of **$2Na^+$:1aa** was measured for the Na^+/glutamine co-transport.

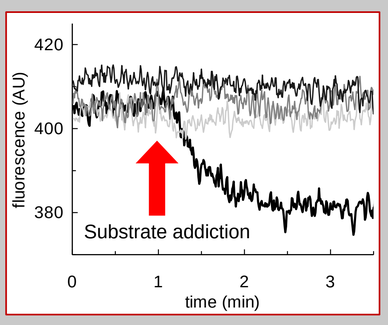


Kinetics of Na^+ transport measured with fluorometric assay.
Data are plotted according to Michaelis Menten equation and a Km of 15.0 ± 1.2 mM.

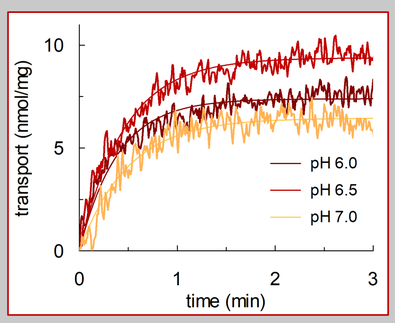
4. H^+ UPTAKE IN PROTEOLIPOSOMES



Radiometric and **fluorometric** assays in parallel

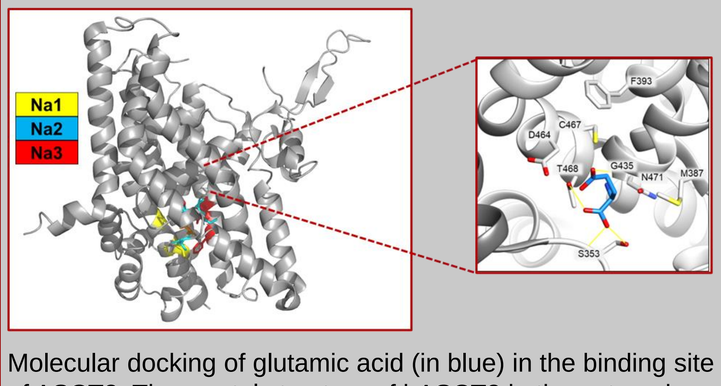


hASCT2 was reconstituted in proteoliposomes containing 10 mM Gln and 0.1 mM pyranine. Addition of Glu (black), Gln (gray), $HgCl_2$ (light-gray, used as specific inhibitor), no Na^+ (dark-gray).



Dependence of glutamate transport on pH using pyranine in fluorometric assay.
From parallel experiments with radiolabeled Glu a stoichiometry of **$1H^+$:1Glu** was measured.

5. Na^+ AND GLU SITES IN 3D STRUCTURE OF ASCT2



Molecular docking of glutamic acid (in blue) in the binding site of ASCT2. The crystal structure of hASCT2 in the outward conformation has been used (PDB ID 6GCT).Three sodium binding sites of ASCT2 are highlighted in the 3D structure.

CONCLUSION AND PERSPECTIVES

The presented results highlight the suitability of a fluorometric assay for measuring the transport activity of an SLC transporter as benchmark. This technique can be employed in those cases in which an ion flux is coupled to the transport of amino acid or other nutrients.
The possibility of collecting results using a **radioactive-free** assay represents a prerequisite for scaling up the assay, as required in the case of automation and drug screening.