Scientific report for COST STSM-Request-BM1406-43535

Applicant / home institute / position

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Proposed Dates of stay: 28-01-2019 to 26-02-2019

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Title: Genetically Encoded Potassium Ion Indicators (GEPII) for *Saccharomyces cerevisiae* and RBL (rat basophilic leukaemia)

I am working on K^+ homeostasis and structure-function analysis of K^+-translocation in yeast (*Saccharomyces cerevisiae*). *S. c.* can survive and grow under different environment from a few µM to hundreds of mM [K^+] while maintaining internal K^+ concentration relatively constant. Trk1 and Trk2 are two specific K^+ translocation systems in *S.c*. Trks are membrane proteins belong to SKT proteins (ion translocation systems that are related to K-channels and were thought to be transporters). We are currently involved in determining the potassium (intracellular and extracellular) concentration in yeast. For intracellular concentration measurement we use atomic absorption spectroscopy and external concentration and fluxes across the plasma membrane are measured by FLISE and MIFE. Recently we started collaborative work on development of genetically encoded potassium ion indicators for yeast with Rainer Schindl and Roland Malli from Med. Uni Graz. I would like to test the application of these sensors in yeast as well as in immune cell line (rat basophilic leukaemia RBL).

Objectives of the STSM:

i) To check the localization of GEPII-constructs targeted to different cellular compartments (cytoplasm, mitochondria, vacuole and nucleus) of yeast.
ii) To estimate the K^+ concentration within these compartments of yeast by FRET.
iii) Developing a strategy to design sensors for immune cells.

Materials and methods:

Yeast strains and media

Haploid *Saccharomyces cerevisiae* strains used throughout this study are listed in Table 1. Strains were grown aerobically at 28 °C with rotational shaking at 180 rpm in liquid medium (Synthetic dextrose arginine phosphate, SDAP and supplemented with the appropriate amino acids and the desired amount of KCl 0.1, 1, 10, 50, 100 and 200mM). pH was adjusted to pH 5.9 with phosphoric acid. All media were prepared with purified water.

Two strains were used for the study, BY4741 (wildtype, Brachmann et al., 1998)) yeast strain 1 and *BY4741-3M (KO)* strain 2 (Zahradka and Sychrova, 2012). Strain 1 is wildtype yeast, strain 2 is KO strain where, *trk1Δ::loxP, trk2Δ::loxP, tok1Δ::loxp* are knocked out. This knockout results in loss of growth, in low K^+ medium (0.1mM), but they can grow when surplus K^+ ions present in medium. Trk1, Trk2 are K^+ uptake transporters in yeast (Trk1 is required for growth in low potassium concentration), tok1 is K^+ efflux channel in yeast.

Both yeast strains were transformed with 4 different plasmids. All plasmids were constructed with derivatives of pYEX-BX (Clontech Laboratories, Mountain View, CA, USA) by using standard molecular biology techniques. In pYEX gene expression is driven by the metallothionein promoter P_{CUP}. For targeting of GEPII in different compartments, specific targeting sequences were used, which were fused to either N termini or C termini of GEPII. For nucleus, it was Long hydrophilic loop (LHL) part of Trk1 (that is targeted to the nucleus when expressed without the transmembrane parts). For mitochondria, it was Su9 (Subunit 9 of the F0-ATPase from *Neurospora crassa*) and for vacuole it was API (soluble vacuolar hydrolase). All strains used for this study are represented in table 1.

Table 1: Different type of *S. cerevisiae* strains

	BY4741(wildtype)-strain1
1.	pYEX-GEPII (cytoplasmic)
2.	pYEX-GEPII/LHL(Nucleus)
3.	pYEX-Su9-GEPII (Mitochondria)
4.	pYEX-API-GEPII (Vacuole)
	***BY4741-3M (KO)*-strain2**
5.	pYEX-GEPII (cytoplasmic)
6.	pYEX-GEPII/LHL(Nucleus)
7.	pYEX-Su9-GEPII (Mitochondria)
8.	pYEX-API-GEPII (Vacuole)

Yeast cell sample preparation:

Cells were grown in SDAP Δleu, ΔUra medium, supplemented with suitable concentration of K+, for (~12-16h) i.e. OD 1-1.5. Cells were centrifuged and 2µl of pelleted cells were placed on clean glass slide and covered with 18mmX18mm coverslip. Cell were observed under 100x magnification objective.

Confocal Microscopy:

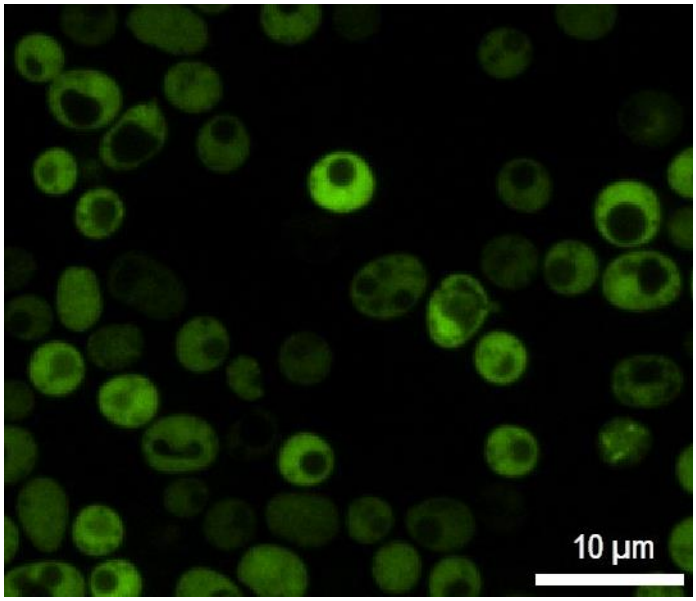
Cellular targeting of GEPII (in cytoplasm, nucleus, mitochondria and vacuole) was analyzed using an array of confocal laser scanning microscope (ACLSM)31. Targeted GEPII's were illuminated using 488nm laser light and emission was collected at 535nm at a binning of 2 using CCD camera (CoolSnapHQ2, Photometrics, Tucson, Arizona, USA).

FRET measurements:

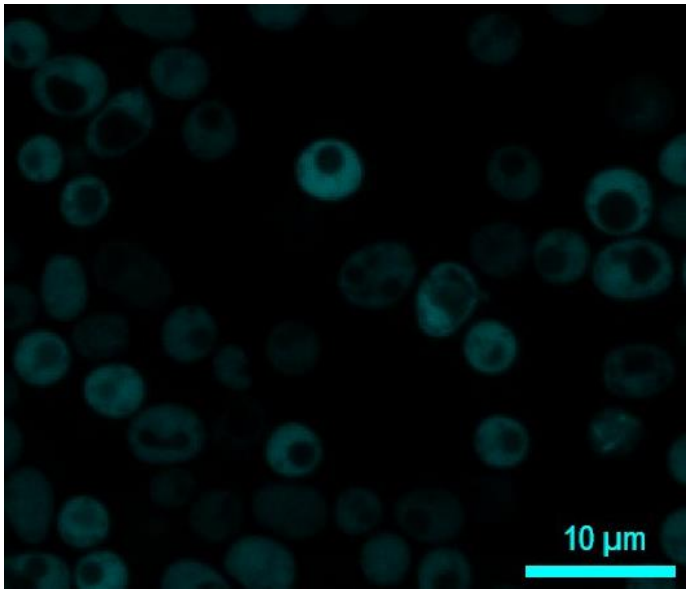
Fluorescence microscopy experiments were performed at iMic inverted and advanced fluorescent microscope using an 100x magnification objective (Zeiss, Gottingen, Germany) with a motorized sample stage (TILL Photonics, Graefling, Germany). Fluorescent proteins were excited at 430nm, emission was collected at 475 and 525nm respectively, using a beam splitter. For control and acquisition the software live acquisition 2 (TILL photonics) was used.

i) The localization of GEPII-constructs targeted to different cellular compartments (cytoplasm, mitochondria, vacuole and nucleus) of yeast, was performed by Confocal Microscopy.

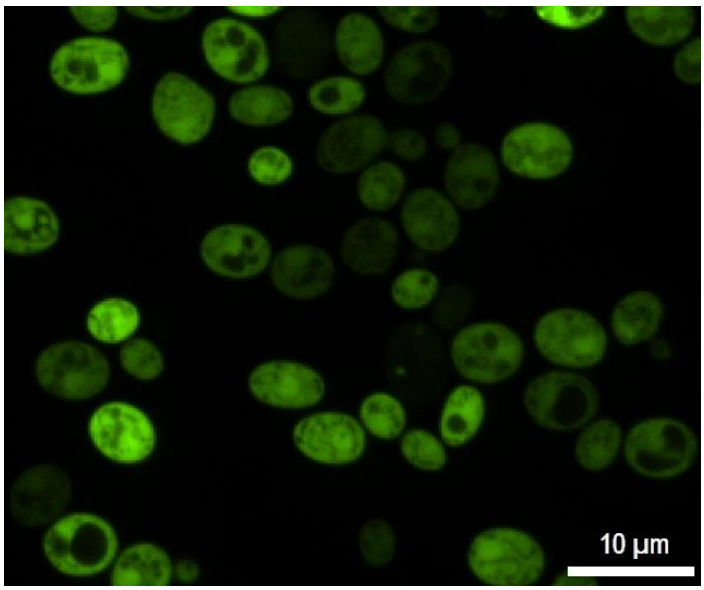
1a. Cytoplasm- Wildtype in YFP filter



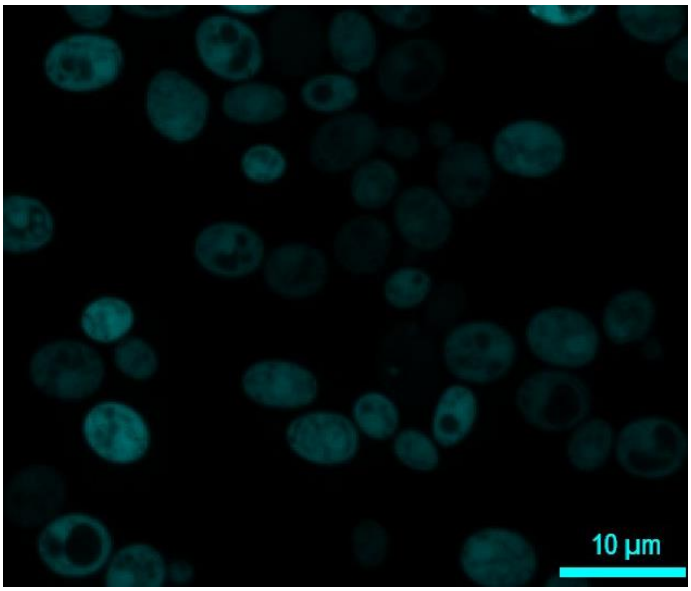
1b. Cytoplasm- Wildtype in CFP filter



1c. Cytoplasm- KO in YFP filter

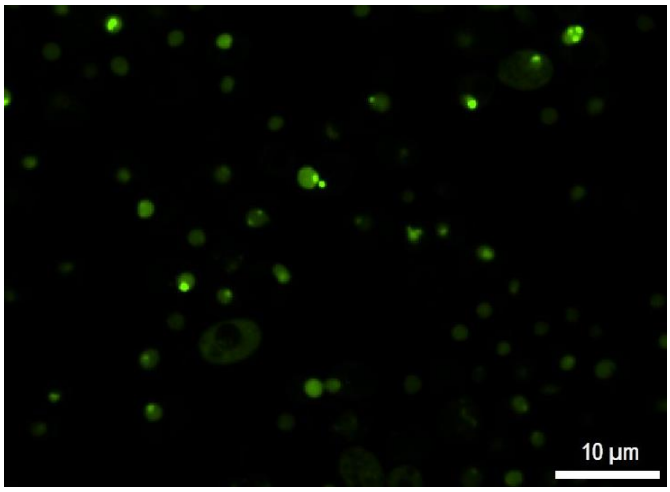


1d. Cytoplasm- KO in CFP filter

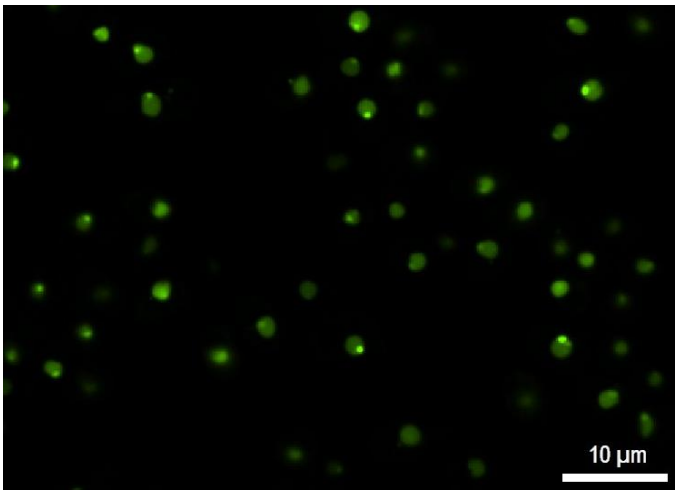


Representative confocal laser microscopy of yeast cells expressing GEPII. Figure 1(a-b) is of yeast cells expressing GEPII in the cytoplasm. Pictures were taken for only one K^+ concentration (cells were grown in 10mM KCl (also written as 10K) medium). Fig. a and c were taken with YFP filter and b and d with CFP filter.

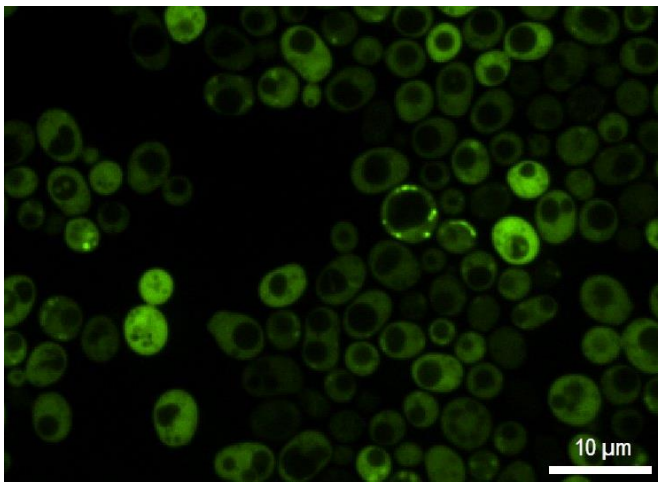
2a. Nucleus- Wildtype



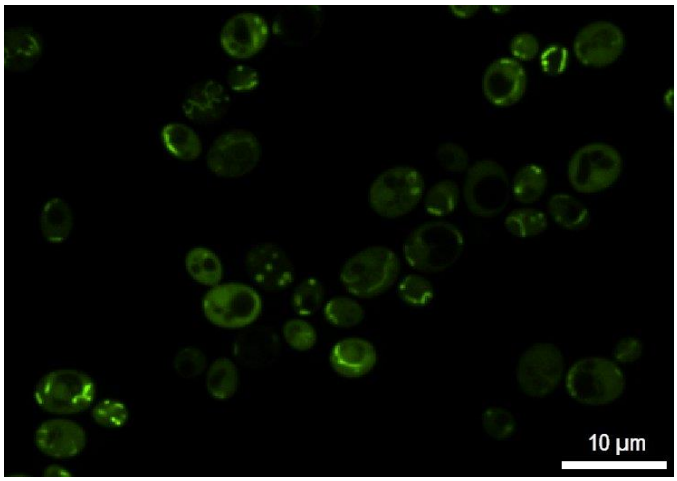
2b. Nucleus- KO



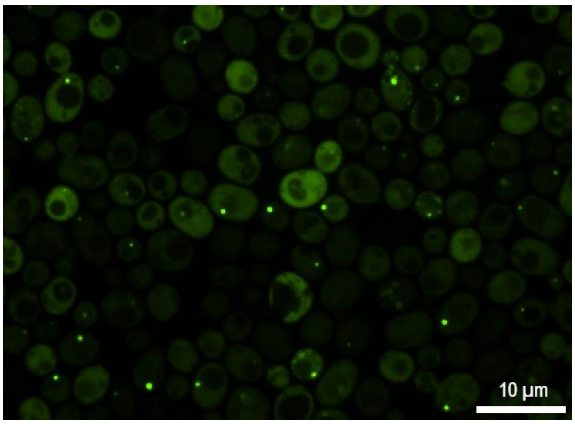
3a. Mitochondria-Wildtype



3b. Mitochondria-KO



4a. Vacuole-Wildtype



4b. Vacuole- KO

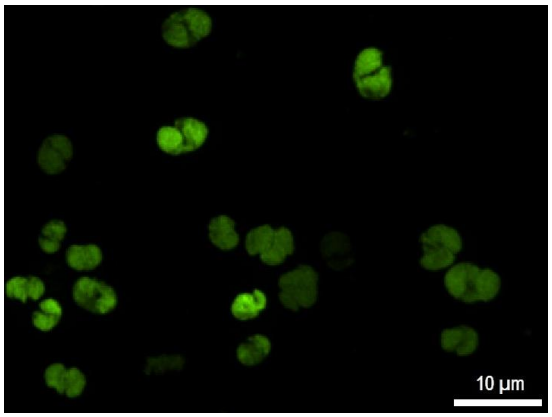


Figure 2(a-b): wildtype yeast and KO yeast expressing GEPII in nucleus, 3(a-b) for GEPII in mitochondria and 4(a-b) for GEPII in vacoule. Representative pictures with only YFP filters are shown.

All 8 different yeast strains were checked microscopically, for targeting of GEPII to the different cellular compartments (cytoplasm, nucleus, mitochondria and vacuole). GEPII was targeted to cytoplasm and nucleus (Fig. 1a-d and 2a-b). In case of mitochondria (Su9/GEPII), for wildtype strain most of CFP florescence was mostly seen in the cytoplasm, rather than in mitochondria (probably due to too high overexpression of the protein). In the KO strain Su9/GEPII was partly targeted to mitochondria but also found in cytosol. In contrst, API/GEPII, supposedly being tergeted to vacuole was only in wt partially found in vacuoleas whereas in the KO strain it was mostly in cytoplasm (Fig. 3a-b and 4a-b respectively). Hence, we did not proceed further with strains expressing GEPII targeted to mitochondria and vacuole and focused on the strains with cytoplasmic and nuclear GEPII.

ii) K^+ concentration within yeast measured by FRET:

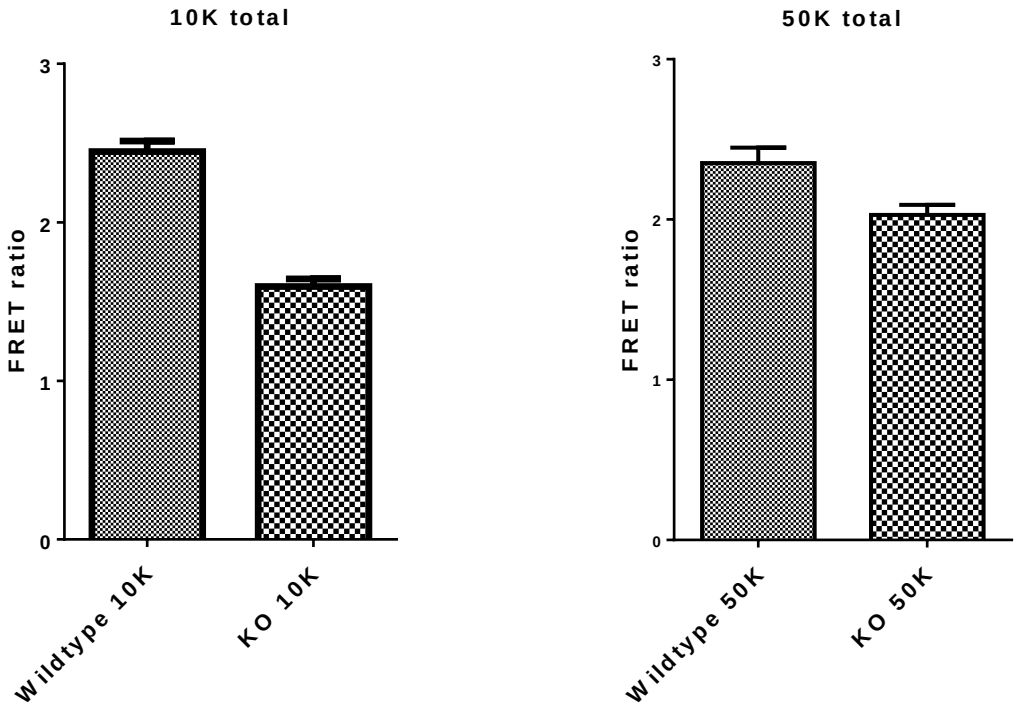


Figure 5: Application of GEPIIs in yeast cells. Basal FRET ratio (± SEM) calculated using GEPII expressed in yeast strains, grown in different K^+ concentration. (n = 3 independent experiments for each/n = 125 cells).

When yeasts were grown in 10K medium, FRET ration for GEPII in wildtype was ~2.5, whereas for KO strain it was ~1.5. A higher FRET ratio corresponds to higher intracellular [K^+]. For Wildtype cells grown in 50K also had a FRET ratio of ~2.5, but in KO cells the FRET ratio was increased to ~2 under this condition (Fig. 5). When cells were grown in medium containing 100 or 200 mM KCl, no difference in FRET ratio was observed between the strains (data not shown).

Pseudocolored FRET ratio-images

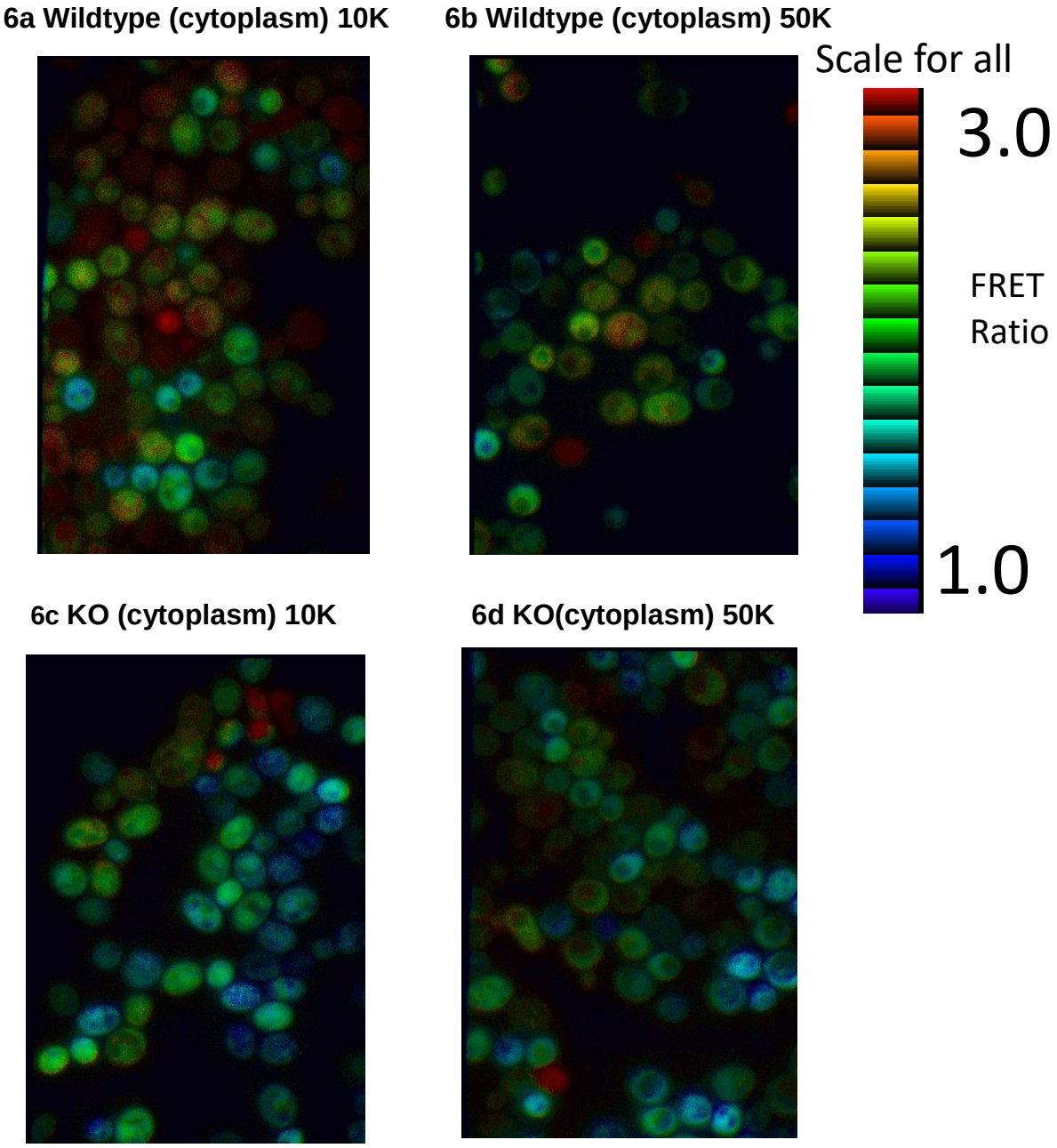
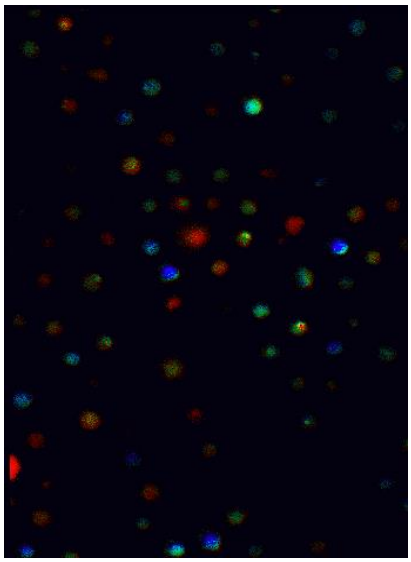
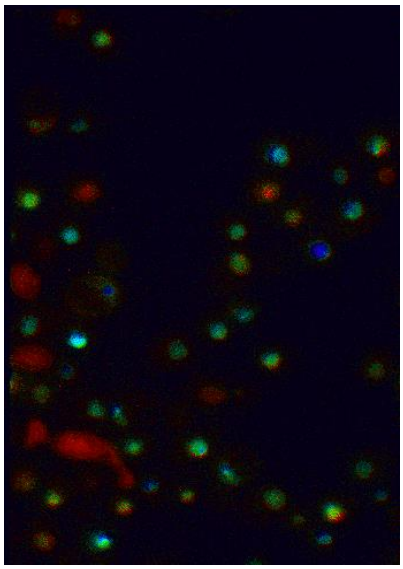


Figure 6: Pseudocolored FRET ratio-images of yeast cells expressing GEPII in the cytoplasm and grown in 10K and 50K respectively. For wildtype cells (Fig. 6a-b) and KO cells (Fig. 6c-d).

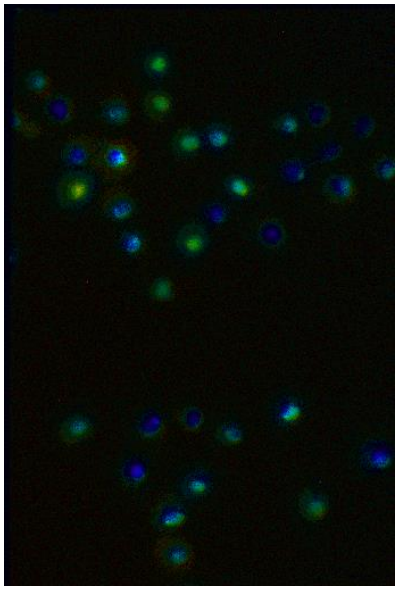
7a Wildtype (nucleus) 10K



7b Wildtype (nucleus) 50K



7c KO (nucleus) 10K



7d KO (nucleus) 50K

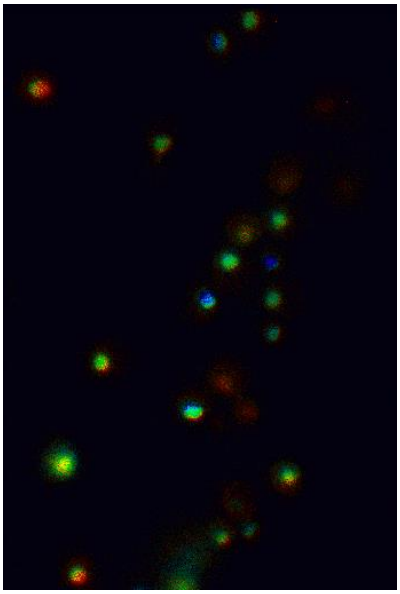


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Figure 7: Pseudocolored FRET ratio-images of yeast cells expressing GEPII in the nucleus and grown in 10K and 50K respectively. For wildtype cells (Fig. 7a-b) and KO cells (Fig. 7c-d).

Color of each cell represents the amount of FRET signal by GEPII (corresponds to the amount of intracellular K concentration). When intracellular [K^+] is high, FRET signal is stronger, cells are green-red in color (Fig. 6a-b). If the K concentration is lower, cells are blue-cyan colored (Fig. 6c), as shown in figure (6-7). Based on color and intensity of these cells, we can see within the cytosol of wildtype cells, grown in 10K and 50K medium (Fig. 6a-b), have higher intracellular FRET than the KO (Fig. 6c). Nuclear GEPII of the wildtype cells, also showed high intracellular FRET (Fig 7a-b), as compared to KO cells, (Fig 7c).

iii) Developing a strategy to design sensors for RBL cells

We have planned cloning method, for constructing RBL specific plasmids. Below is representative plasmid map for one of the constructs (Fig 8). These plasmids will be transfected in RBL cell line. Additionally, we plan to create a cell line with knockout for K channel. Further, we would like to quantify the effect of K channel knockout and its effect in the intracellular concentration within these cells. This will give us a tool to analyze the effect of intracellular K^+ concentration differences in knockout strain and wildtype RBL (immune cell line) and K signaling and its regulation during immune response.

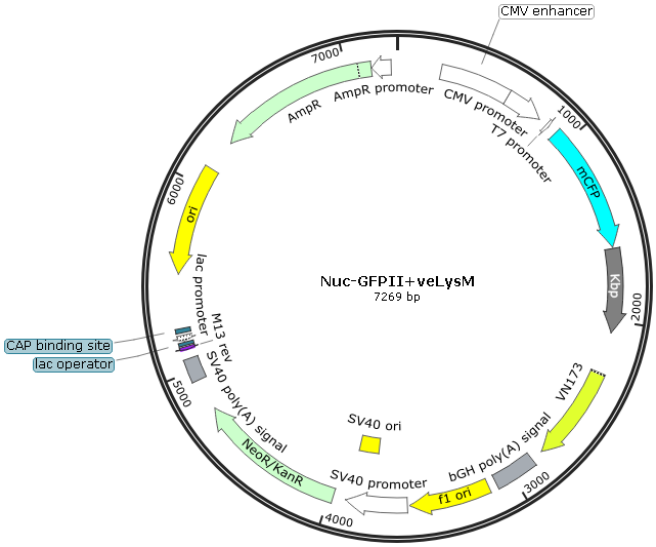

Figure 8: Representative plasmid map for RBL.

Conclusion: Within the STSM, we could prove the applicability of Kbp (potassium binding protein) based sensors (GEPII) in yeast cells. The GEPIIs, enabled us to monitor [K^+] changes in wildtype cells and in cells from a *Δtrk1,2,tok1* knockout strain. The results confirmed that *Δtrk1,2,tok1* cells contain less intracellular potassium, as compared to wildtype cells. In future we plan to continue with GEPII work with Roland Mali and Rainer Schindl in order to quantitatively measure changes of intracellular [K^+] in real time. Also, we plan to do the K^+ concentration measurements in RBL expressing K^+ sensors (GEPII).

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