

Continuous multiparametric monitoring of cell metabolism in response to transient overexpression of the sirtuin deacetylase SIRT3

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Abstract The analysis and visualisation of research data in an environment which is most similar to living conditions belong to the most challenging claims of present scientific research endeavours. To date, the effect of protein function on cell metabolism is most commonly assessed from a series of end point analyses, which finally allows an approximate estimation on how a specific effect takes its course. In the study presented herein, we demonstrate how the combination of transient transfection and a biosensor chip system gives the opportunity to analyse the effect of a specific protein on cell metabolism in living cells through real-time monitoring of metabolically relevant parameters, such as oxygen consumption, acidification rate and cell adhesion. In addition, this method allows online monitoring of the time course of metabolic changes due to changes in expression levels of metabolic regulative proteins from the time of transfection to maximum overexpression. The methodology presented herein was assessed for the transient overexpression of the sirtuin deacetylase SIRT3, a mitochondrial key element in the regulation of energy metabolism, metabolic disease, cancer and ageing.

Keywords Enzymatic activity monitoring · Biosensing · Biomonitoring · Cellular respiration

Introduction

The real-time analysis of single protein function and mode of action in living cells under a minimum of background artefacts is still one major challenge in life science. Our study presents a novel approach, which allows for the continuous real-time analysis of protein function and the effect of single proteins on cell metabolism over a defined period of time, in a closely monitored environment that mimics to the best a predetermined physiological milieu. For this purpose transiently transfected cells were integrated into a biosensory chip analysis system (“Bionas”), which has recently been presented as an innovative tool for real-time in vitro monitoring of metabolic parameters such as glycolysis, respiration and cell adhesion (Thedinga et al. 2007). This is rendered possible through measurement of the extracellular acidification rate with pH-sensitive sensors, recording of cellular oxygen consumption with modified clark-type sensors and the assessment of cell impedance with special “IDES” sensors (interdigitated electrode structures; Ceriotti et al. 2007).

The analysis of cellular metabolism specific parameters were carried out subsequent to transient transfection of defined genes that were highly expressed from eukaryotic vector constructs into the Hek293T, H1299 and HeLa cell lines. The methodology presented herein not only allows for a prolonged monitoring of metabolic changes subsequent to transfection till the achievement of maximum protein activity, it also facilitates the time- and cost-efficient assessment of SNPs and other mutation/deletion associated protein

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modifications that may affect protein activity, stability or its intracellular localisation.

In this report, we demonstrate the highly efficient combination of transient transfection with the biosensor chips (Bionas) methodology, which allows for a fast and reproducible analysis of single protein effects on cell metabolism in living cells. As the key role of SIRT3 was just recently proved again by a current article in nature (Hirschey et al. 2010) SIRT3 presented an ideal candidate for the assessment of this method due to its mitochondrial key roles in regulation of energy metabolism (Ahn et al. 2008; Hallows et al. 2006; Hirschey et al. 2010; Shi et al. 2005).

Materials and methods

Plasmids and antibodies

In our analyses the following constructs have been used: hSIRT3-Flag (kindly provided by E. Verdin, The Gladstone Institute, San Francisco, CA, USA; pcDNA3.1; North et al. 2005, Schwer et al. 2002). Site-directed mutagenesis (QuikChange Mutagenesis Kit; Stratagene, Cedar Creek, TX, USA) was carried out to generate the hSIRT3H248Y-FLAG construct. SIRT3 wt and inactive mutants were further cloned into the pEGFP-C1 vector (Clontech Laboratories, Saint-Germain-en-Laye, France) via EcoRI restriction sites. pGFPmax (Amaxa/Lonza, Köln, Germany) was used as an additional control for transfection efficiency. All constructs were verified by direct DNA sequencing. Antibodies that were used for immunoblotting: anti-Flag M2 (Sigma-Aldrich, Deisenhofen, Germany), anti-SIRT3 (Imgenex, San Diego, CA, USA). Western blots were performed according to standard protocols on nitrocellulose membranes (Biorad, Hercules, CA, USA) and visualised by enhanced chemiluminescence (GE Healthcare, Buckinghamshire, UK).

Cell culture and transfection

The cell lines that were used included Hek293T (DSMZ, Braunschweig), H1299 (ATCC) and HeLa (DSMZ, Braunschweig) cells, which were cultured in Dulbecco's modified eagle medium (DMEM; PAA, Pasching, Austria), supplemented with 10% FBS (PAA, Pasching, Austria), 1% penicillin/streptomycin (Gibco Invitrogen, Carlsbad, CA, USA) and nonessential amino acids (Biochrom AG, Berlin, Germany) within a CO₂ incubator at 37°C with 5% CO₂. Cells were transfected with either Nanofectin (PAA, Pasching, Austria) or nucleofection for Amaxa-electroporation (Amaxa/Lonza, Köln, Germany) with the nucleofection solution R (Lonza, Köln, Germany) because

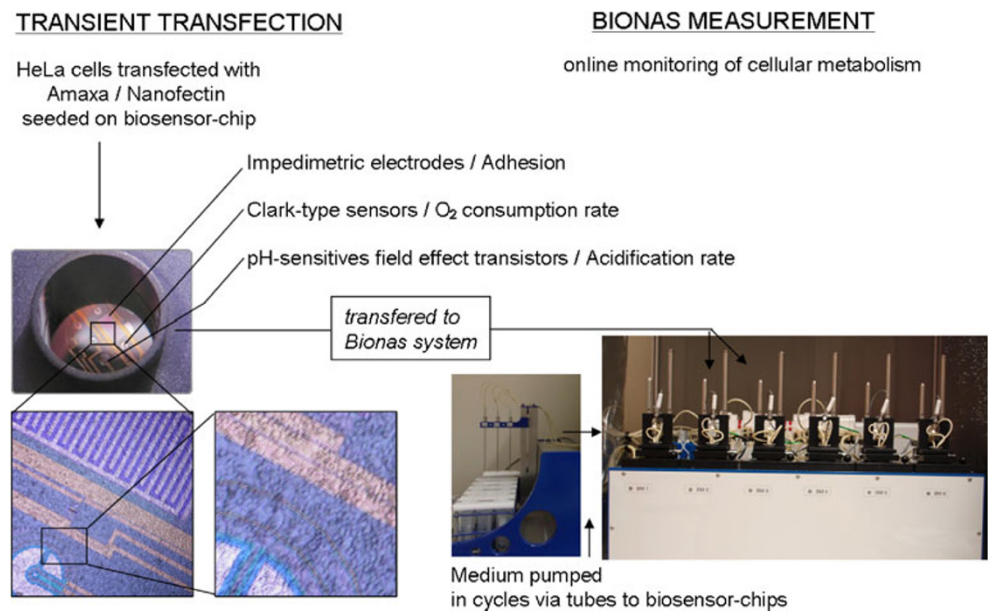
these methods yielded the best transfection efficiencies out of all tested methods (nucleofection and Nanofectin versus lipofection and calcium-phosphate transfection). Nanofection was performed according to the manufacturers' instructions, with the following modifications for use in the biosensor chips: HeLa cells were transfected in six well plates at 70% to 80% confluence with 200 µl transfection reagent per well (200 µl: diluent (150 mM NaCl), 1.5 µg plasmid DNA and 9.6 µl Nanofectin), followed by medium exchange 5 h after transfection with DMEM with 5% FBS. After transfection cells (20 h) were trypsinized and seeded at a density of 3×10^5 cells on poly-D-lysine (Sigma, Deisenhofen, Germany) coated biosensor chips (Bionas, Rostock, Germany) in 500 µl DMEM with 5% FBS for 4 h prior to Bionas analysis. Amaxa nucleofections were performed according to the manufacturers' instructions using 1×10^6 cells per transfection out of which one third ($\sim 3.3 \times 10^5$) were transferred onto poly-D-lysine coated biosensor chips in 500 µl Optimem (Gibco Invitrogen, Carlsbad, CA, USA) supplemented with 5% FBS and 1% penicillin/streptomycin for 4 h before Bionas kinetic measurements were started.

Real-time monitoring of cellular metabolism in living cells with the biosensor chip system

Metabolic chips SC1000 (Bionas, Rostock, Germany) were washed and disinfected according to the manufacturers' instructions and coated with 100 µl poly-D-lysine (Sigma, Deisenhofen, Germany) for 5 min, which was followed by three washes with autoclaved ddH₂O and subsequent drying for 2 h. Before each experiment the tube system of the Bionas 2500 analysing system (Bionas, Rostock, Germany) was cleaned and disinfected with 70% EtOH and then rinsed with PBS and conditioned with running medium according to the manufacturers' instructions.

The coated chips were covered with either transfected or untransfected cells, which were then analysed on the Bionas 2500 system for 20–24 h after transfection for direct comparison of respiration and glycolysis or 4 h after transfection for kinetic analyses (Fig. 1). Cells were seeded and grown to approximately 80–90% confluence on the chip surface as required for online monitoring. In the Bionas 2500 analysing system the running medium (DMEM without carbonate buffer and only weakly buffered with 1 mM Hepes (Sigma, Deisenhofen, Germany) and reduced FBS and glucose (2.5% and 1 g/l, respectively)) was continuously exchanged in 8 min cycles (4 min exchange of medium and 4 min without flow) during which the parameters were measured. The metabolic sensor chips (SC1000) are equipped with Clark-type oxygen sensors, which allow for the monitoring of oxygen consumption and ion-sensitive field-effect transistors to record pH changes and impedimetric interdigitated

Fig. 1 Scheme of combination of transient transfection and Bionas system for online monitoring of the effect of single proteins on cellular metabolism



electrode structures for the measurement of cell impedance (Ehret et al. 2001).

Results and discussion

The combination of transient transfection with the real-time monitoring of cellular metabolism with biosensor chips allows for a rapid analysis and visualisation of single protein effects on cellular metabolism for an extended period of time in living cells in a closely monitored environment that mimics to the best a physiological milieu. The methodology presented herein provides a number of advantages with regard to the monitoring of protein function: to date such analyses were usually carried out as end point analyses on cellular material that was previously destroyed by detection markers or fixation procedures and which usually required high cell numbers while yielding unsatisfactory information on cellular response kinetics.

Determination of the best suitable cell line

For the establishment of this method and its further application it was essential to establish highly reproducible transient transfections in a cell line which showed sufficient viability in the Bionas system for long-term kinetic experiments. For this reason cell lines with longer duplication time seemed to be most appropriate. In a first step we tested the H1299, HEK293 T and HeLa cells, some of which had already been used earlier on the Bionas system (Harlos et al. 2008; Schatzschneider et al. 2008). We identified HEK293T and HeLa to be the most suitable cell lines for the combination of transient transfection being followed by subsequent analyses on

the Bionas biosensor chips. The best transfection efficiencies and long-term behaviour was observed with the HeLa cells, both for nanofection and Amaxa-nucleofection (60–70% and 50–60%, respectively). For all experiments transfection efficiencies were tested with a SIRT3-GFP construct.

The most suitable proteins for real-time monitoring of the impact of single proteins on cellular metabolism present of course proteins with known or at least assumed functions in cellular metabolism, especially with regulatory effects on energy metabolism. For this reason we decided to use SIRT3, an essential mitochondrial deacetylase (class III histone deacetylase), with known effects on cellular metabolism, especially on cellular respiration (Ahn et al. 2008; Shi et al. 2005).

Protein expression kinetics in relation to cell metabolism

In order to prove the validity of real-time monitoring of transiently transfected cells with subsequent analysis on the Bionas biosensor chips, the kinetics of SIRT3 wt overexpression was visualised both via Western blot and concomitant monitoring on the Bionas system (Fig. 2). We have been able to show that SIRT3 wt overexpression leads to an increase in respiration by up to 35% when compared to cells that were transfected with the inactive SIRT3 mutant and versus untransfected cells (Fig. 2a), which was confirmed by Western blots analysis (Fig. 2c). Respiration changes were however delayed by 1–2 h when compared to changes on the protein level. Maximum SIRT3 overexpression and increase of cell respiration were achieved 18 to 20 h post transfection.

After identification of the time point for maximum SIRT3 effect on respiration we carried out further comparative

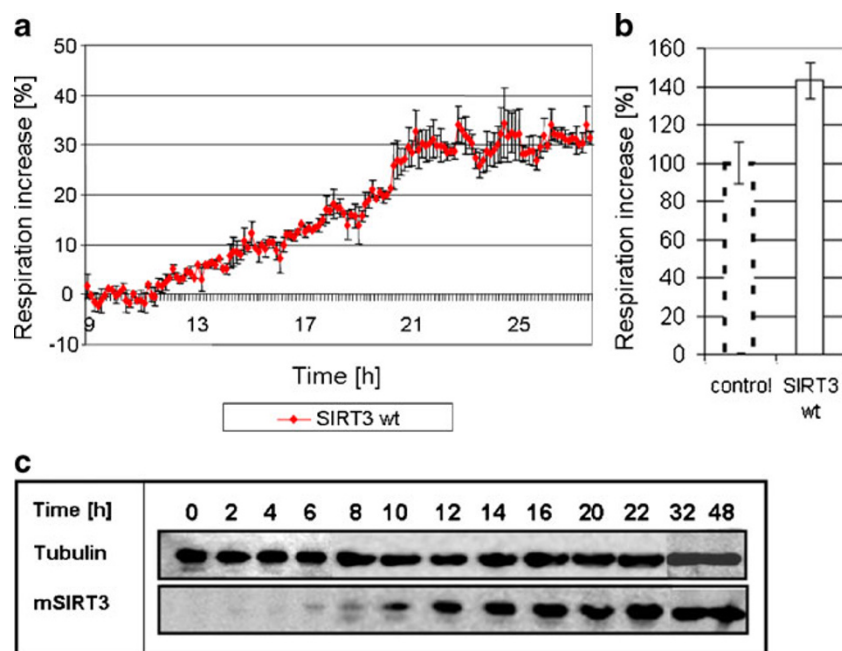


Fig. 2 Changes in respiration kinetics in relation to SIRT3 overexpression. **a** SIRT3 overexpression starts to increase respiration approximately 12 h after transfection with a maximum increase by 30–35% around 21 h after transfection (baseline=controls; presented increase of respiration of cells transfected with SIRT3 was the result of the percent rise of SIRT3 transfected cells after distraction of the percent rise of the controls (cells transfected with the inactive SIRT3 mutant and versus untransfected cells). **b** 20 h post transfection the metabolic rates of cells transfected with SIRT3 wt or controls

transfected with the SIRT3_{H248Y} inactive mutant or untransfected cells were compared ($N=3$ independent experiments/7 independent measurements). **c** Western blot signals of overexpressed mitochondrial SIRT3 (mSIRT3-Flag) via Flag antibody detection shows first signals 6 h post transfection and a signal maximum intensity 16 to 20 h after transfection. An increase of SIRT3 protein expression concurs with an increase in respiration, which was shown in two independent experiments (four independent measurements)

analyses on the Bionas system starting measurements after this time (22 h) post transfection for several hours in order to further determine the level of respiration increase (Fig. 2b) and glycolysis together with impedance, whereby the latter two did not show any changes (data not shown) in relation to SIRT3 expression. These comparative measurements (started 22 h after transfection) yielded to the same results as the kinetic analyses: While SIRT3 wt overexpression had no significant influence on glycolysis we could verify the increasing effect on respiration with mean levels of about 40% (Fig. 2b).

This observed increase of respiration due to SIRT3 overexpression shows that SIRT3 is an appropriate candidate for the establishment of this new method. The observed delay of 1 to 2 h that were measured for cellular respiration when compared to the time course towards maximum SIRT3 signals as demonstrated by Western blot may be explained by direct and indirect ways how SIRT3 may activate respiration (Fig. 3): as published most recently, SIRT3 is directly activating complex I of the respiration chain (Ahn et al. 2008) and may also act indirectly on the transcriptional level via promotion of PGC-1 α expression, which in turn activates mitochondrial respiration (Shi et al. 2005). Another possibility how SIRT3 may affect energy metabolism may be via

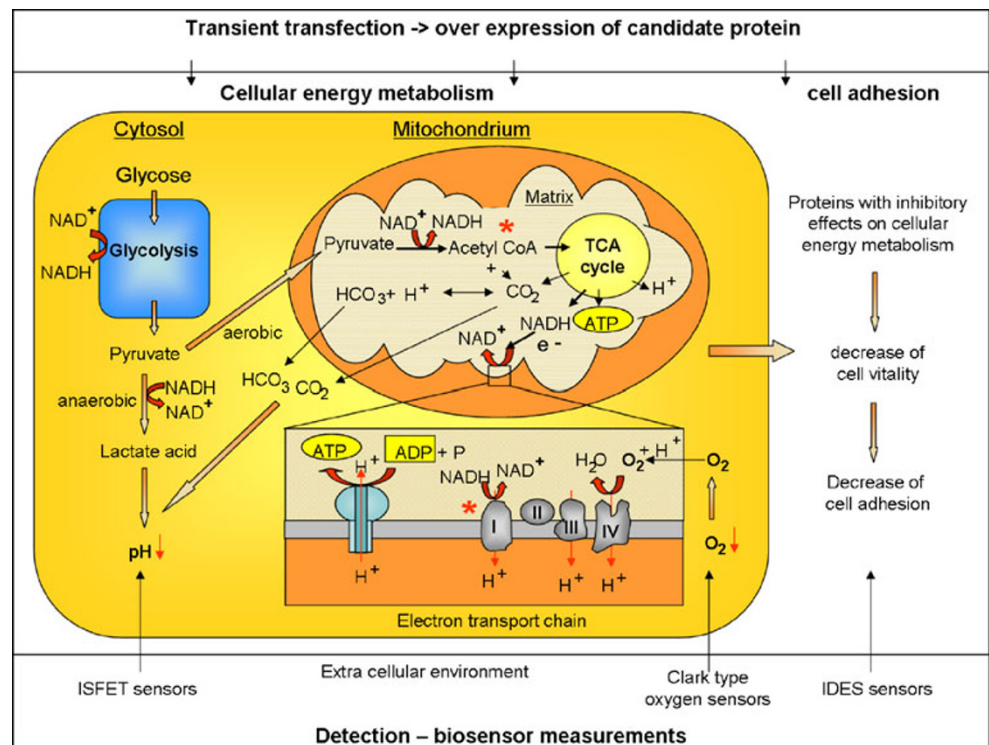
an increase of ATP and glucose synthesis through activation of the mitochondrial acyl-CoA-synthetase (Hallows et al. 2006; Schwer et al. 2006) and GDH (Lombard et al. 2007; Schlicker et al. 2008) by enabling amino acids to be used as fuels for citric acid cycle and gluconeogenesis.

The analysis of cellular metabolism is carried out via measurements of metabolically relevant parameters, which are most accessible for noninvasive online monitoring such as oxygen consumption or extracellular acidification, thus allowing conclusions towards cellular energy metabolism, i.e. respiration and glycolysis, respectively (Fig. 3).

Changes of extra cellular acidification reflect changes in energy metabolism due to oxygen consumption by respiration and glycolysis byproducts. In addition, cellular impedance reflects cellular adhesion, morphology and confluence and allows for conclusions on cellular vitality (Fig. 3), as intact cell membranes on the electrodes are determining the current flow and thus the IDES biosensors signals.

The correctness of the obtained results is also being confirmed by recent data in the literature, reporting SIRT3 overexpression to go along with an increase of cellular respiration by 80% (Shi et al. 2005), which was paralleled by an increase of respiration by 30% to 40%, however taking into consideration a transfection efficiency of about

Fig. 3 Overview on parameters that are being used in the monitoring of cellular energy metabolism and adhesion via biosensor chips. Noninvasive measurements of extracellular pH (ISFET sensors), oxygen (Clark-type sensors) and impedance (IDES sensor) allow conclusions towards cellular energy metabolism (i.e. glycolysis, respiration and cell adhesion, respectively). *Single asterisk* indicates points of direct SIRT3 activating activity



50–60%. Taking into account this transfection efficiency, the reported increase of respiration by 30% to 40% needs to be doubled, which would then lead to exactly the same values that have been reported by Shi et al.

As the standard variations of the Bionas measurements of the transient transfected cells account for about 10% in our experiments, proteins of interest should lead to an increase in respiration or glycolysis of more than 10%, assuming a transfection efficiency of 50–60% in order to get significant results. For proteins with minor effects (<10%) on key parameters of cellular metabolism (respiration and glycolysis, as measured via the Bionas system), this method is probably not appropriate.

One suggestion for the further optimization of the method presented herein would be the construction of transparent biosensor chips, as they would allow the real-time monitoring of transfection efficiency, which would make parallel control approaches unnecessary. Parallel measurements of transfection efficiency only give an indirect estimation of the number of transfected cells on Bionas system. The direct evaluation of transfection efficiency on each chip would allow for the calculation of a probe specific “correction factor”, which takes into account probe specific transfection variations, which would lead to a reduction of standard errors. This in turn would increase the “sensitivity” of the method and expand the spectrum of candidate proteins to be assessed.

As it is possible to compare metabolic changes in cells after transfection with both, wild type or mutants of the protein of interest, one additional feasible application for this method could consist in the analysis of the impact of SNPs or point mutations on cellular functions of the protein of interests referring to changes of metabolism, adhesion or survival with respect to protein activity, stability or localisation. In our example we used an inactivating mutation in the active domain of the SIRT3 protein as a negative control, which showed the same metabolic signals as transfections with the empty vector or as seen in untransfected cells.

An additional advantage of the presented methods herein is its possibility to perform further analyses on the influence of single or combinational overexpression of specific proteins under the simultaneous treatment with defined pharmacological agents in order to determine whether these proteins have an effect on treatment response in addition to their effects on cell metabolism.

Conclusion

Taken together, the combination and optimisation of transient transfection with the real-time monitoring of cellular metabolism with biosensor chips allows the fast and efficient characterisation of the influence of single overexpressed proteins on metabolic parameters in living

cells for kinetic experiments in a closely monitored environment with the possibility for further analyses of protein effects on treatment responses or regenerative effects subsequent to treatment. This methodology therefore presents a promising tool for a wide variation of scientific applications.

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Conflict of interest The authors declare that they have no conflict of interest.

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