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Intracellular pH imaging in cancer cells *in vitro* and tumors *in vivo* using the new genetically encoded sensor SypHer2

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ABSTRACT

Background: Measuring intracellular pH (pH_i) in tumors is essential for the monitoring of cancer progression and the response of cancer cells to various treatments. The purpose of the study was to develop a method for pH_i mapping in living cancer cells *in vitro* and in tumors *in vivo*, using the novel genetically encoded indicator, SypHer2.
Methods: A HeLa Kyoto cell line stably expressing SypHer2 in the cytoplasm was used, to perform ratiometric (dual excitation) imaging of the probe in cell culture, in 3D tumor spheroids and in tumor xenografts in living mice.
Results: Using SypHer2, pH_i was demonstrated to be 7.34 ± 0.11 in monolayer HeLa cells *in vitro* under standard cultivation conditions. An increasing pH_i gradient from the center to the periphery of the spheroids was displayed. We obtained fluorescence ratio maps for HeLa tumors *in vivo* and *ex vivo*. Comparison of the map with the pathomorphology and with hypoxia staining of the tumors revealed a correspondence of the zones with higher pH_i to the necrotic and hypoxic areas.
Conclusions: Our results demonstrate that pH_i imaging with the genetically encoded pH_i indicator, SypHer2, can be a valuable tool for evaluating tumor progression in xenograft models.
General significance: We have demonstrated, for the first time, the possibility of using the genetically encoded sensor SypHer2 for ratiometric pH imaging in cancer cells *in vitro* and in tumors *in vivo*. SypHer2 shows great promise as an instrument for pH_i monitoring able to provide high accuracy and spatiotemporal resolution.

1. Introduction

Intracellular pH (pH_i) is known to be an important regulator of many cell functions. In normal cells, intracellular pH is lower than extracellular pH (pH_e), with the pH_i and pHe values lying mostly in the range 7.0–7.2 and 7.3–7.4 respectively. Cancer cells are generally associated with higher values of pH_i 7.12–7.65 and lower pH_e 6.2–6.9. Such a reversed intra-extracellular pH gradient is considered to be a hallmark of neoplastic tissue, assisting the progression of the cancer [1,2]. An elevated pH_i allows cell proliferation and the evasion of apoptosis, provokes a metabolic switch from oxidative phosphorylation to aerobic glycolysis (Warburg effect) and possibly promotes genetic instability and the multidrug resistance (MDR) of cancer cells. An increased pH_i and a decreased pH_e coordinately enhance invasion and metastasis. Therefore it appears that the measurement of pH_i in tumors could represent an important method for monitoring the progression of cancers and the responses of cancer cells to various treatments.

Given the significant role of pH_i in tumor development, it is crucially important to be able to measure it with high accuracy and spatiotemporal resolution. Fluorescence imaging based on pH-sensitive fluorescent probes offers excellent opportunities as a highly-sensitive, low cost technique for real-time, non-invasive pH determination in cells and tissues. The majority of commercially available probes are small organic molecules that have to be introduced into the cells or tissues exogenously. The most widely used of these are derivatives of fluorescein (BCECF, BCPCF, fluorescein, fluorescein sulfonic acid, carboxyfluorescein) and benzoxanthene (SNAFL, SNAFR, SNARF) [3].

Despite the variety of pH sensitive fluorescent dyes available, measuring intracellular pH remains problematic, especially in living tissues. These shortcomings include problems with the intracellular delivery; with self-redistribution of the dyes and their leakage from the cells; their interactions with other molecules in the cell, and their own cytotoxicity [4]. As a result, the current applications for such

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synthetic probes are limited to the assessment of pH_i in cell cultures, in dissociated spheroids and tissues and in tissue slices following incubation with the probe [5–9].

Although some of these dyes are able to enter cells *in vitro*, the mapping of pH_i in solid tumors *in vivo* remains impracticable and, so far, only pH_e measurements in tumors have been carried out with the use of this type of synthetic probe. For example, Mordon et al. ratiometrically measured pH_e *in vivo* in subcutaneously grafted lymphoid leukemia P388 tumors in mice, using 5,6-carboxyfluorescein, 5,6-*CF* [10]. Robey et al. determined pH_e in MDA-MB-231 tumor xenografts following the injection of SNARF-1 free acid [11]. In the paper by Hight et al. pH_e was quantified with SNARF-4 F in dye-perfused surgically-resected tumor specimens [12].

Therefore, it is desirable both to engineer new indicators for intracellular pH measurement and to develop appropriate methods for their use *in vivo*. In this context, pH-reporters based on green fluorescent protein (GFP) represent promising instruments for overcoming the limitations of the synthetic dyes [13,14]. Being genetically encoded, they can be directed to any particular compartment within a cell, or allowed to distribute themselves by diffusion within the cytosol. Consequently, they enable the subcellular measurement of pH with unrivaled specificity. The stable expression of GFP-based probes opens up possibilities for continuous pH monitoring in living cells and tissues.

In this issue, Matlashov et al. [15] report on an improved pH-sensitive ratiometric indicator SypHer2 based on the cpYFP fluorophore. It was generated from the H_2O_2 indicator HyPer-2 [16] by mutation of the H_2O_2-sensing cysteine residue to serine (supplemental Fig. S1), causing a total loss of sensitivity to H_2O_2. SypHer2 has two excitation peaks, at 420 nm and 500 nm, and one emission peak at 516 nm, similar to HyPer [17], however, in mammalian cells, compared to SypHer [18], it has a 2 to 3 fold brighter fluorescence signal.

The purpose of the study was to develop a method for pH_i sensing in living cancer cells *in vitro* and tumor xenografts *in vivo*, using this new genetically encoded indicator SypHer2. Ratiometric (dual excitation) imaging of the probe was performed in cell culture, in 3D tumor spheroids and in tumor xenografts in living mice. Quantitative assessment of the *in vitro* pH_i was made possible by using a calibration curve. Additionally, histopathology and hypoxia in the tumors were characterized, to assist in interpreting the ratiometric imaging data.

2. Materials and methods

2.1. SypHer2 spectral characterization

The SypHer2 protein was extracted and purified from competent E.coli XL1-Blue cells transfected with pQE30-SypHer2 plasmids. Individual bacterial clones were picked and grown overnight on solid LB-agar plates at 37oC followed by 24 h at room temperature. Cells were lysed using B-PER reagent (Thermo Fisher Scientific), then equal aliquotes of the cell lysates were added to buffers (25 mM Tris–HCl and 150 mM NaCl) with pH values 7.1, 7.6, 8.1, 8.6, 9.0. Fluorescence excitation spectra were recorded using Cary Eclipse (Varian) fluorimeter in 350–510 nm range (530 nm emission). Fluorescence emission spectra in 440–620 nm range were recorded with 420 nm excitation. The obtained excitation and emission spectra of SypHer2 are presented in Fig. 1A.

Measuring of the excitation spectra at different pH showed that the excitation peak at 420 nm decreases with pH proportionally to the increase in the peak at 500 nm, allowing ratiometric measurement of pH (Fig. 1B).

2.2. Monolayer and 3D cell cultures

A HeLa Kyoto cell line, stably expressing SypHer2 (HeLa-SypHer2) in the cytosol, was used. The cells were cultured in DMEM supplemented with 10% FBS (Hyclone), 2 mM glutamine (PanEco), 10 μ/mL penicillin and 10 mg/mL streptomycin. For fluorescence microscopy the cells were seeded (1×10^5 in 2 mL) into glass-bottomed 35 mm FluoroDishes, and incubated overnight at 37 °C, 5% CO_2 and 80% relative humidity. Then the cells were washed with PBS and placed into Henk's solution for imaging.

The protocol for 3D cultures (tumor spheroids) was modified from the plate manufacture's protocol. A cell suspension of HeLa-SypHer2 cells in complete growth medium was seeded into the wells of a round bottom 96-well Ultra-Low Attachment plate (Corning, USA) at a density of 100–150 cells/200 μL/well. Complete culture medium was added on day 3. Once spheroid formation had been completed, on the day 7 they were carefully washed with PBS and transferred into glass-bottom FluoroDishes with PBS for investigation.

2.3. Animals and tumors

All animal protocols were approved by the Ethics Committee of Nizhny Novgorod State Medical Academy. Experiments were performed on female athymic nude mice purchased from the Pushchino animal nursery (Pushchino, Russia). Mice of 20–22 g body weight were inoculated subcutaneously in the left flank with HeLa cells expressing cytosolic SypHer2 (2×10^6 in 200 μL PBS). Imaging started 7 days after the cell injection, when the tumors had reached 3–4 mm in diameter. The animals were monitored on days 7, 10, 14 and 18. Before fluorescence imaging the mice were anesthetized intramuscularly with a mixture of Zoletil (40 mg/kg, 50 μL, Virbac SA, Carros, France) and 2% Rometar (10 μL and 10 mg/kg, Spofa, Czech Republic). To reduce signal attenuation, a skin flap over the tumor was repeatedly opened for image acquisition and closed with a 6–0 suture immediately afterwards.

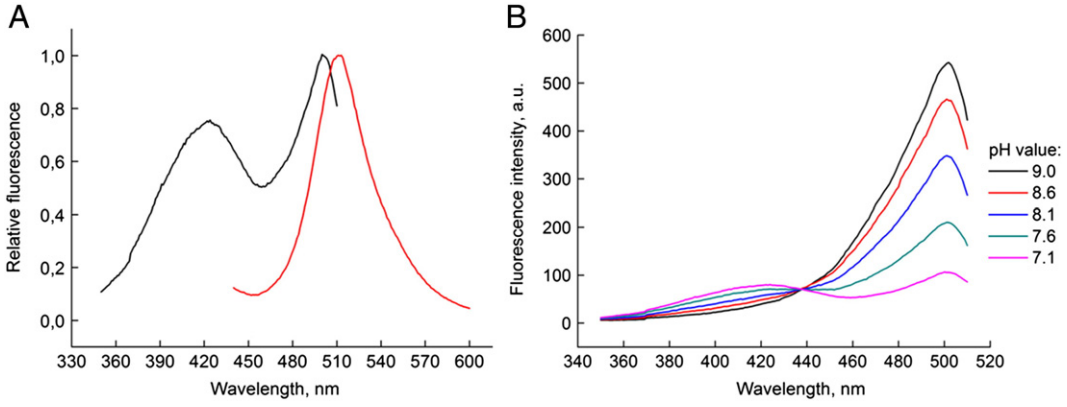

Fig. 1. A) Fluorescence excitation (em. 530 nm) and emission (ex. 420 nm) spectra for SypHer2 protein from crude lyzates registered at pH 7.1. B) Excitation spectra of SypHer2 at different pH.