Optical clearing for fluorescence visualization of dCas9/FP expression in tumor subcutaneous xenografts in mice

RESEARCH CENTER OF BIOTECHNOLOGY RAS

Gerel A. Abushinova[1,2], Liliya G. Maloshenok[1,2], Victoria V. Zherdeva[1]

1- Research Center of Biotechnology RAN, A.N. Bach Institute of Biochemistry, Moscow;
2- Vavilov Institute of General Genetics RAN, Moscow, Russian Federation

A549 cells were established for expressing double chimeras (SttdCas9-EGFP and NmdCas9-mCherry, clone E9) under inducible Tet-O doxycycline promoter. Tumor xenografts were established and maintained in athymic mice that were given doxycycline via gavage followed by fluorescence imaging using a planar system. It was shown that the highest fluorescence signal in tumor xenografts was registered on the 3rd day after induction of chimeric protein expression. Subsequently fluorescence detection was carried out on the 3rd day. To improve the contrast, a 0.7 M solution of gadobutrol was used. FI of red chimera expressed in tumor xenograft was amplified two-fold in vivo by applying 0.7 M gadobutrol due to the optical clearing (OC) of the skin. The MRI study reflected the perfusion of the tumor and coincided with the area of fluorescence.

At the previous stage of the work, genetically encoded chimeric constructs were constructed using the third generation lentiviral vector FU-tet-o-hOct4 (Addgene Plasmid # 19778, USA) as a precursor. In these constructs, under an inducible doxycycline-activated promoter, there are the dCas9 and FPs genes, connected by a linker and equipped with NLS sequences. The pair of FPs: EGFP and mCherry were selected due to their ability to engage in Forster resonance energy transfer (FRET)as a donor and acceptor, respectively. St dCas9-EGFP and NmdCas9-mCherry genetic constructs were obtained. Cells expressing St dCas9-EGFP and Nm dCas9-mCherry showed normal morphology with predominantly nuclear dual fluorescence. Before FP chimera induction with Dox there was no fluorescent signal detectible in cells proving tight regulation of chimera expression. FP expression was observed in vivo 1 day post induction and fluorescent signal was progressively decreasing during the course of 4-5 days following the withdrawal of Dox (Fig.1).

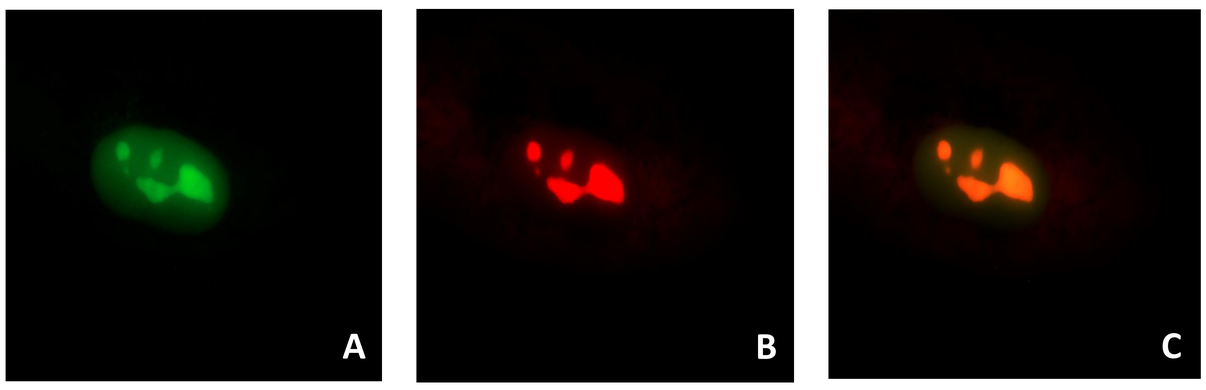

Figure 1 - Fluorescence microscopy of A549 StdCas9-EGFP-NmdCas9-mCherry 48 h after Tet- induced expression of chimeras by doxycycline (1 μg/ml). Zeiss Axio Observer Z1, DI-AxioCam-HRm, x100 (Scale (μm/pixel): 100x/1.4 px = 0.063 micron), A - green cube Ex. BP 470/40, BS FT 495/ Em. BP 525/50; B - red cube: Ex. BP 550/25 BS FT 570/Em. BP 605/70; C- the ooverlay of images (A) and (B).

Well-established cell clones expressing double chimera orthologues (StdCas9-EGFP and NmdCas9-mCherry, clone E9) (2.5-5 million cells) were inoculated in DBPS: Matrigel at a 1: 1 ratio subcutaneously (s.c.) into nu/nu mice (n=3). After tumor stabilization over 2 weeks mice were given doxycycline using gavage twice at the dose of 200 μg/kg. Fluorescence intensity was measured on the 1st ,3rd , 6th day after doxycycline (Dox) induction using a planar fluorescence visualization system. Signal intensity changes were determined by image analysis using manually drawn ROI (Fiji/ImageJ). (Fig.2).

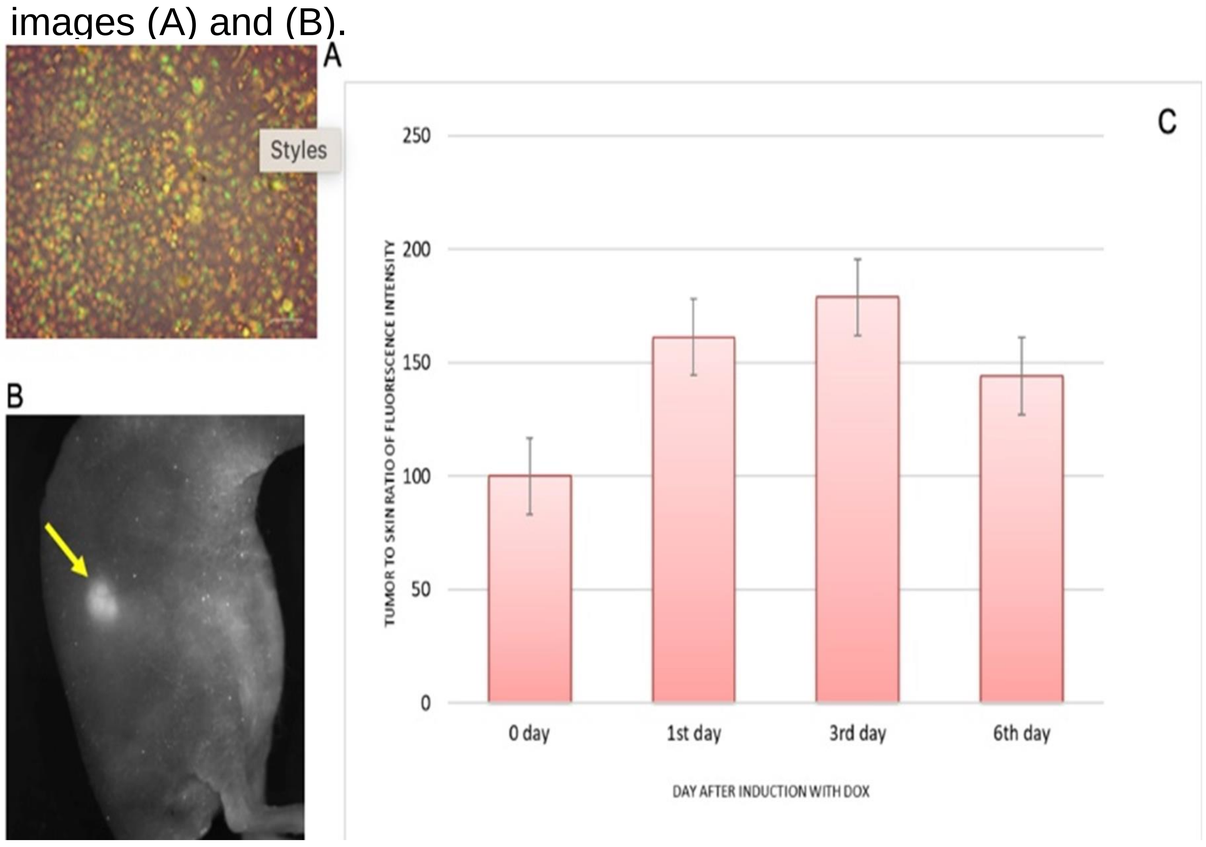

Figure 2 - A -expression of chimeric proteins in E9 cell culture, induced by the doxycycline (2 μg / ml) on the 5th day after induction (white-green-red channel merge). B - Normalized fluorescence measured in E9 tumor (A549 St dCas9 EGFP-Nm dCas9-mCherry) after the induction of Tet-on doxycycline-dependent expression of mCherry-fused dCas9 ortholog on the 3rd day. Excitation: 502-547 nm, fluorescence: 570-640 nm, (iBox imaging system). C- Selection of ROI normalized by skin autofluorescence in a representative animal on 0, 1st 3rd and 6th days.

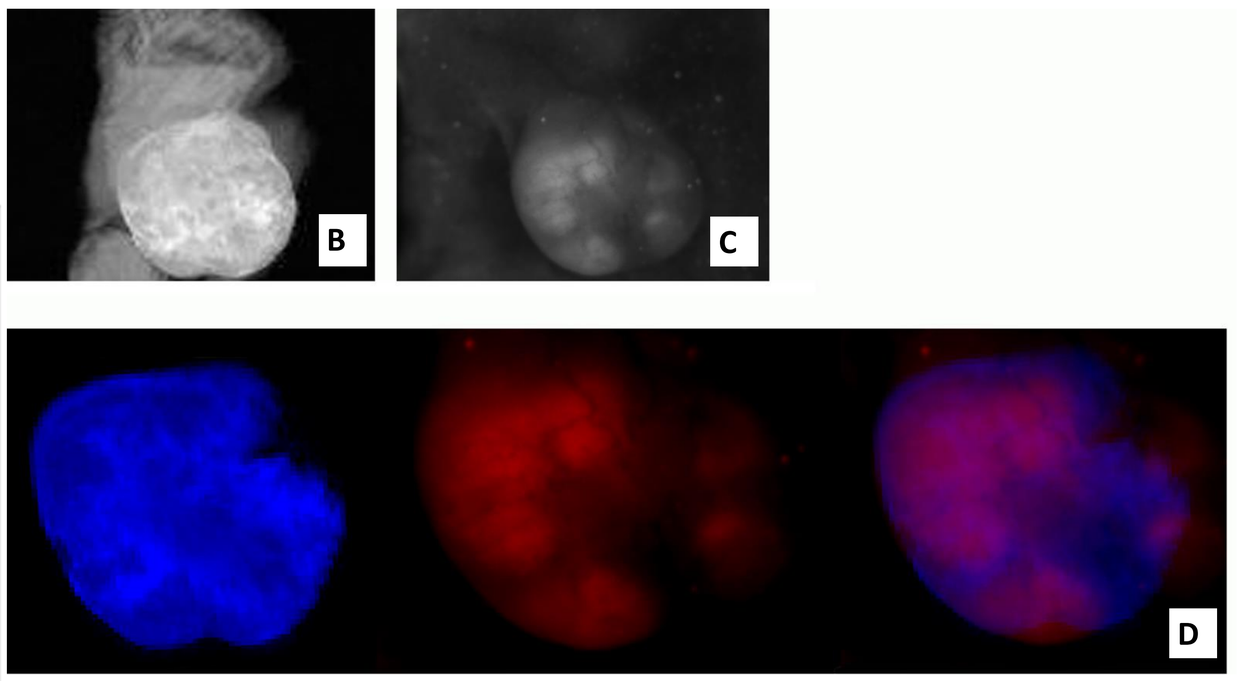

Figure 4 – A - 1T 3D GRE MR images (TR/TE=60/2.9 ms, FA 20o, NEX=5) 30 min after the application of 0.7M GB, maximum intensity projection image obtained using a stack of four MRI slices before OC; B - fluorescence of Nm dCas9-mCherry ortholog chimera (Supercontinuum laser (Fianium, UK): ex 540 nm, LP550nm, BP 590-620 nm, DCS-120 (B&H, Germany); C – a pseudo color image showing the overlay (C) of the MR-image (blue) and FI (red) of E9 tumor in the animal injected with 0.15 mmol/kg gadobutrol. Scale = 5 mm

Results: 2. In experiments involving E9 xenografts grown in athymic mice we observed that the highest FI was achieved on the 3rd day after induction of chimeric protein expression. To improve the contrast, a 0.7 M solution of gadobutrol was found to be useful for optical clearing of the skin surface resulting in overall increase of FI. I.V. injection of gadobutrol assisted in detection of regional tumor perfusion which delineated areas with inducible dCas9-FP chimera expression (Fig. 5).

Conclusions: The use of optical clearing approach enabled high-contrast imaging of dual (red and green fluorescent) chimeric dCas9-based proteins expression in tumor xenografts have been demonstrated. MR contrast agent gadobutrol improved both the intensity and contrast of FI as well as mapping of tumor perfusion by MRI.

Acknowledgment: This work was supported by the grant of Russian Science Foundation №22-14-00205