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

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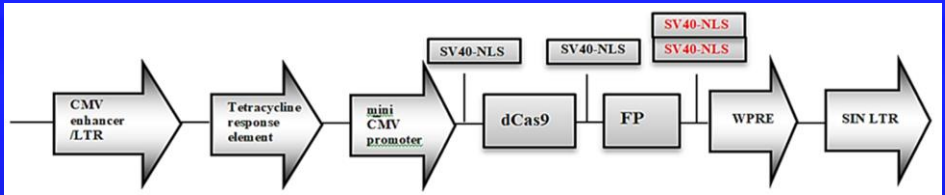
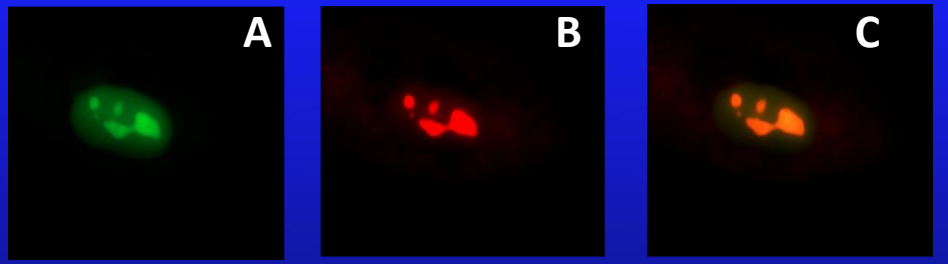
Tet-on system engeniering & fluorescence microscopy



Scheme of a genetically engineered construct St dCas9-EGFP and NmdCas9-mCherry

- FU-tet-o-hOct4 (Addgene Plasmid # 19778, USA) doxycycline-inducible Tet-On system.
- 2 NLS -fused catalytically inactive mutant dCas9 orthologs from Streptococcus thermophilus (St) and Neisseria meningitides (Nm) and (FP) marker were chosen at the ratio of 1:1.
- EGFP and mCherry were selected due to their ability to engage in Forster resonance energy transfer (FRET)
- FU-tet-o-linker construct was designed to carry a polylinker with unique restriction sites for dCas9 and FP orthologs in various combinations.
- FU-Tet-o-StdCas9-EGFP and FU-Tet-o-NmdCas9-mCherry were engineered by standard protocols to enable lentiviral particle production

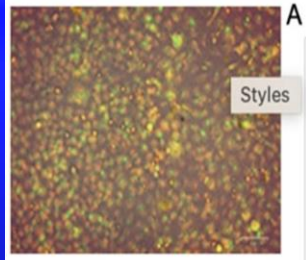


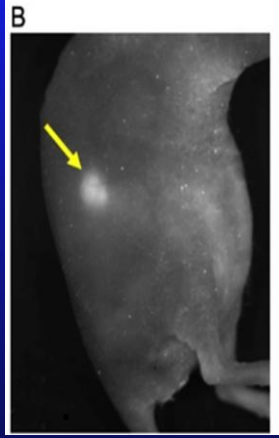
Fluorescence microscopy of A549 StdCas9-EGFP-Mn mCherry 48 h after Tet- induced expression of chimeras by doxycycline (1 µg/ml).

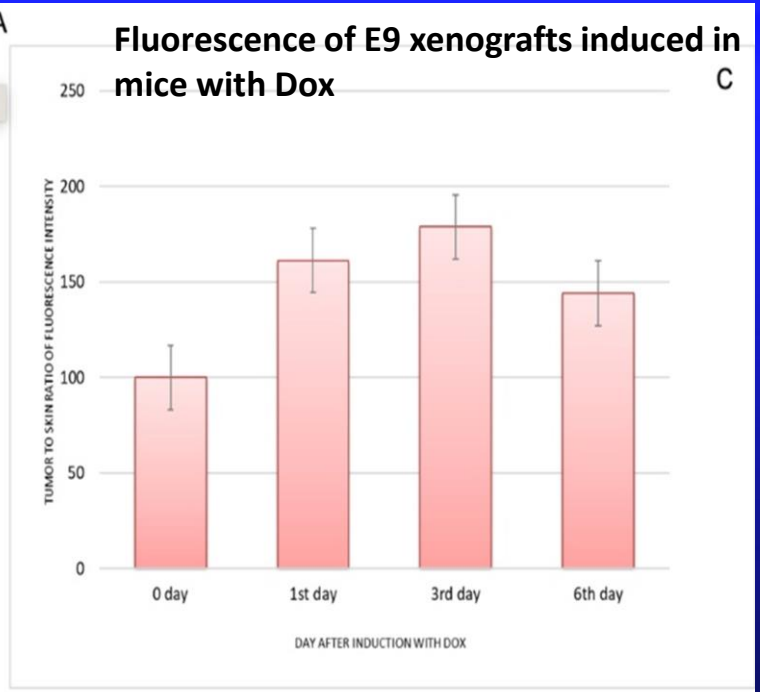
Zeiss Axio Observer Z1, DI-AxioCam-HRm, x100 (Scale (µm/pixel): 100x/1.4 px = 0.063 micron),

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)

Planar imaging of s.c. tumor





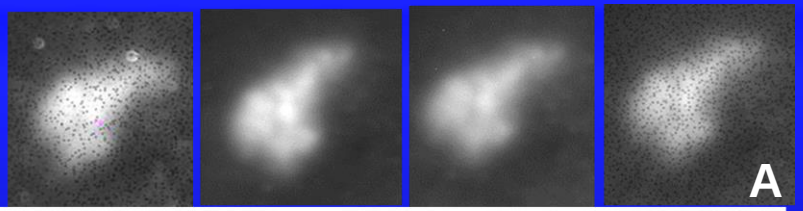


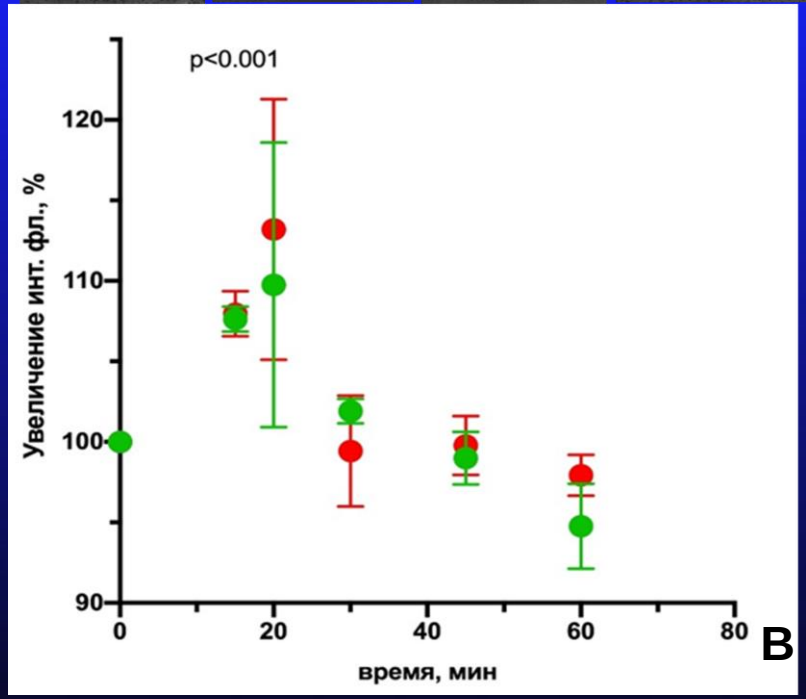
Well-established cell clones expressing double chimera orthologues (StdCas9-EGFP and NmdCas9-mCherry, clone E9) (2.5-5 million cells) were inoculated in DBPS: Matrigel at a 1: 1 ratio subcutaneously (s.c.) into nu/nu mice (n=3). After tumor stabilization over 2 weeks mice were given doxycycline using 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).

A -expression of chimeric proteins in E9 cell culture at passage 10, induced by the doxycycline (2 µg / ml) on the 5th day after induction (white-green-red channel merge Ex.480 / 17nm, Em: 517/23 nm/ Ex.556 / 20 nm, Em: 615/61 nm (Zoe cell imager).

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: before induction (day 0), on the 1st, 3rd and 6th days. Excitation: 502-547 nm, fluorescence: 570-640 nm, (iBox imaging system).

C- Selection of ROI normalized by skin autofluorescence in a representative animal.

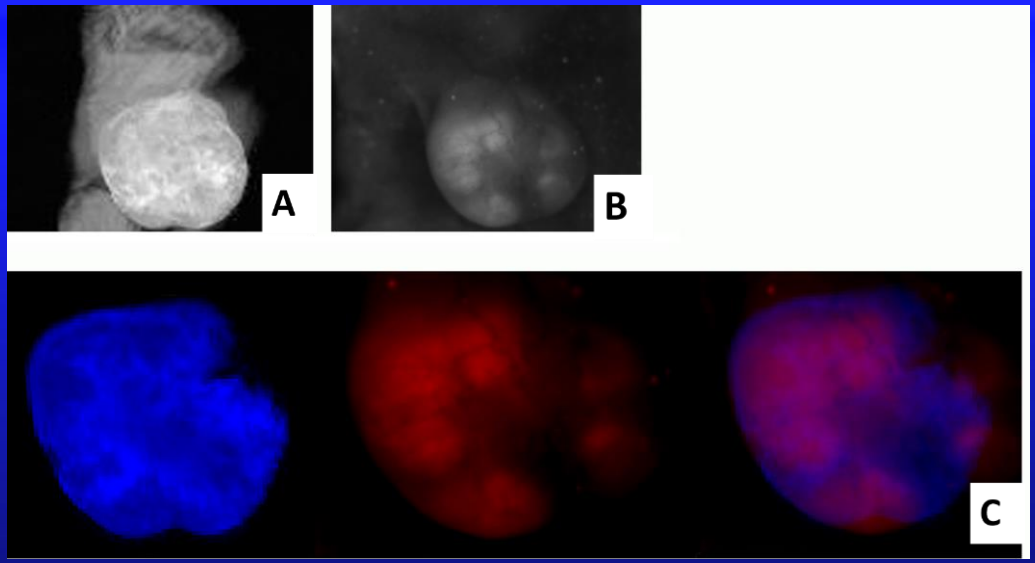




Planar imaging of s.c. tumor using optical clearing with 0.7 gadobutrol

- Fluorescence imaging (A) and skin-normalized fluorescence in the E9 tumor (B) at 3rd day after tet- induction using 0.7 M gadobutrol as an optical clearing (OC) agent (before optical clearing, 15 min after OC, 30 min after OC, after 45 min after OC, 60 min after OC) (ex 502-547 nm, em 570-640 nm, iBox UVP)

B- P values (Wilcoxon-Mann-Whitney test, n = 6-12 data points) are shown above the bars. Data are presented as mean ± standard deviation



MRI & Fluorescence imaging of s.c. E9 xenograft

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); E – 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

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

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.

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