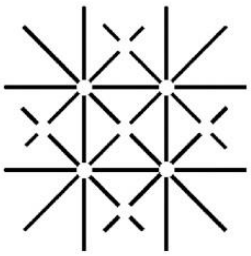

Uni Basel

The generation of transgenic PhOTO-Bow embryonic stem cells for direct multicolor lineage tracing

Master Thesis, M.Sc. in Nanosciences

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Abstract

Establishing new lineage tracing tools is important for providing new ways to study developmental biology. One of the state-of-the-art tools is the Brainbow transgenic multicolor labeling system which marks dividing cells with multiple distinct colors upon CRE recombination. By combining this global labeling system with the PhOTO approach developed by the Pantazis lab, the Brainbow was modified to a new version called PhOTO-Bow. This tool will enable both global and sparse direct labeling of single cells. Here we generated and tested the functionality of mouse embryonic stem (ES) cells with two copies of the PhOTO-Bow integrated into defined regions of the genome using CRISPR/Cas9. We successfully created transgenic PhOTO-Bow ES cells and analyzed their fluorescent profile after recombination using flow cytometry and microscopy. With further investigation, the PhOTO-Bow can be used as an accurate long-term lineage tracing tool.

Contents

1 Introduction

1.1 Lineage Tracing

Methods which enable to investigate cell behavior *in vivo* are of high importance in developmental biology. One of those methods is lineage tracing. By adding a tracker to a founder cell and trace it over time, relationships can be made between the founder cell and its progeny. This method may be used to visualize stem cells and their differentiated progeny or to achieve more information about how cancerous cells spread[1]. There are several features which a lineage tracer should possess. First, it should not influence the environment or even the characteristics of the cells. Second, all the progeny of the marked cell must inherit the label and none of the unrelated neighboring cells are allowed to inherit it. And, as the label has to be passed over several cell cycles, it should have the ability to be maintained in each marked cell[2]. By using lineage tracers, cells in early-stage embryos can be marked and followed to later phases to identify the importance and fate of those founder cells. Before fluorescent lineage tracers were used, scientist followed the cells through direct observation by using a microscope. The main drawback of this method is that the cells are not easily distinguishable, thus only a low number of cells can be followed. Additionally, more complex systems like embryos have to be accessible as for example in zebrafishes where the development is occurring externally and not enclosed as in mammalian systems[3]. A way to distinguish cells and enable lineage tracing in more complex organisms/organs in 3D is by inserting a trackable label into the genome. This label can nowadays be integrated at defined regions of the genome through the CRISPR-Cas9 system[1,2]. There are several loci in the genome which are called safe-harbor sites. Those sites have almost no effect on the global gene expression and as they are open for transcription a continuous expression level of an insert can be maintained. Two safe-harbor sites are the Rosa26 locus on chromosome 6 and the H11 locus on chromosome 11[4,5].

1.2 Fluorescent Proteins

A genetic label to make a cell trackable can be a DNA sequence encoding for a fluorescent protein (FP). A FP can be expressed ubiquitously or such can be coupled to another protein and thereby allow for quantitative imaging[6]. The main feature of a FP is that it can be excited through a laser and afterwards emits light of a higher wavelength. The underlying mechanism behind that is some molecules can absorb the energy of photons and excite through this an electron to a higher potential level. Subsequently, the molecule will spontaneously return to the ground electronic state through emitting light of lower energy as some of the energy is lost through conversion to heat[7]. The molecule which causes the fluorescence in FPs is the imidazoline ring

from the chromophore located in the center of the 3-D protein. FPs consist of 11 beta-sheets that are folded into a barrel structure and thus form a hydrophobic environment for the imidazoline ring. Slight changes in the chemical environment of the imidazoline ring have an effect on the fluorescence profile of the FP[6]. The first FP was derived from the jellyfish Aequorea Victoria named green fluorescent protein (GFP). The 3-D structure of GFP and the enclosed chromophore is shown in Figure 1[8]. By collecting more FPs from other bioluminescent animals and changing a few amino acids, different kind of new FPs with enhanced properties were derived. Some of the new FPs are mNeonGreen a more brighter GFP, mOrange2 an orange FP and mKate2 a far-red FP[9–12]. These days, there are enough FPs to cover up the entire visible spectrum. Which FP should be used for an experiment is determined by the maturation time, excitation and emission spectrum, photostability and brightness[6].

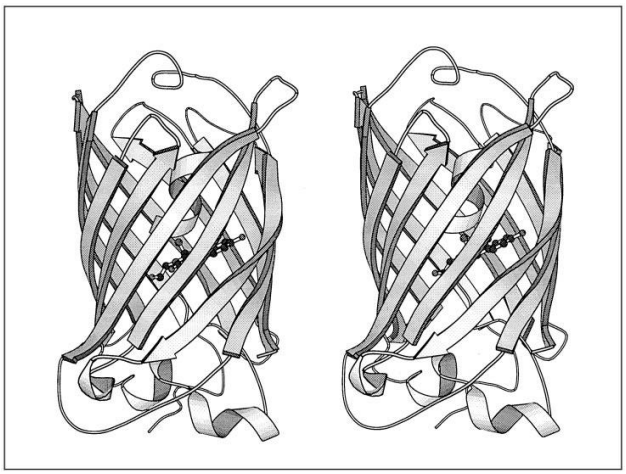

Figure 1: View of GFP and the enclosed chromophore (ball and stick). The protein takes the tertiary structure of a beta-barrel and thus forms a hydrophobic environment for the chromophore. The figure is taken from Ref[8].

1.3 Multicolor Genetic Labeling

By combining multiple FPs more sophisticated imaging experiments can be performed. Recombination is the process where DNA sequences are rearranged and thus may change the FP expression if multiple FPs are encoded in the genome. Recombination can lead to an insertion, excision, inversion or translocation of a DNA sequence. There are several methods which allow through a site-specific recombination, expression of multiple FPs in a cell. The FLP-FRT recombination technique which is primarily used in fruit flies depends on the heat shock controlled enzyme Flippase and the flippase recognition targets[1,2]. The FLP-FRT system has been used in the multicolor imaging tool Raeppli where cells in the wing imaginal disc of Drosophila were visualized through the color combination of four distinguishable FPs[13]. Another recombinase

technology is the Cre-Lox recombination which is derived from bacteriophage P1. As in the previously mentioned system, an interaction between an enzyme and two identical sites is needed, in this case the protein Cre (that causes recombination) and the loxP (locus of X-over P1) sites[14]. There are several lox sites that differ in sequence. They all consist of 34 base pairs and the first and last 13 base pairs are palindromic. The middle 8 base pairs spacer region, which gives the direction of the sequence, settles if there will be an excision (both sites show in the same direction) or inversion (one site shows in the other direction)[15]. The genetic cell-labeling technique Brainbow uses the idea of combining multiple lox sites and a few spectrally distinguishable FPs to generate hundreds of color combinations. For this, multiple copies of the Brainbow construct need to be present in the genome and Cre recombined. Through combinatorial probability, different patterns of recombined FPs will be expressed and thus lead to cells with unique color combinations. Some of the diverse Brainbow strategies and their influence on cells are shown in Figure 2[16,17].

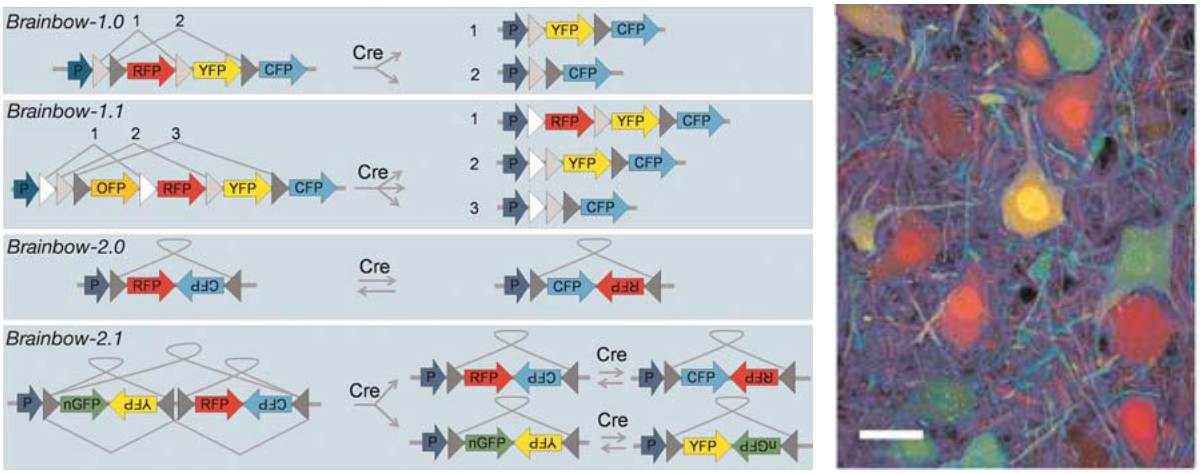

Figure 2: Left panel: Four different Brainbow constructs and their expression after Cre recombination. The number and direction of the lox sites decide if a sequence is excised or inverted. Right panel: Fluorescent brain stem of a Cre recombined transgenic Brainbow mice. Scale bar, 100 µm. Figures were taken from Ref[16].

1.4 Photoactivatable Fluorescent Proteins

Besides normal fluorescent proteins that excite light of a certain wavelength when they are excited, two other types of FPs were developed that can be photoactivated. These include non-fluorescing proteins which under near-UV light start to fluorescence (photoactivation) and fluorescing proteins which change their emission spectrum upon near-UV light illumination (photoconversion). The second type of FPs are of particular interest as they are visible before and after photoactivation[18]. One of those photoconvertible proteins is Dendra2 which is derived from the octocoral

Dendronephthya sp. The chromophore inside of the protein can irreversibly switch from green to red fluorescence after irradiation with a 405 nm laser[19]. This happens through the forming of a C-C double bond in the chromophore and cleavage of the protein backbone[20,21]. Photoconversion can be used on thin cell surfaces and 3-D structures but as the near-UV laser is not axially confined, multiple cells will be photoconverted. A way to photoconvert one precise cell in a 3-D volume is by using the primed conversion method, where two lasers lines cross and enable a precise three dimensional photoconversion[18]. First, the 488 nm "priming" laser excites the chromophore which subsequently relaxes into an intermediate state. In this state, the chromophore can again be excited through a 730 nm "converting" laser and at the end, the chromophore will be irreversibly converted into a red FP. This method enables to photoconvert only the cell of interest which is crucial in lineage tracing[22,23].

1.5 PhOTO-Bow

One lineage tracing tool which uses photoactivatable proteins is the PhOTO (photoconvertible optical tracking of nuclear and membrane dynamics in vivo) Zebrafish developed by the Pantazis lab. These transgenic zebrafishes possess the photoconvertible FP Dendra2 and a ubiquitously expressed blue FP (Cerulean). By fusing one FP with the H2B sequence and adding a fatty acid substrate as membrane signaling to the other FP, cells will have a both global and a sparse marker that allows for photoconversion of single cells. In Figure 3 a few cells of a PhOTO Zebrafish embryo were photoconverted. In this case, the green Dendra2 is targeted to the membrane and cerulean (blue) is targeted to the nucleus. The photoconverted cells show over multiple cell divisions a red membrane labeling[24].

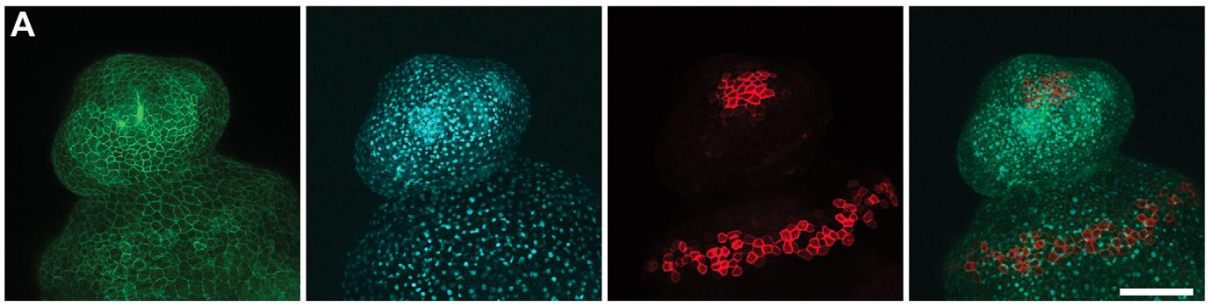

Figure 3: PhOTO Zebrafisch embryo microscopy image. A few cells were photoconverted and imaged the next day. The photoconverted Dendra2 which is membrane labeling is visible even after several cell divisions. Left to Right: unconverted Dendra2 (green), nucleic Cerulean (blue), photoconverted Dendra2 (red), all three channels merged. Scalebar, 150 µm. The Figure is taken from Ref[24].

The Pantazis lab has also developed another construct called the PhOTO-Bow (PB) that is based on the same idea of combining a global and single cell labeling as a

lineage tracing method (unpublished data). The PB consists of a Dendra2 FP, fused to H2B to generate a nuclear photoconvertible tracker and a Brainbow construct to create a multicolor labeling upon recombination. The Dendra2 is ubiquitously expressed, but the FPs encoded in the Brainbow can only be expressed after Cre recombination. This regulation is caused by a lox-Stop-lox DNA sequence at the beginning of the Brainbow construct which stops the transcription of the following sequence. By adding this Stop cassette no FP from the Brainbow construct will be expressed until Cre recombination. The FPs encoded in the Brainbow are mOrange2, mNeonGreen and mKate2. The excitation and emission spectra of the four FP are plotted in Supplementary Figure 1. Each of the FPs from the Brainbow construct is fused with a woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) to enhance the stability of mRNA and subsequently attached to a farnesylation group as a signal for membrane labeling. Between the FPs are different loxP sites so that after recombination only one FP can be expressed. The PB plasmid is flanked by two homologues arms to allow CRISPR/Cas9 based integration in their designated safe-harbor site. In the work presented here the ubiquitously expressed loci H11 or Rosa26 were used. A schematic of the PB is drawn in Figure 4 and the detailed map of the PB plasmid is shown in Supplementary Figure 2. The PB allows single-cell lineage tracing of the cells of interest after photoconversion (PhOTO Zebrafish) and thus the cells will be trackable until recombination of the PB has taken place (Brainbow method). In this way, the PB combines the advantages of the two different lineage tracing methods.

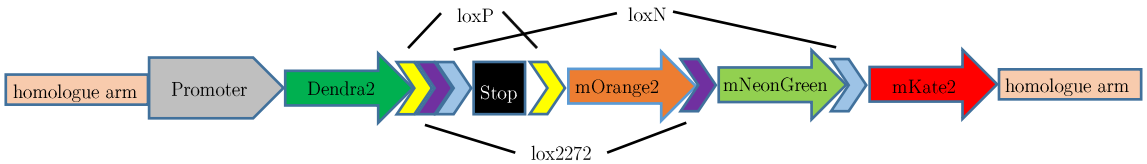

Figure 4: Schematic of the PhOTO-Bow construct. The first lineage tracing method in this construct is the photoconvertible Dendra2 which is fused to H2B for nuclear targeting. The second method is a Brainbow variation. Three FPs are fused to WPRE and farnesylation groups and divided through unique lox sites. After Cre recombination either mOrange2, mNeonGreen or mKate2 will be expressed. The arms which flank the central construct are homologues to a safe-harbor site to allow CRISPR/Cas9 based integration.

1.6 Aim of the project

The principal aim of this master thesis project was to create a mouse embryonic stem cell (mESC) line that has two copies of the PB construct integrated into define regions of the genome. After Cre recombination, each PB construct would express one of the

three FPs in order to generate a total of six different color combinations: green-green, green-orange, green-red, orange-orange, orange-red and red-red. Having the possibility of expressing multiple color variations is important in lineage tracing as it permits to visualize and distinguish several cells. Since the PB also includes a ubiquitously expressed H2B-Dendra2 sequence, this will give us the opportunity to label single cells before Cre recombination has taken place providing an advantage over multi-labeling lineage tracing tools that currently exist. A schematic of the functionality of two integrated PB constructs is drawn in Figure 5. Proving the power of the combined lineage tracing method will eventually lead into using the PhOTO-Bow in developmental biology.

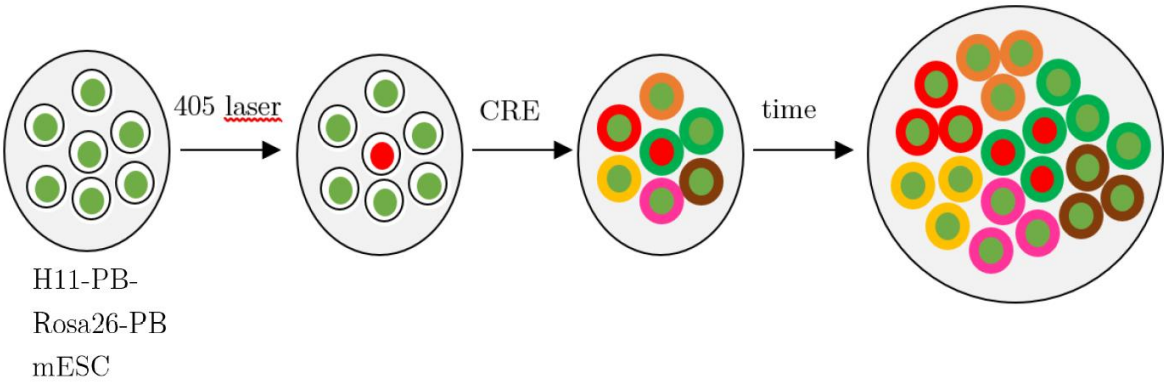

Figure 5: Schematic of the functionality of two integrated PhOTO-Bow constructs. As the transgenic cells express a nuclear targeting Dendra2 (green), one specific cell can be labeled with a near-UV laser by photoconverting the Dendra2 (red). Upon Cre recombination, the cells will recombine one of the six possible color combinations which are membrane labeling. As time passes, the progeny are going to express the same membrane label as their founder cells.

2 Material and Methods

2.1 Cell Culture

The experiments were done with the R1 mESC line and with two clones of the R1 H11-PB line in which one copy of the PhOTO-Bow is integrated in the H11 locus. All cells were cultured on tissue culture treated dishes coated with a 0.1% gelatin solution. The maintenance medium was made using DMEM High Glucose/glutaMAX (Thermo Fisher) with 15% FBS (PAN Biotech), 2 mM L-Glutamine (Gibco), 1x NEAA (Gibco), 0.1 mM beta-mercaptoethanol (Sigma), 1x Pen-Strep (Thermo Fisher), 100 U/mL Recombinant Murine LIF (PeproTech), 3 µM CHIR99021 GSK3 inhibitor and 1 µM

PD0390125 MEK inhibitor[25] and replaced every day. When the cells reached 80% confluency (every other day) they were split 1:10 using 0.05% trypsin (Thermo Fisher).

2.2 Cloning

All the PCR experiments were made in a Mastercycler® pro (eppendorf) using the Herculase II Fusion (Agilent) protocol. Inverse PCRs were made with the CloneAmp HiFi PCR Premix (Takara) protocol. The primers used for cloning the DNA sequences of pCAGGS, mKate2, mNeonGreen, mOrange2 and CRE in their designated vectors are listed in the Supplementary Table 1. The PCR products were purified with MinElute PCR Purification Kit (QIAGEN). The plasmid vectors pCAGGS-mKate2, pCAGGS-mNeonGreen, pCAGGS-mOrange2 and pCAGGS-CRE were generated with In-Fusion® HD Enzyme Premix 5x (Takara) protocol. The cloned vectors were restriction digested with several enzymes (New England BioLabs) and checked through gel electrophorese with a 1 kB DNA Ladder (New England BioLabs). Additionally, the clones were Sanger sequenced (microsynth).

2.3 Transformation and plasmid purification

The desired vectors were amplified by transforming Stable Competent E. Coli (New England BioLabs) cells through the standard High Efficiency Transformation Protocol (New England BioLabs). E. Coli colonies were afterwards collected and grown overnight in LB-ampicillin at 37 °C, rotating at 180 r.p.m. The plasmids were purified using a miniprep (Plasmid Miniprep - Classic, ZYMO Research) or maxiprep kit (BenchPro 2100, Invitrogen). The final plasmid concentration was measured with the spectrophotometer NanoDrop 2000c (Thermo Scientific).

2.4 sgRNA plasmid

The mESCs were transfected with the PhOTO-Bow by using a modified Palindromic single guide RNA plasmid (sgPal7, Supplementary Figure 3) from the self-cloning CRISPR protocol[26,27]. The sgPal7 was modified by adding the 20 base pairs ACTCCAGTCTTTCTAGAAGA which is a sequence on the Rosa26 locus and are needed as the target specification for the CRISPR/Cas9 system. First inverse PCR was performed as described in section 2.2 and the primers in Supplementary Table 1 to create a linear DNA sequence. Afterwards the old methylated sgPal7 template was cut with DpnI (New England BioLabs). Next, the PCR product was purified with the GenElute PCR Clean-Up Kit (Sigma-Aldrich), phosphorylated at the 5' end with T4 Polynucleotide Kinase (New England BioLabs) and subsequently ligated with T4 DNA

Ligase (New England BioLabs). Finally, the vector was transformed into Stable Competent E. Coli (New England BioLabs).

2.5 The generation of transgenic PhOTO-Bow mESCs

The transfection was made using the Lipofectamine 3000 (Thermo Fisher) protocol. Three plasmids were simultaneously transfected into mESCs: PB construct with arms homologous to the Rosa26 locus, a Cas9 plasmid with Blasticidin resistance and the new sgPal7 plasmid with the sgRNA for Rosa26. The sgPal7 plasmid has a Hygromycin B resistance sequence. After the transfection, the cells were selected for Hygromycin B and Blasticidin resistance by adding the antibiotics for 2 days. The positively transfected cells were first bulk sorted for green fluorescence emitted by Dendra2 expression, cultured and then single cell sorted for green fluorescence in 96-well plates. The single cell clones were cultured and genotyped to check for the correct integration of the PB transgene in the Rosa26 locus.

2.6 Fluorescence-Activated Cell Sorting

The fluorescence was measured with the FACS analyzer LSR Fortessa SORP (Special Order and Research Product, Becton Dickinson). For this, they were trypsinized, resuspended in fresh mESC medium and kept on ice before sorting. Three different laser/filter settings were needed to distinguish the different colors as described in Table 1. After the transfection as described in section 2.5 the cells were bulk and single cell sorted with an Influx cell sorter (Becton Dickinson) by gating for green fluorescence.

Table 1: Laser and filter setting of the FACS machine.

Name	**Laser Excitation**	**Band Pass**	**Long Pass**	**Color**
488 (C)	488 nm	530/30 nm	505 nm	Dendra2, mNeonGreen
561 (E)	561 nm	586/15 nm	570 nm	tdTomato, mOrange2
561 (C)	561 nm	670/30 nm	635 nm	mKate2

2.7 Genotyping

The DNA of the cell clones was harvested by lysing the cells with a lysis buffer (100 mM Tris pH 8.0, 5 mM EDTA, 0.2% SDS, 200 mM NaCl and fresh 200 µg/ml proteinase K). Cells in a 24-well dish were washed with PBS and 500 µl of the lysis buffer was added to each well. After an incubation time of an hour at 37 °C, the lysate was transferred to an Eppendorf tube containing 500 µl isopropanol. The tube was vigorous vortexed until a DNA precipitate was visible. Next, the DNA was centrifuged at 10'000 r.p.m. for 5 minutes to pellet the DNA. Afterwards the pellet was washed

with 75% ethanol, centrifuged again, air dried and resuspended in 100 µl TE buffer. The DNA was vortexed and incubated at 55 °C overnight to fully resuspend it in the buffer. The PCRs for the genotyping were made as in section 2.2 and by using 1 µl DNA and the primers in Supplementary Table 2.

2.8 Transfection

All the transfection reactions were made with either Lipofectamine 2000 Transfection Reagent (LF2K, Thermo Fisher) or with Lipofectamine 3000 Transfection Reagent (LF3K, Thermo Fisher). The cells were transfected in the beginning as in the LF2K or LF3K protocol but afterwards changed from a transfection with adherent cells to one with cells in suspension. For this, a well with 80% cell confluency was trypsinized and 1:20 passaged into a well from a 24-well plate were the LF2K or LF3K reagents were already added. The cells were pipetted up and down to achieve a higher transfection efficiency by mixing the cells with the reagents.

2.9 Microscopy

The microscopy images of the cells were made with a Zeiss LSM 780 (ZEISS) laser scanning confocal microscope. The microscope was equipped with an incubator box set at 37 °C and 5% CO_2. Transfected cells were cultured on a µ-Slide 8 Well (ibidi) with maintenance medium without the 2i inhibitors. The objective used was a C-Apochromat Apo 40x/1.1 W Corr M27 (ZEISS). For long-term imaging, the immersion fluid Immersol W (2010) (ZEISS) was needed. The FPs were excited with the lasers 488 nm, 561 nm, 594 nm and photoconverted with 405 nm. The filters were set between 491 - 545 nm, 560 - 569 nm and 600 - 640 nm to collect only the emission light of the corresponding FP. Image analysis was made with the program ZEN (grey and blue edition, ZEISS).

2.10 quantitative Polymerase Chain Reaction

The RNA of the cell clones was isolated using the TRIzol Reagent (Thermo Fisher) protocol. The RNA was DNase I (New England BioLabs) treated and the cDNA was synthesized with the SuperScript III Reverse Transcriptase (Thermo Fisher) protocol. In the end, 100 µl of ddH_2O was added. Two qPCRs were run on the LightCycler 96 (Roche) with LightCycler 480 Multiwell Plate 96 (Roche) and LightCycler 480 Sealing Foil (Roche). Primers were used for the genes GAPDH (housekeeping gene), Dendra2 PB, H11 locus, Rosa26 locus, Oct 4 and Nanog. Both times four different PB clones and wt cells were used during the qPCRs. Each cell line was tested for the six genes by performing technical triplicates. For the first qPCR, the 5x HOT FIREPol EvaGreen

qPCR Supermix (Solis BioDyne) protocol was used. The second qPCR was run with the KAPA SYBR FAST qPCR Master Mix (2x) (Kapa Biosystems) protocol. The ratio to compare the genes was calculated using the following formula:

$$\boldsymbol{ratio = 2^{-(\Delta C_t gene - \Delta C_t GAPDH)}}$$

3 Results

3.1 PhOTO-Bow

The multicolor genetic labeling tool PhOTO-Bow (PB) labels cells with a ubiquitously expressed nuclear photoconvertible Dendra2 and upon Cre recombination, it marks the membrane of the cells with mOrange2, mNeonGreen or mKate2. The goal is to generate mESC lines with two copies of the PB construct in their genome to enable recombination of six color combination. When we have accomplished the establishment of this cell lines we will check the performance of the multi-color labeling upon recombination and test the functionality for direct lineage tracing of single cells. The two homologues arms which flank the PB can be used for CRISPR/Cas9 based integration and thus precisely target the loci H11 (H11-PB) or Rosa26 (Rosa26-PB). The first step was modifying the sgPal7 (Supplementary Figure 3) plasmid with a sgRNA for the Rosa26 locus, through inverse PCR. The correct plasmid was checked by Sanger sequencing and is depicted in Supplementary Figure 4. Three different cell lines were targeted with the Rosa26-PB (Supplementary Figure 2) construct: R1 wt mESCs and two mESC lines named H11-PB Clone (Cl)2 and Cl5 which are already heterozygous for the PB at the H11 locus. Figure 6 shows that the two H11-PB clones are emitting green fluorescence from the Dendra2 protein which is encoded in the PB construct.

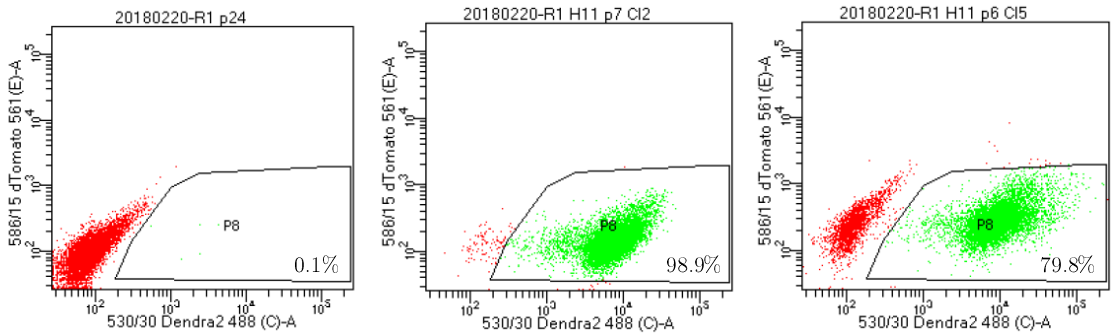

Figure 6: FACS measurement of wt cells and two H11-PB cell lines Cl2 and Cl5 which are heterozygous for the PB at the H11 locus. wt cells (left) exhibit compared to the other two no green fluorescence.

PB with Rosa26 homologous arms (Rosa26-PB), sgPal with Rosa26 sgRNA and a Cas9 vector were transfected into wt cells and the H11-PB Cl5 line through lipofection. The positively transfected cells were selected using antibiotics and the cells with green Dendra2 fluorescence were bulk sorted according to Figure 7. wt cells were additionally transfected to estimate the integration efficiency (15.3%) of the PB construct in the Rosa26 locus.

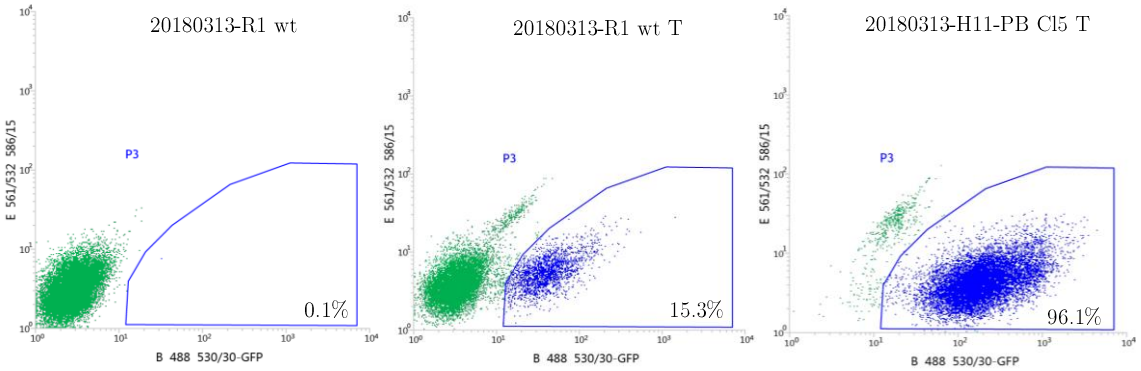

Figure 7: FACS bulk sort of cells transfected with Rosa26-PB, sgPal with Rosa26 sgRNA and a Cas9 vector. The transfected wt cells (middle) have compared to non-transfected wt cells (left) green fluorescence.

Single cell clones were sorted from the two bulk sorts, wt Rosa26-PB transfected and H11-PB Cl5 Rosa26-PB transfected, to generate clonal targeted ESC lines for genotyping. In Figure 8 the single cell sorts are depicted and in both cases the cells which had the brightest fluorescence were collected. H11-PB Cl5 transfected cells were sorted from gate P3 and wt transfected cells from gate P4.

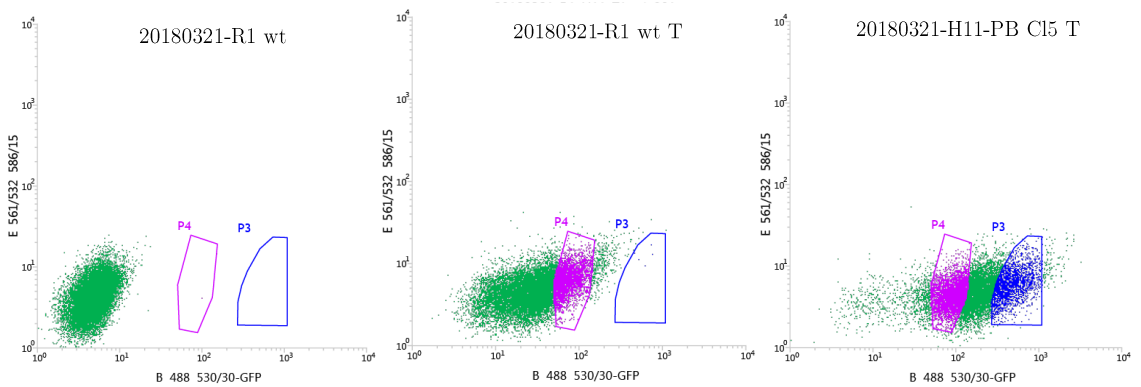

Figure 8: FACS single cell sort of the two bulk sorts, wt Rosa26-PB transfected and H11-PB Cl5 Rosa26-PB transfected. Cells from gate P4 were sorted as Rosa26-PB clones (middle) and cells from gate P3 were sorted as H11-PB clones which might have Rosa26-PB (right). wt cells were used as control (left).

The single cell clones were afterwards checked for the correct integration of the PB in the Rosa26 locus by genotyping. Five clones were collected: Rosa26-PB Cl1, 3 and 4

from the wt transfected cells and H11-PB-Rosa26-PB Cl39 and 41 from the H11-PB Cl5 transfected cells. For the genotyping four primer combinations were used: (1) Rosa26 fw - Rosa26 rev are located on the homologues arms of the Rosa26-PB and thus on the Rosa26 locus, they amplify the Rosa26 locus, (2) Rosa26 fw - CMV to Rosa26 rev amplify a sequence from the homologues arm to the promoter of the PB, (3) Dendra2 PB - mOrange2 rev are located in the center of the PB, (4) WPRE fw - Rosa26 rev amplify a sequence from a WPRE group to the second homologues arm. The results of the four primer combinations used on the cell lines wt, H11-PB Cl2 and Cl5, Rosa26-PB Cl1 and Cl3 and H11-PB-Rosa26-PB Cl39 are shown in Figure 9. The primers (1) who are located on the homologues arms but also on the Rosa26 locus were amplified by all clones. The primer combinations (2) and (4) which have one primer located on a homologues arm and one primer on the PB were only amplified by Rosa26-PB clones. This result doesn't prove that the Rosa26-PB cell lines have integrated the Rosa26-PB in the Rosa26 locus, just that it is integrated in the genome. An additional primer on the Rosa26 locus which is not on the homologues arms and a primer on the homologues arm would need to amplify a sequence to prove a correct integration. The primer combination (3) was not amplified clearly.

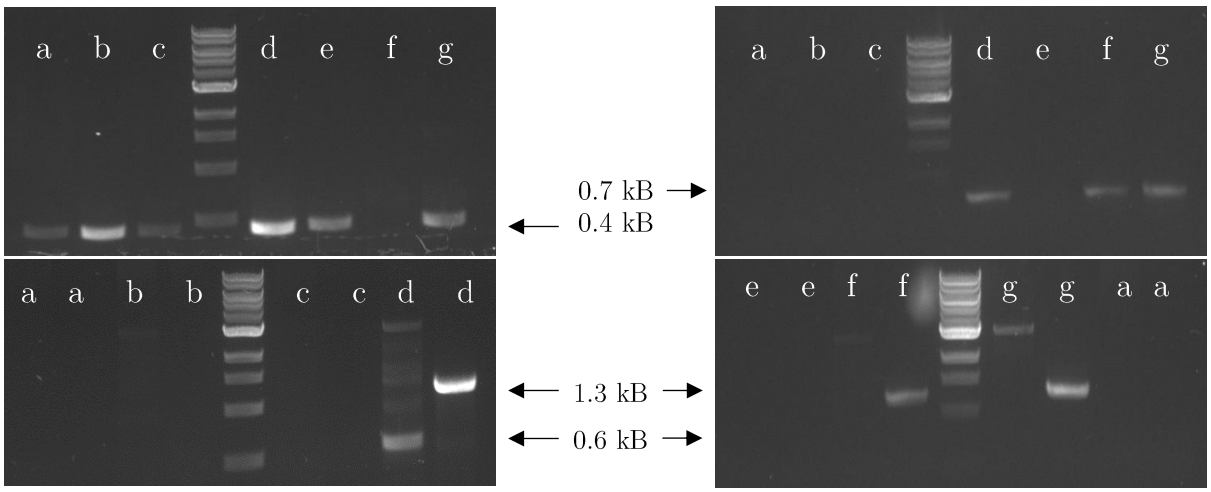

Figure 9: Genotyping results of the clones wt (a), H11-PB Cl2 (b), H11-PB Cl5 (c), Rosa26-PB Cl1,1 (d), Rosa26-PB Cl1,2 (e), Rosa26-PB Cl3 (f), H11-PB-Rosa26-PB Cl39 (g) by gel electrophoresis. Four different primer combinations were used: Rosa26 fw - Rosa26 rev (upper left), Rosa26 fw - CMV to Rosa26 rev (upper right), Dendra2 PB fw - mOrange2 rev and WPRE fw - Rosa26 rev (next to each other in the bottom row). a, b and c have only the band that corresponds to an intact Rosa26 locus.

Rosa26-PB Cl1 was separated on two cell lines Cl1,1 and Cl1,2 because two different populations were measured according to Figure 10. The cell line Rosa26-PB Cl4 was confirmed during the genotyping but the FACS results showed a very small population of green fluorescent cells as shown in Figure 10. The remaining fluorescent cells were

bulk sorted, however they lost the fluorescence after one month which may be caused through a only transient transfection of the Rosa26-PB. The cell line H11-PB-Rosa26-PB Cl41 showed a very drastic change of the fluorescent population over a period of two months. The transfected Rosa26-PB may have integrated into a locus which is needed intact for the survivability of a cell. In Supplementary Figure 5 the cell lines wt, H11-PB Cl2 and Cl5, Rosa26-PB Cl3 and H11-PB-Rosa26-PB Cl41 were measured for green fluorescence over 3 months with a FACS analyzer. As some clones may show unusual behavior, multiple single-cell clones need to be sorted to ensure the collection of stable PB clones.

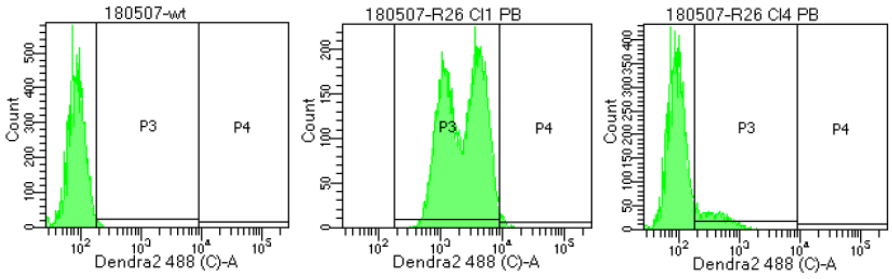

Figure 10: FACS measurement of two Rosa26-PB clones. Rosa26-PB Cl1 (middle) was separated into two separate cell lines Cl1,1 and Cl1,2. Even though Rosa26-PB Cl4 (right) was confirmed by genotyping as positive transfected, the number of green fluorescent cells was very low. Cells in gate P3 were bulk sorted, however lost the fluorescence after one month. wt cells (left) were used as a control.

3.2 Control Colors

The three different FPs which are encoded in the PB and can be expressed through Cre recombinase (mNeonGreen, mOrange2 and mKate2) were needed as vectors to adjust first the FACS analyzer and afterwards the confocal microscope to their corresponding fluorescence. A correct setting of the fluorescent spectra was important to distinguish which FP was recombined. The FPs together with the farnesylation group and the woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) were amplified from the PB vector. A plasmid which had a pCAGGS promoter (a combination of CMV enhancer and chick b-actin promoter) was used as a target for the FPs by combining them with In-Fusion cloning. In Figure 11 the FPs and the DNA strand with pCAGGS promoter were amplified by a PCR. Because mNeonGreen and mOrange2 had similar primers, the correct FP was verified with restriction digestion as shown in Figure 12. The three plasmids which encoded the individual FPs were afterwards transfected in mESCs to translate the FP and thus produce fluorescence.

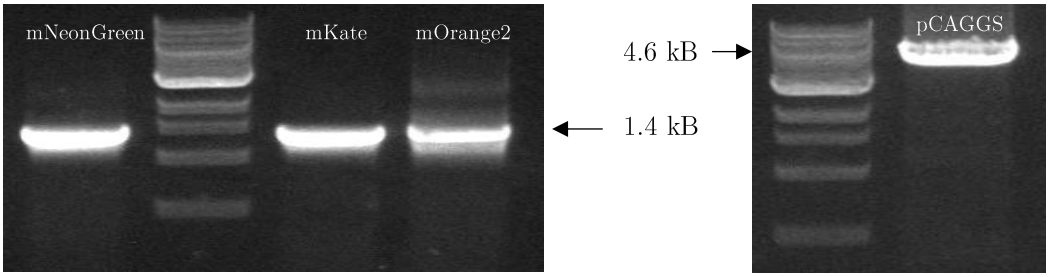

Figure 11: Gel electrophoresis result of the FPs from the PB construct and the pCAGGS vector. All three FPs have the same size.

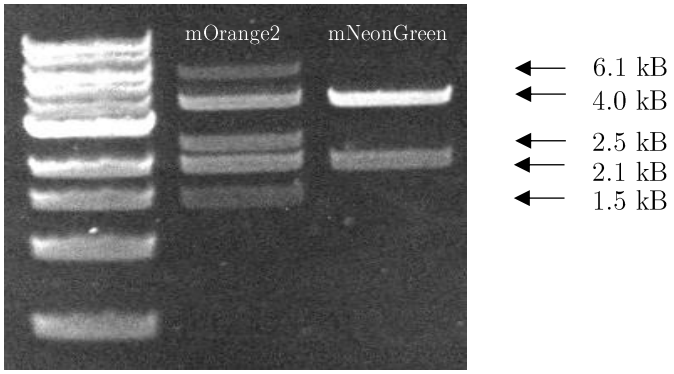

Figure 12: Gel electrophoresis result of mOrange2 and mNeonGreen. Three different restriction enzymes were used to distinguish mOrange2 from mNeonGreen. All three cut mOrange2 but one is a noncutter for mNeonGreen.

3.3 FACS compensation configuration

wt cells were transfected with the plasmids containing the individual FPs and analyzed by FACS to observe if the fluorescence is similar and thus not correctly distinguished. The cause of such a problem arises through the overlap of the emission spectra of the FPs. If the FACS is perfectly adjusted to the fluorescence spectra, it could analyze the percentage of recombined PB cells. By setting the compensation control of the FACS analyzer as in Supplementary Table 3, the transfected cells were counted to the correct channel. The influence of the compensation configuration is shown in Supplementary Table 4 for a monochrome transfection and in Supplementary Table 5 for a dichromatic transfection. The plots from the dichromatic transfection are shown in Supplementary Figure 6. After enabling the compensation control the FACS analyzer counts the FP cells almost only in the correct channel.

3.4 Transfection optimization

In order to get multiple recombination patterns upon Cre recombination the transfection efficiency of a CRE expressing plasmid needs to be high. wt cells, H11-PB Cl2 and Cl5 and all the Rosa26-PB cell lines were transiently transfected with plasmids by LF2K or LF3K reagents to determine the approach for highest transfection

efficiency. The plasmids used encoded FPs or CRE, they were co- or single-transfected and subsequently quantified with a FACS analyzer. All the results are numbered in Supplementary Table 6. The main findings were that transfecting cells with the LF2K or LF3K reagents when they were in suspension was much more efficient than transfecting them as an adherent layer (4). Co-transfecting two vectors had no drawback thus transfecting non-fluorescent CMV-CRE can be transfected together with a FP (3). The amount of Lipofectamine should be at least double the volume of the amount of total plasmid used per transfection (1+2). Vectors who code for a FP can be detected one day after transfection (7).

3.5 Imaging mNeonGreen, mOrange2 and mKate2

Before the Cre recombined cells were imaged, wt cells were transfected with the plasmids containing the individual FPs to adjust the range of the microscope detectors. This step was important to ensure that mOrange2, mKate2 and green FPs (Dendra2 and mNeonGreen) are visualized through different channels and thus enables one to decide which FP was recombined. In Figure 13 each FP is detected only in one specific channel.

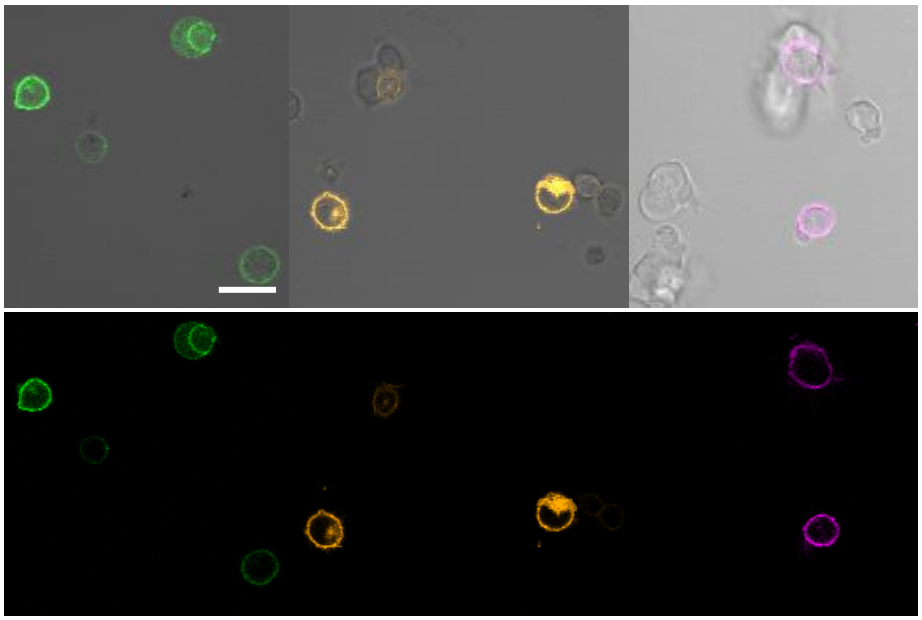

Figure 13: wt cells transfected with plasmids encoding mNeonGreen (left), mOrange2 (middle) or mKate2 (right) were imaged with a confocal microscope. In the upper row all channels are on and in the bottom row only the channel corresponding to its FP. Scale bar, 20 µm.

H11-PB-Rosa26-PB Cl39 cells which express Dendra2 were transfected with the mNeonGreen plasmid to check if the nuclear green FP can be distinguished from the membrane bond green FP. Cells with two integrated PB constructs can have upon Cre recombination the color combination green-green. If the two green FPs are not

distinguishable than there would be only five possible color combinations which determine a recombination event. According to Figure 14, the two FPs can be visually separated through a thin gap between the two labels. Additionally, as Dendra2 is photoconvertible and mNeonGreen not, they can be distinguished by photoconverting Dendra2 with a 405 laser.

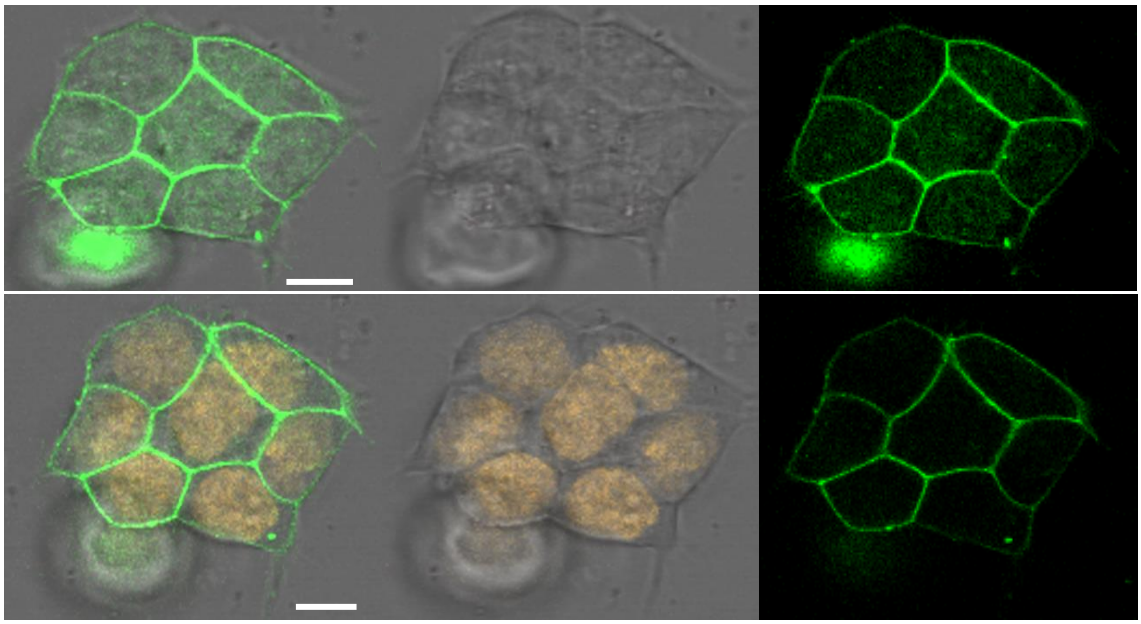

Figure 14: H11-PB-Rosa26-PB Cl39 was transfected with the mNeonGreen vector and imaged with a confocal microscope. Before the photoconversion, Dendra2 had the same color as mNeonGreen (upper row). After the photoconversion, Dendra2 gets the orange color and is in the middle of the cell because it has a Histone H2B labeling (bottom row). Scale bar, 10 µm.

The cell line H11-PB-Rosa26-PB Cl39 can have upon CRE recombination six different color combinations, because of the two integrated PB constructs. The FPs which will be recombined are membrane targeting. H11-PB-Rosa26-PB Cl39 was co-transfected with plasmids of two different FPs to check if the microscope can distinguish two membrane labels at the same time and location. After the image acquisition, the microscope successfully overlapped the two different colors and even provided a new color for the combination. In Figure 15 the color white was given to cells in which the green and red channel overlap, pink for the overlap of the orange and red channel and yellow for the overlap of the orange and green channel.

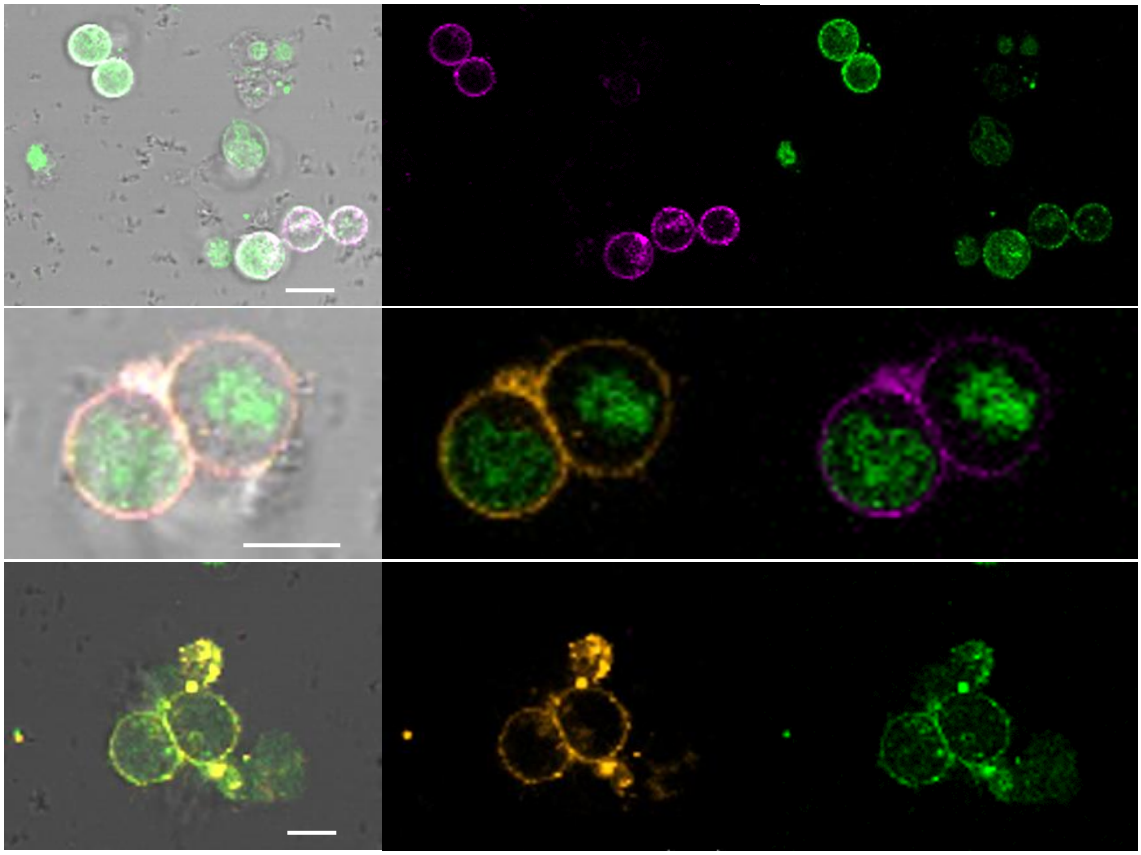

Figure 15: H11-PB-Rosa26-PB Cl39 was co-transfected with two different FPs (first row = mKate2 + mNeonGreen, second row = mOrange2 + mKate2, third row = mOrange2 + mNeonGreen) and were imaged with a confocal microscope. In the first column, the combination of the channels and their detected light result in a new color. White for green-red, pink for orange-red and yellow for orange-green fluorescence. Even if two different membrane labels are at the same position they can be distinguished. Scale bar, 10 µm.

3.6 CRE Recombination

The lox sites which separate the FPs in the PB lead to site-specific recombination by the enzyme CRE. Two different CRE expressing vectors were used to activate the recombination of the PB. The first one had a CMV promoter and the second one was generated through In-Fusion cloning by combining the CRE protein from the CMV-CRE plasmid and the pCAGGS promoter from the pCAGGS Eos plasmid. The correctly generated clone was found through restriction digestion as shown in Figure 16. A second CRE plasmid was made to check if the efficiency of Cre recombination is influenced by the promoter (CMV or pCAGGS) inside the plasmid.

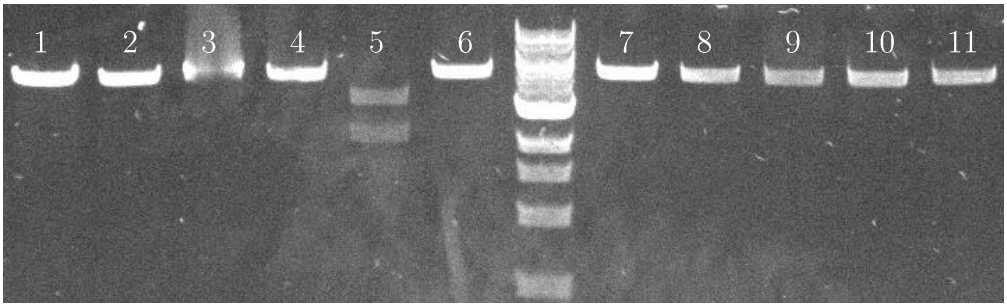

Figure 16: Gel electrophoresis result of the second CRE expressing plasmid generated through In-Fusion cloning. Through restriction digestion clone five was confirmed as a plasmid with the correct pCAGGS promoter and CRE protein.

Rosa26-PB and H11-PB ESCs were transfected with one of the two CRE expressing vectors. Those cells showed an increased amount of green fluorescence. This was visible with the FACS analyzer as depicted in Figure 17 and under the confocal microscope as shown in Figure 18. Some of the cells in Figure 18 have a brighter nucleic Dendra2 (green) and thus were photoconverted to verify if the brightness is not caused by a combination of Dendra2-mNeonGreen. This might happen because the 2A self-cleaving peptide situated in between them doesn't always cut, but after the photoconversion, no traces of green fluorescence were left. Most of the cells showing these bright nuclei also appear to have undergone recombination. Therefore, it is possible that after recombination of the PhOTO-Bow, the transgene becomes smaller and could influence the efficiency of transcription and translation of Dendra2. The plots in Figure 17 prove that the higher fluorescence isn't related to autofluorescence.

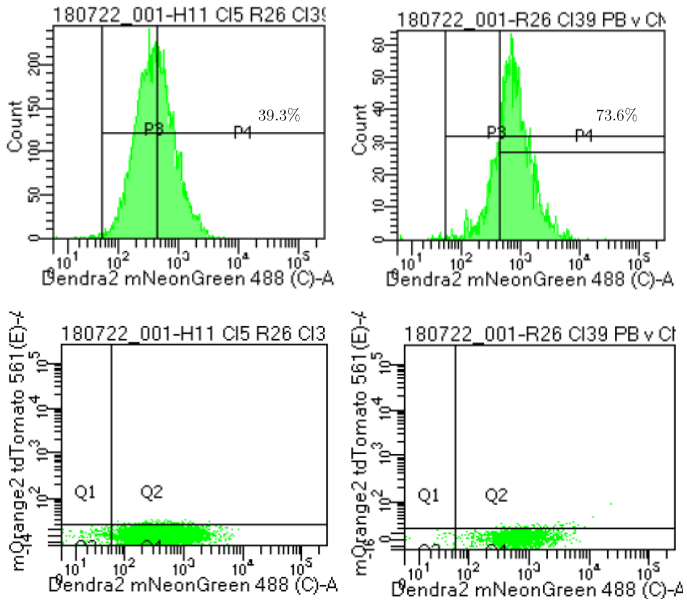

Figure 17: FACS measurement of H11-PB-Rosa26-PB Cl39 without (left) and with CRE (right). Adding CRE to a clone with a PB construct leads to a higher number of cells with brighter Dendra2. In this case 34.3% (73.6% - 39.3%) of the cells emitted brighter green fluorescence.

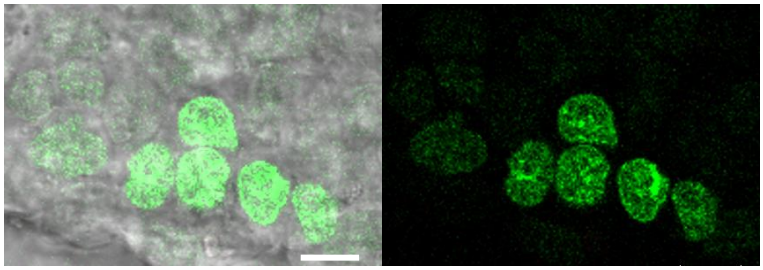

Figure 18: H11-PB Cl5 was transfected with a plasmid encoding CMV-CRE and imaged with a confocal microscope. Some of those cells showed a brighter nucleic green fluorescence. Because the five brighter cells are clearly separated from each other, the green fluorescence can only be emitted by Dendra2 and not mNeonGreen which is membrane labeling. Scale bar, 10 µm.

H11-PB Cl5 was Cre recombined and as this cell line has only one PB construct, each cell can have only one kind of membrane labeling upon Cre recombination. However, the emission spectrum of mOrange2 overlaps with the one from mKate2 which can lead to false interpretation of the Cre recombination. If the channels/detector ranges are not set correctly, some cells seem to have recombined mOrange2 and mKate2 proteins as seen in Figure 19. Setting the detector ranges as in section 3.5 is important for distinguishing which FP recombined.

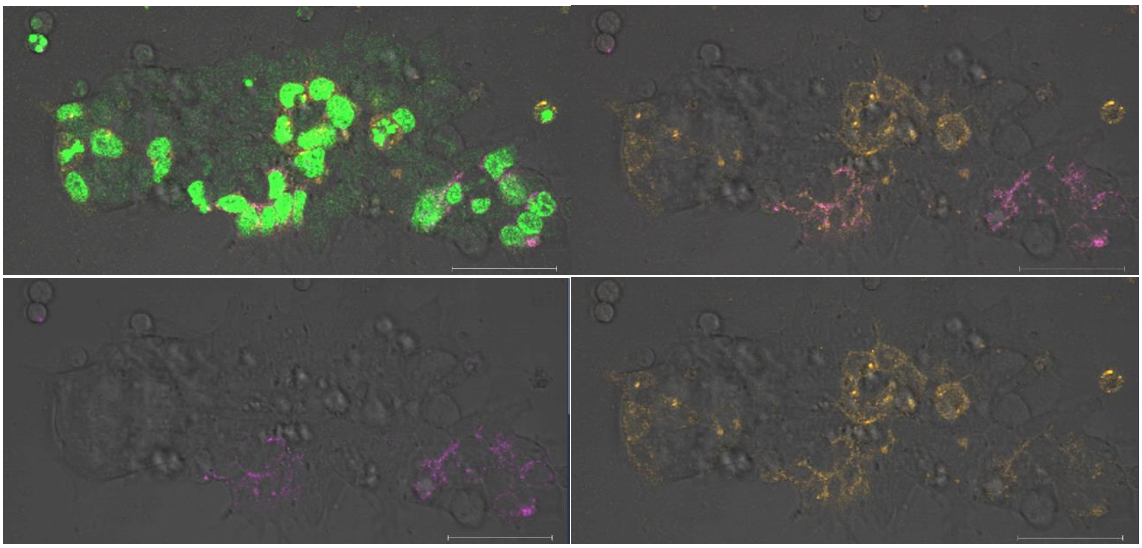

Figure 19: H11-PB Cl5 was transfected with a plasmid encoding CMV-CRE and imaged with a confocal microscope. This are the only images where the detector ranges are not correctly adjusted. The incorrect detection led to mOrange2 recombined cells which fluoresced in the orange and red channel. Scale bar, 50 µm.

Figure 20 shows that the CRE recombined FP mKate2 is labeling the membrane as same as in the experiment (Figure 13) where the plasmid which encoded mKate2 was transfected in cells.

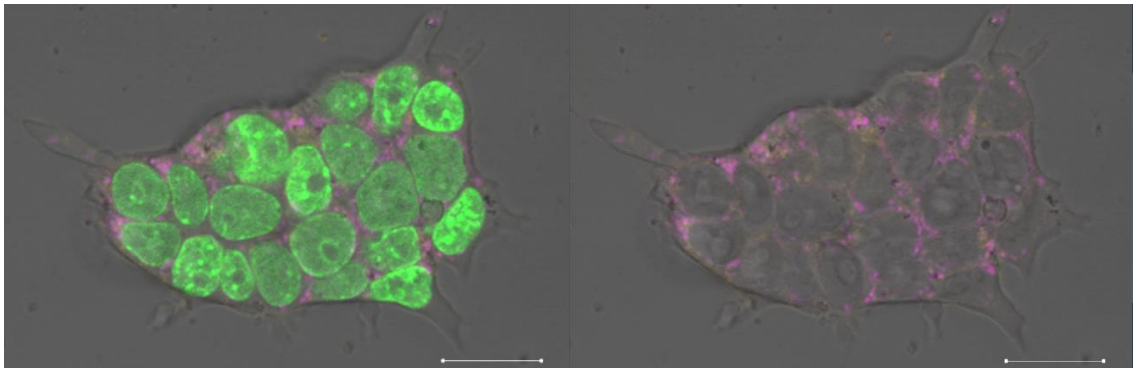

Figure 20: Rosa26-PB Cl3 was transfected with a plasmid encoding CMV-CRE and imaged with a confocal microscope. After the recombination mKate2 proteins were translated by the cells and because of the farnesylation group bound to the cell membrane. Scale bar, 20 µm.

Additionally, the combination of Cre recombination and photoconvertible Dendra2 was tested. Therefore, a time-lapse series was run to prove that expression and visibility of the recombined FPs happen before the disperse of photoconverted Dendra2. H11-PB-Rosa26-PB Cl39 was transfected with a plasmid encoding CMV-CRE and four hours later used for the time-lapse imaging over a period of 48 hours. Right before starting the time-lapse experiment, one cell was photoconverted with a near-UV laser and showed afterwards a nuclear orange label. Some recombined cells were expressing the membrane label 25 hours after transfection with the plasmid, meanwhile the photoconverted Dendra2 was still clearly visible. At the end of the time-lapse was the photoconverted Dendra2 distributed over too many cells and thus hindered the single-cell tracking. The one photoconverted cell and its progeny did not recombine as the transfection efficiency is not high enough. To visualize a photoconverted cell which recombines one of the color combinations, multiple cells would need to be photoconverted to increase the probability of such an event. The time needed until Cre recombined FPs are labeling the membrane is short enough to overlap with the single-cell tracking by photoconverted Dendra2. Figure 21 and Figure 22 show the time-lapse series from the same location, once with bright field and once without, in order to ease the visualization of the colors.

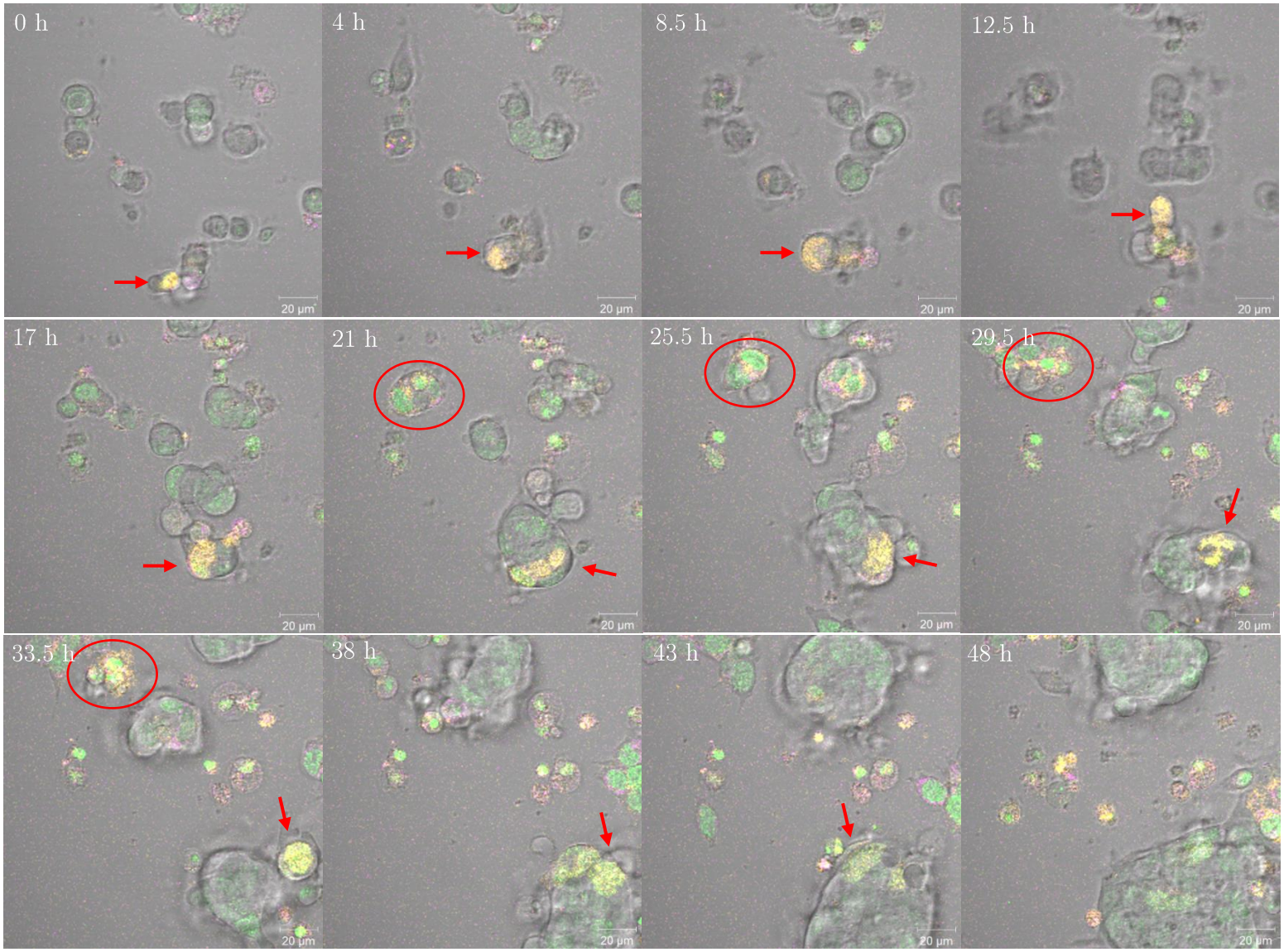

Figure 21: 48-hour time-lapse series of H11-PB-Rosa26-PB Cl39 cells transfected with a plasmid encoding CMV-CRE imaged on a confocal microscope. The cells were transfected 4 hours before the time-lapse and the cell photoconverted (red arrow) right before the time-lapse started. 25 hours after transfection, the FPs mKate2 and/or mOrange2 are labeling the membrane of a few cells (red circle). The recombination of the PBs and the labeling of the membrane is occurring before the photoconverted Dendra2 is not trackable anymore.

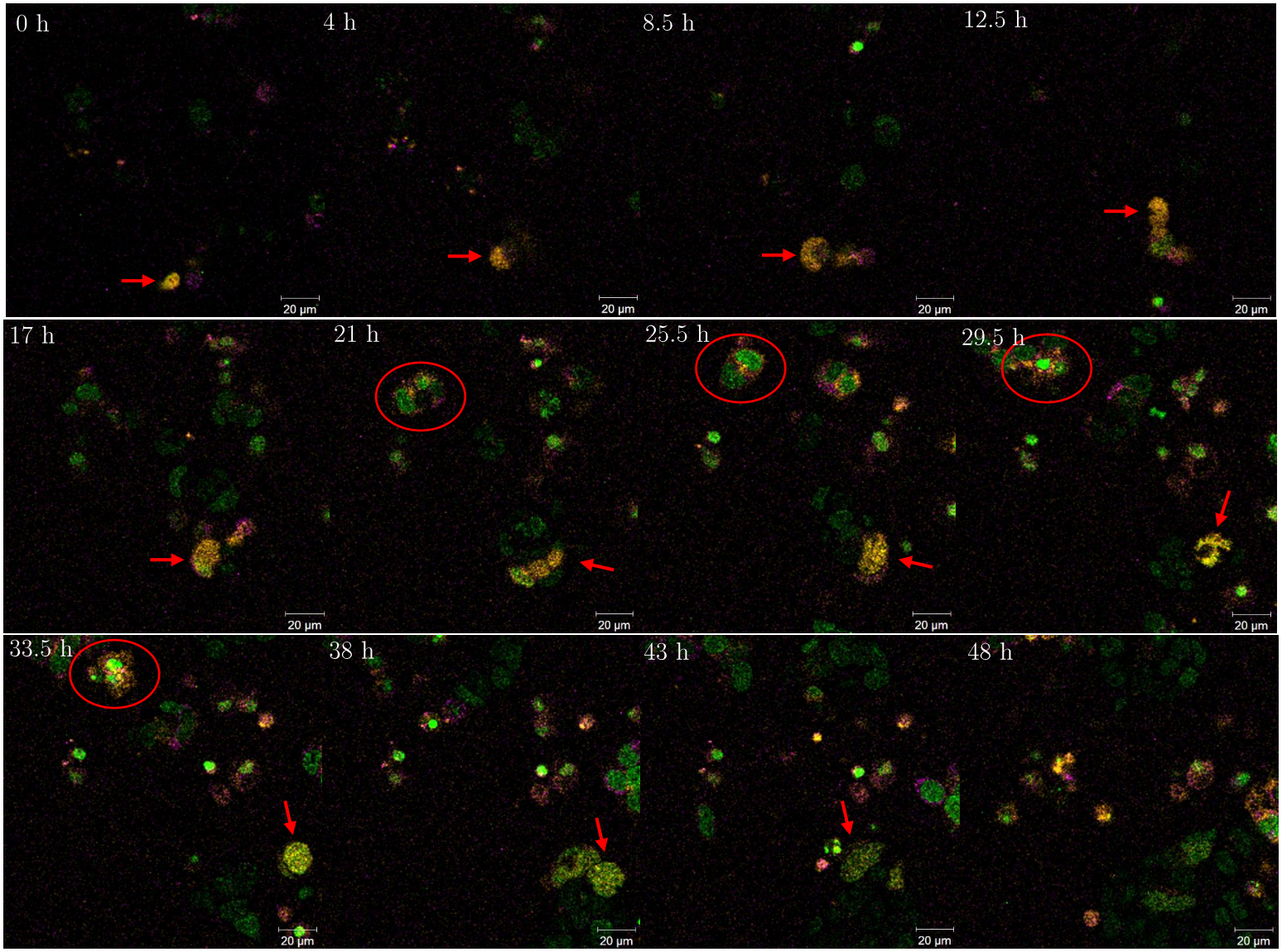

Figure 22: 48-hour time-lapse series of H11-PB-Rosa26-PB Cl39 cells transfected with a plasmid encoding CMV-CRE imaged on a confocal microscope. The cells were transfected 4 hours before the time-lapse and the cell photoconverted (red arrow) right before the time-lapse started. 25 hours after transfection, the FPs mKate2 and/or mOrange2 are labeling the membrane of a few cells (red circle). The recombination of the PBs and the labeling of the membrane is occurring before the photoconverted Dendra2 is not trackable anymore.

We previously characterized the H11-PB-Rosa26-PB Cl39 to carry two copies of the PB transgene which should enable labeling with two colors simultaneously after recombination. At the end of the time-lapse series, we found cells that showed both mOrange2 and mKate2 membrane labeling as depicted in Figure 23. Achieving six different color combinations was not possible as the green membrane label from the mNeonGreen protein was not detected. Only three different combinations were successful recombined: orange, red and orange-red. A possible reason for the non-detectable mNeonGreen would be a mutation at the lox2272 8-base pairs sequence which disables correct splicing of mOrange2 and thus the production of mNeonGreen.

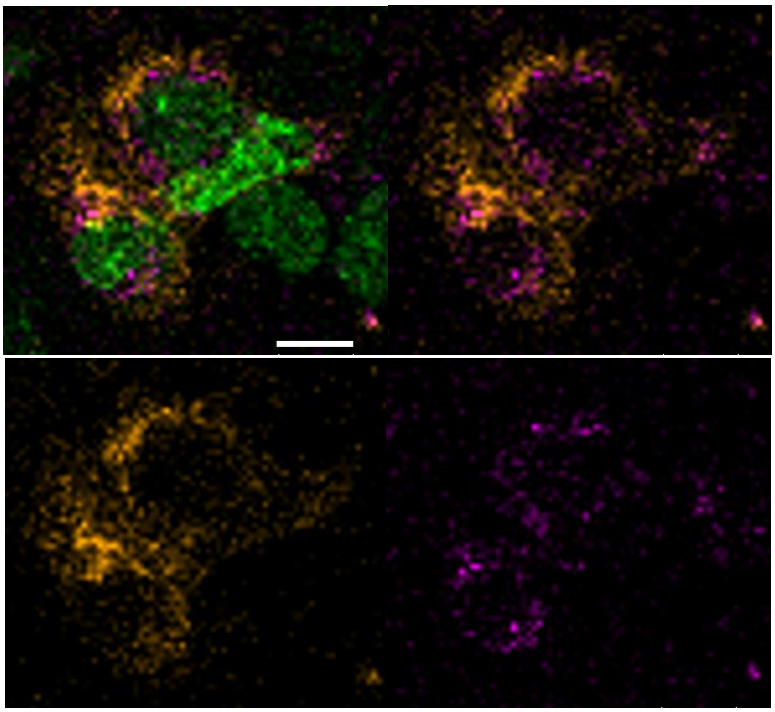

Figure 23: H11-PB-Rosa26-PB Cl39 transfected with a plasmid encoding CMV-CRE and imaged after 52 hours on a confocal microscope. One of the PB constructs recombined mOrange2 whereas the second PB recombined mKate2. Scale bar, 10 µm.

The four cell lines Rosa26-PB Cl1,2, H11-PB-Rosa26-PB Cl39, Rosa26-PB Cl3 and H11-PB Cl5 which were transfected with CRE and used during the time-lapse experiment where afterwards FACS analyzed. The gates were placed in such a way to visualize the number of orange (P7) and red (P8) fluorescing cells. This is possible as mOrange2 and mKate2 have different fluorescence spectra. In Figure 24 Rosa26-PB Cl1,2 showed that the orange and red fluorescing cells are separated during the analyzing because those cells can recombine only one of the FPs. Cells with two integrated PB constructs as H11-PB-Rosa26-PB Cl39 can have upon Cre recombination orange fluorescing cells that overlap with red ones. The FACS results

match with the images taken with the confocal microscope. In Supplementary Figure 7 are shown the results for Rosa26-PB Cl3 and H11-PB Cl5.

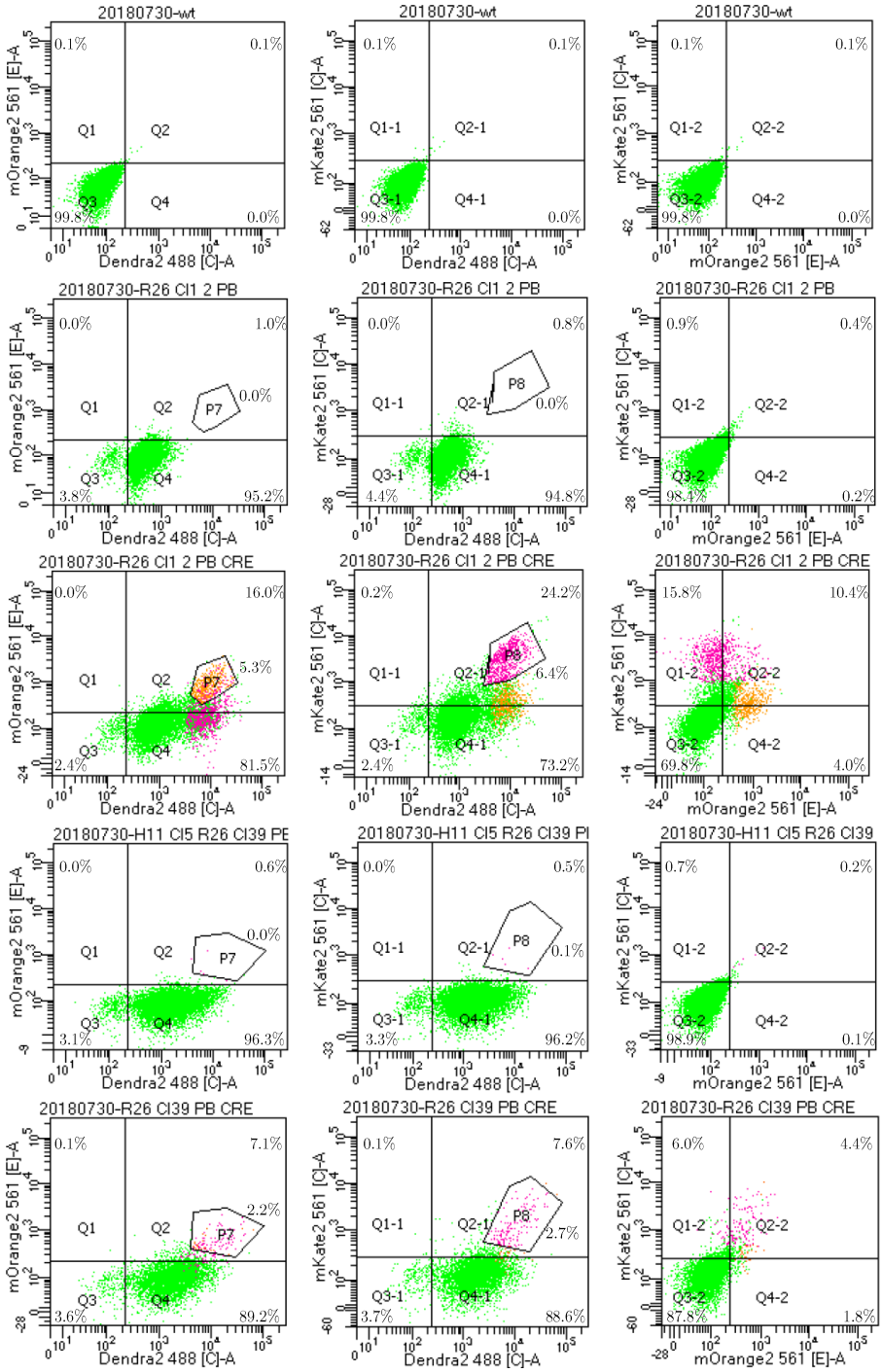

Figure 24: Rosa26-PB Cl1,2 and H11-PB-Rosa26-PB Cl39 were FACS analyzed three days after transfection with a CMV-CRE encoding plasmid. wt cells were used as a control. Cells in gate P7 correspond to mOrange2 expressing cells and in gate P8 are the mKate2 expressing cells. In the plots from the fifth row, the H11-PB-Rosa26-PB Cl39 cells are overlapping in both gates and thus express both FPs.

3.7 qPCR

Transgenic cell lines with two PB constructs integrated in the two safe-harbor sites H11 and Rosa26 would ubiquitously express their insert. Differences in the expression level of the loci would influence the color combination generated upon Cre recombination. If the differences are visible there will be more than the assumed six recombination patterns. For example, the combination orange-red fluorescing cells will have to be split into strong orange-weak red and weak orange-strong red fluorescing cells. Two qPCR experiments were set to compare the expression levels of the genes Dendra2 from the PB, H11 locus, Rosa26 locus, Oct 4 and Nanog in the different transgenic cell lines. They were compared to the housekeeping gene GAPDH. The first qPCR was run with the cell lines Rosa26-PB Cl1,1, Cl1,2, Cl3 and H11-PB-Rosa26-PB Cl41 and in the second one H11-PB Cl5, Rosa26-PB Cl1,2, Cl3 and H11-PB-Rosa26-PB Cl39. Both times wt cells were used as a control. All cells with a PB construct were expressing Dendra2 and the clones with two PB constructs had a much higher expression level compared to the other clones. The genes Oct 4 and Nanog which are involved in the self-renewal of undifferentiated embryonic stem cells were shown by all the cells at similar levels. By adding a differentiated cell line into this experiment, it would be possible to confirm that mESCs have a higher expression of self-renewal genes compared to differentiated cells. The expression level of the H11 locus is according to the results around 5x higher than of the Rosa26 locus. If this has an influence on the recombined colors and their combination patterns, needs to be investigated.

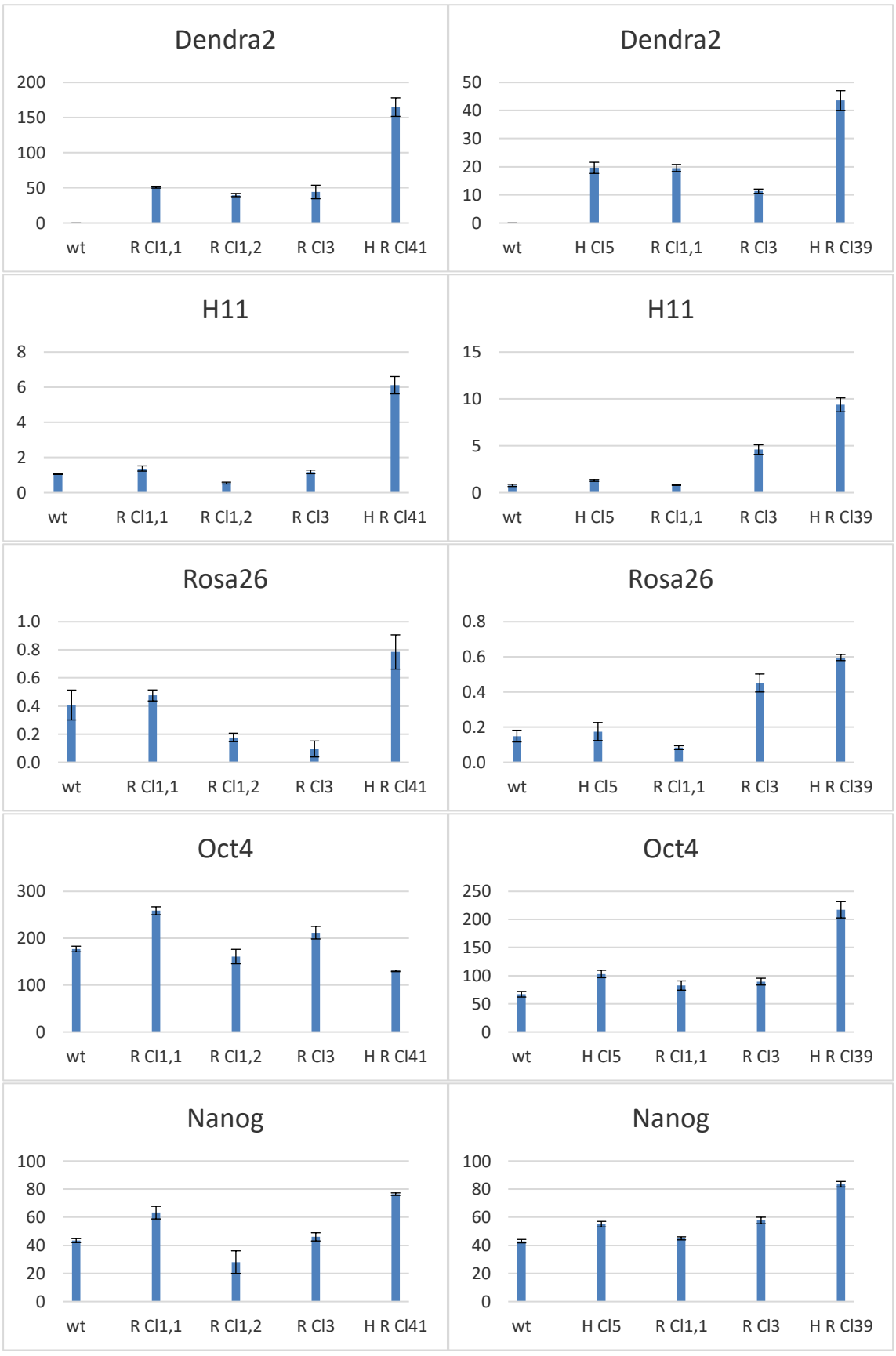

Figure 25: The expression level of five genes was compared to the one for GAPDH through qPCR. Two experiments were performed. The first one in the left column with ΔC_tGAPDH = 29.3 $\pm$ 1.9 and the second one in the right column with ΔC_tGAPDH = 25.8 $\pm$ 1.4. On the x-axis are listed the clones, the y-axis is the dimensionless ratio. R = Rosa26-PB, H = H11-PB.

4 Discussion and Conclusion

Lineage tracing, a method used in developmental biology were cells are followed over time, is progressing by incorporating more and more advanced tools. The PhOTO-Bow is one tool that combines a global and single cell labeling in one lineage tracing technique. A transgenic mESC line H11-PB with one PB construct in its genome already existed. The goal of this project was to create transgenic cells which have an additional PB in the Rosa26 locus. The Rosa26-PB plasmid was successfully incorporated in the Rosa26 locus by using CRISPR/Cas9. During the selection process of the correctly targeted Rosa26-PB clones, we noticed that some clones may show transient expression of Dendra2 and thus needed more clones. However, finding clones with two PB constructs was difficult because it was not possible to sort for green fluorescence since the cells were expressing already Dendra2 from the H11-PB. A way to ease the harvesting of cells which could recombine six different colors and have a single-cell tracker (Dendra2) would be by using the H11-PB ESCs and a slightly modified Rosa26-PB. The modification would be a ubiquitous expression of mOrange2. H11-PB cells transfected with this modified Rosa26-PB can be sorted after the orange fluorescence. The new clone would still have the nuclear green Dendra2 but also an orange membrane label that can be changed through recombinases. The main drawback is that the color combination orange-orange cannot be related to Cre recombination anymore as the cells already show an orange label. Genotyping the transfected cells is nevertheless mandatory to check for the correct incorporation of the plasmid.

Even though multiple experiments were made with vectors to achieve a high transfection efficiency, recombination of PB cells with a CRE encoding plasmid was always under 20%. In the case of H11-PB-Rosa26-PB where two PB constructs are integrated it would be great to have a recombination efficiency of 60%. That way each color combination would be visible in 10% of the cells if the recombination of the six colors is equally likely.

Unfortunately, the FACS plots don't match perfectly with the microscopy images because the FACS analyzer used different lasers and filters. But the FACS results showed as same as the microscopy images that red, orange and red-orange cells were recombined after transfection with the CRE vector. If mNeonGreen would be much brighter than Dendra2 and they thus be separable from each other, it might be possible to distinguish all six color combinations. It would be useful to separate the color combinations by FACS because then it would be possible to calculate the probability of the recombination events.

It is beneficial being able to not only mark all the cells with a label but also to trace one specific cell during its lineage. The time-lapse result shows that the combination of the global lineage tracing technique Brainbow/CRE recombinase and

the sparse lineage tracing technique PhOTO/photoconversion worked. The visibility of the red photoconverted Dendra2 lasted longer as the time needed for recombining the membrane label, so it is possible to trace the cell over the entire period. The founder cell which was genetically modified with CRE showed the same color as its progeny. This means that if the progeny of the photoconverted founder cell show a red membrane, the founder cell recombined mKate2.

In summary, mESCs were generated that had two PB copies in two different loci of the genome. We showed that this enables the simultaneous recombination of two different FPs mKate2 and mOrange2 in one cell. However, the third FP mNeonGreen was not visible, either there were recombinational difficulties or simply the FP was not detected as it emits at the same spectrum as the nuclear green Dendra2. The separation and thus visualization of nuclear and membrane labeling can be improved by differentiating the mESC into a different lineage as for example cardiomyocytes or neurons which have a larger nuclear-cytoplasmic ratio.

The combination of the two different tracing techniques, photoconversion and CRE recombination, were successfully performed. Main fields were the PhOTO-Bow can be used as a labeling tool would be early developmental biology and cancer research. In the first stages of development, the number of cells is very low, so it is more advantageous to have a defined number of color combinations. Using the Brainbow method would hinder the separation of the colors/cells because the cells could express difficult to distinguish color combinations. Cancerous PB cells could recombine one of the defined membrane labels and thus help in tracing and analyzing metastases. Those progeny should express the same membrane label as their founder and shrink down the amount of work for finding the correct lineage trace.

5 Acknowledgment

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6 References

1. Woodworth, M. B., Girskis, K. M. & Walsh, C. A. Building a lineage from single cells: genetic techniques for cell lineage tracking. *Nat. Rev. Genet.* **18,** 230–244 (2017).
2. Kretzschmar, K. & Watt, F. M. Lineage Tracing. *Cell* **148,** 33–45 (2012).
3. Conklin, E. G. Mosaic development in ascidian eggs. *J. Exp. Zool.* **2,** 145–223 (1905).
4. Hasegawa, Y. *et al.* Novel ROSA26 Cre-reporter Knock-in C57BL/6N Mice Exhibiting Green Emission before and Red Emission after Cre-mediated Recombination. *Exp. Anim.* **62,** 295–304 (2013).
5. Ruan, J. *et al.* Highly efficient CRISPR/Cas9-mediated transgene knockin at the H11 locus in pigs. *Sci. Rep.* **5,** 14253 (2015).
6. Kremers, G.-J., Gilbert, S. G., Cranfill, P. J., Davidson, M. W. & Piston, D. W. Fluorescent proteins at a glance. *J Cell Sci* **124,** 157–160 (2011).
7. Guilbault, G. G. *Practical Fluorescence, Second Edition.* (CRC Press, 1990).
8. Tsien, R. Y. The Green Fluorescent Protein. *Annu. Rev. Biochem.* **67,** 509–544 (1998).
9. Shaner, N. C. *et al.* Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nat. Biotechnol.* **22,** 1567–1572 (2004).
10. SHCHERBO, D. *et al.* Far-red fluorescent tags for protein imaging in living tissues. *Biochem. J.* **418,** 567–574 (2009).
11. Shaner, N. C. *et al.* A bright monomeric green fluorescent protein derived from Branchiostoma lanceolatum. *Nat. Methods* **10,** 407–409 (2013).
12. Shaner, N. C. *et al.* Improving the photostability of bright monomeric orange and red fluorescent proteins. *Nat. Methods* **5,** 545–551 (2008).

13. Kanca, O., Caussinus, E., Denes, A. S., Percival-Smith, A. & Affolter, M. Raeppli: a whole-tissue labeling tool for live imaging of Drosophila development. *Development* **141,** 472–480 (2014).

14. Livet, J. *et al.* Transgenic strategies for combinatorial expression of fluorescent proteins in the nervous system. *Nature* **450,** 56–62 (2007).

15. Weissman, T. A. & Pan, Y. A. Brainbow: New Resources and Emerging Biological Applications for Multicolor Genetic Labeling and Analysis. *Genetics* **199,** 293–306 (2015).

16. Weissman, T. A., Sanes, J. R., Lichtman, J. W. & Livet, J. Generating and Imaging Multicolor Brainbow Mice. *Cold Spring Harb. Protoc.* **2011,** pdb.top114 (2011).

17. Welling, M., Ponti, A. & Pantazis, P. Symmetry breaking in the early mammalian embryo: the case for quantitative single-cell imaging analysis. *MHR Basic Sci. Reprod. Med.* **22,** 172–181 (2016).

18. Dempsey, W. P. *et al. In vivo* single-cell labeling by confined primed conversion. *Nat. Methods* **12,** 645–648 (2015).

19. Adam, V., Nienhaus, K., Bourgeois, D. & Nienhaus, G. U. Structural Basis of Enhanced Photoconversion Yield in Green Fluorescent Protein-like Protein Dendra2. *Biochemistry* **48,** 4905–4915 (2009).

20. Chudakov, D. M., Lukyanov, S. & Lukyanov, K. A. Tracking intracellular protein movements using photoswitchable fluorescent proteins PS-CFP2 and Dendra2. *Nat. Protoc.* **2,** 2024–2032 (2007).

21. Nienhaus, K., Nienhaus, G. U., Wiedenmann, J. & Nar, H. Structural basis for photo-induced protein cleavage and green-to-red conversion of fluorescent protein EosFP. *Proc. Natl. Acad. Sci.* **102,** 9156–9159 (2005).

22. Mohr, M. A. & Pantazis, P. Primed Conversion: The New Kid on the Block for Photoconversion. *Chem. – Eur. J.* **24,** 8268–8274 (2018).

23. Mohr, M. A. *et al.* Rational Engineering of Photoconvertible Fluorescent Proteins for Dual-Color Fluorescence Nanoscopy Enabled by a Triplet-State Mechanism of Primed Conversion. *Angew. Chem. Int. Ed.* **56,** 11628–11633 (2017).

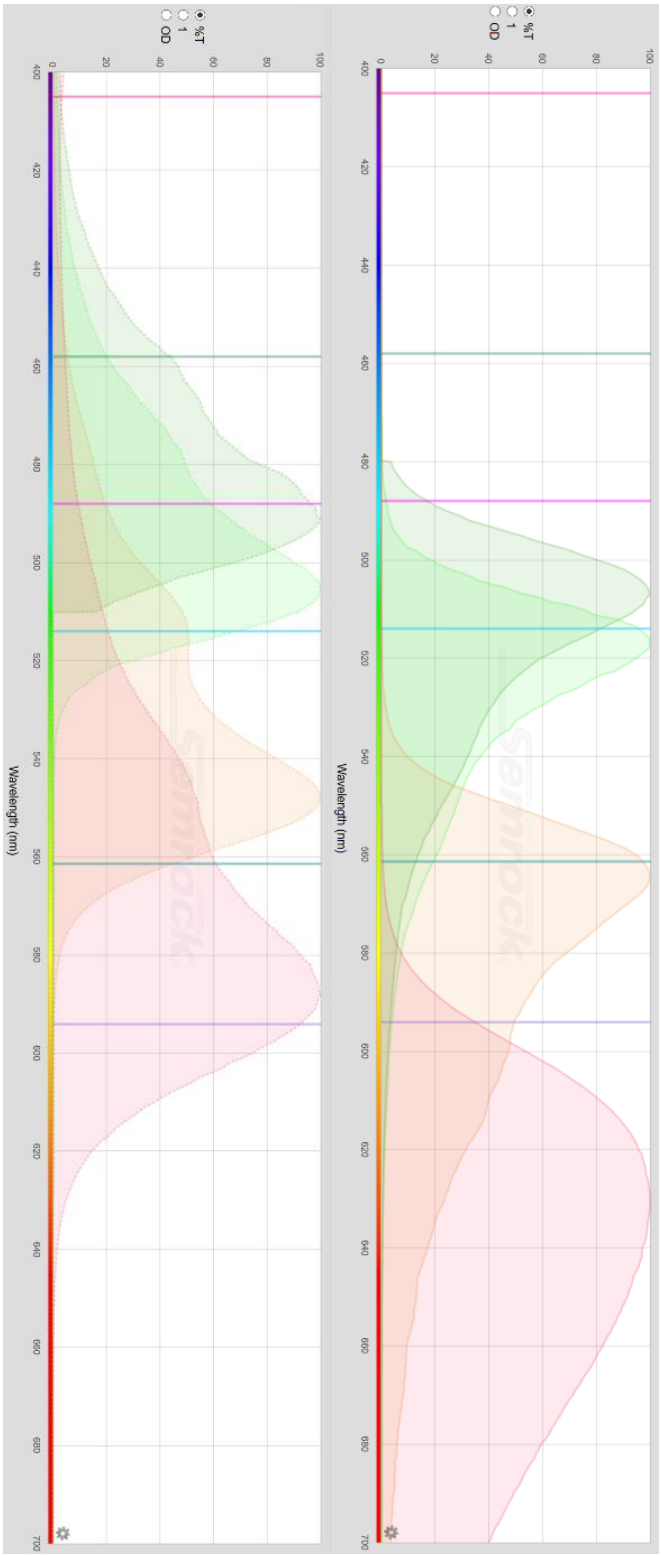
24. Dempsey, W. P., Fraser, S. E. & Pantazis, P. PhOTO Zebrafish: A Transgenic Resource for In Vivo Lineage Tracing during Development and Regeneration. *PLOS ONE* **7,** e32888 (2012).

25. Ying, Q.-L. *et al.* The ground state of embryonic stem cell self-renewal. *Nature* **453,** 519–523 (2008).

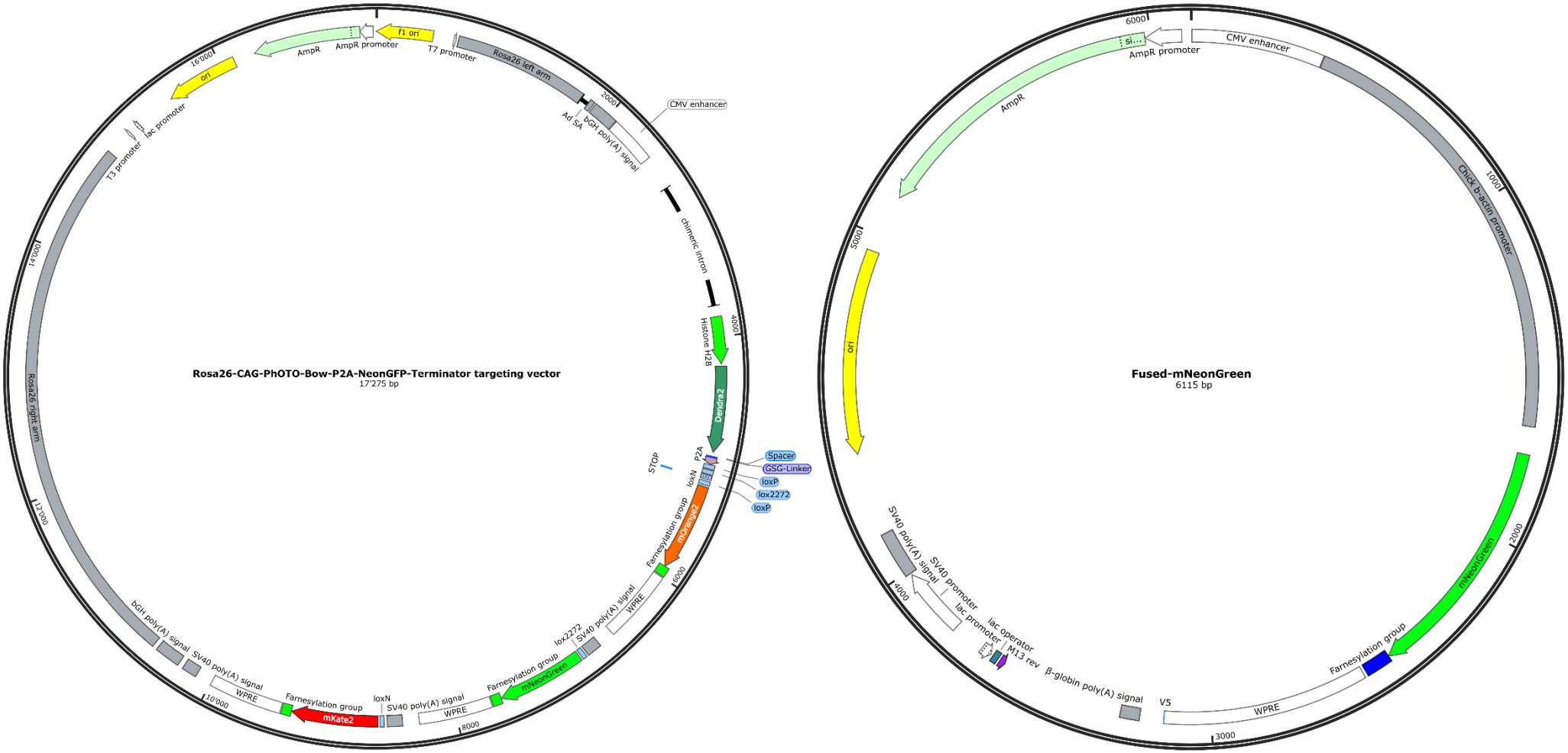
26. Arbab, M. & Sherwood, R. I. Self-Cloning CRISPR. *Curr. Protoc. Stem Cell Biol.* **38,** 5B.5.1-5B.5.16 (2016).

27. Arbab, M., Srinivasan, S., Hashimoto, T., Geijsen, N. & Sherwood, R. I. Cloning-free CRISPR. *Stem Cell Rep.* **5,** 908–917 (2015).

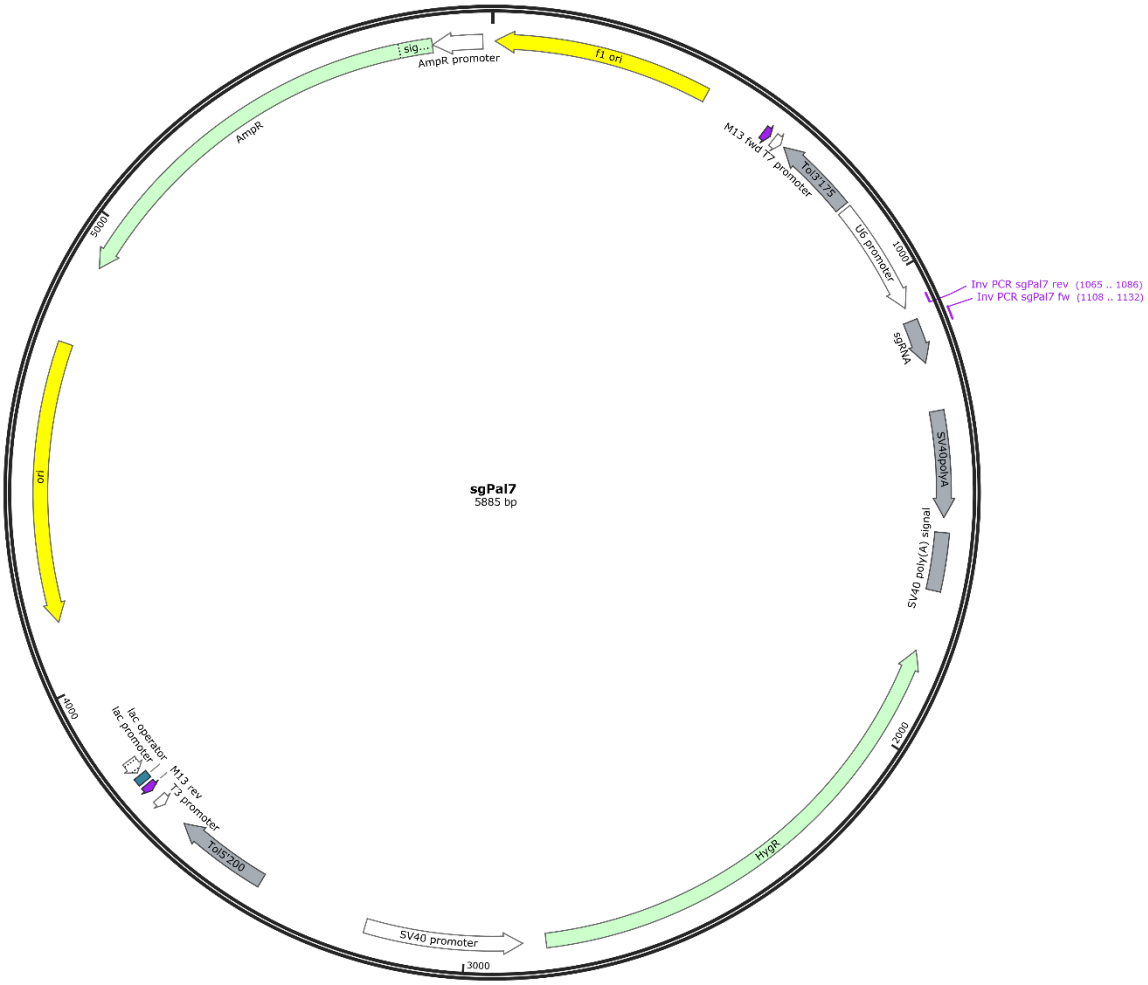
7 Appendix



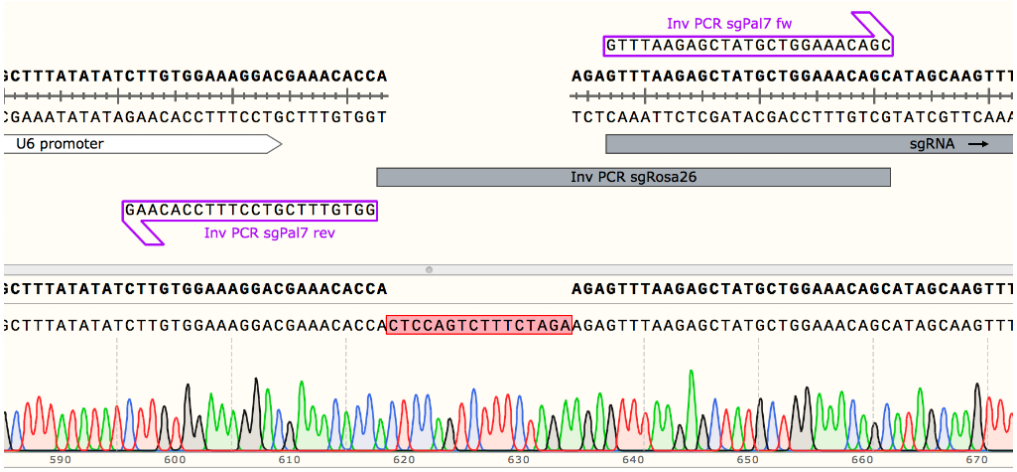
Supplementary Figure 1: Excitation (left) and emission (right) spectra of the fluorescent proteins coded in the PhOTO-Bow (dark green = Dendra2, light green = mNeonGreen, orange = mOrange2, red = mKate2). The vertical lines are laser lines (from left to right: 405, 458, 488, 514, 561, 594).



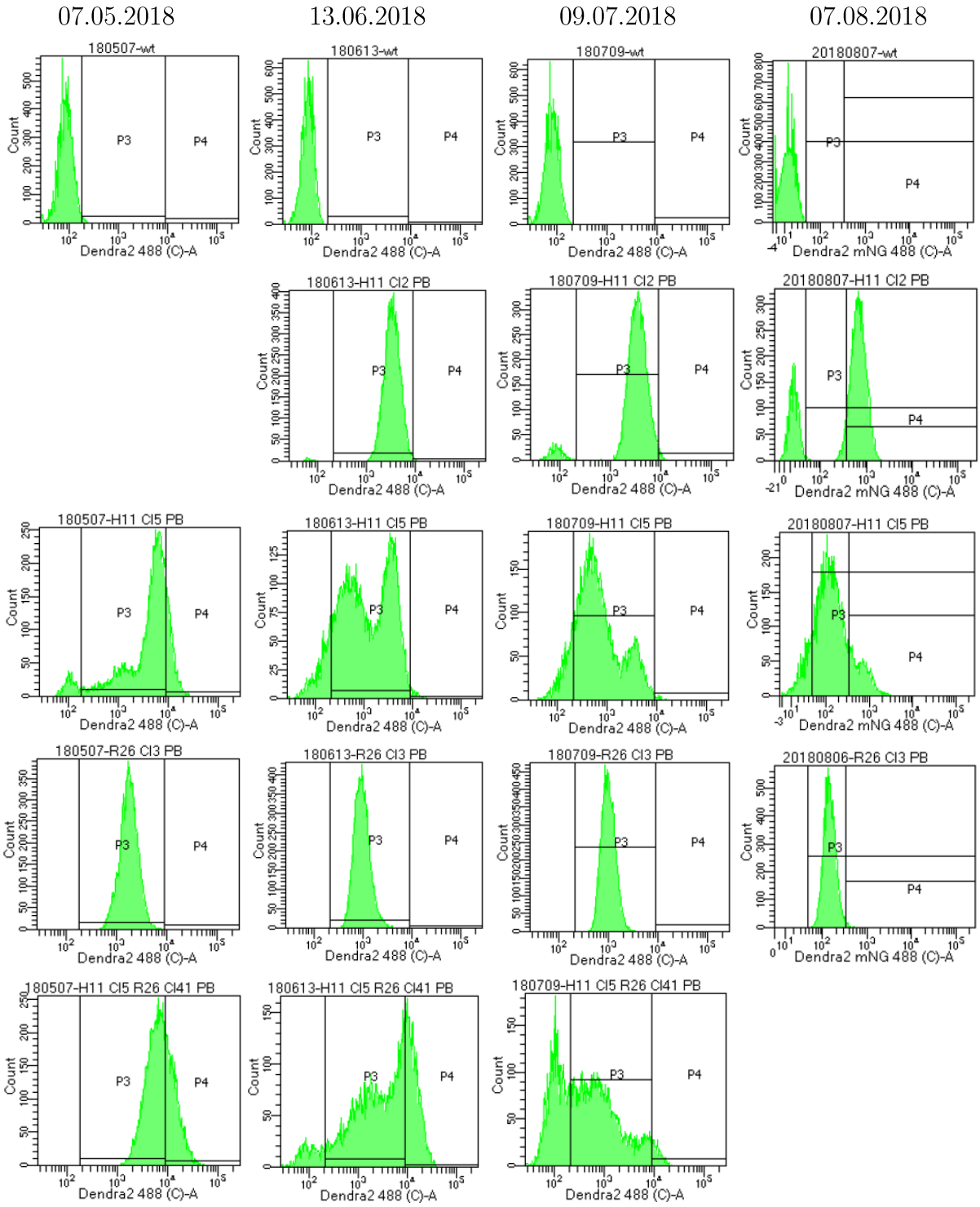
Supplementary Figure 2: Overview of the PhOTO-Bow (left) and one of the generated control color vectors (right). The PB has two homologues arms corresponding to the Rosa26 locus, Dendra2 attached to a Histone H2B label for nuclear green-to-red fluorescence and three FPs that are combined with a farnesylation group and WPRE for membrane labeling. The three FPs are separated through lox sites to enable CRE recombination. The control color vectors have the same FP-farnesylation group-WPRE combination and the pCAGGS promoter.



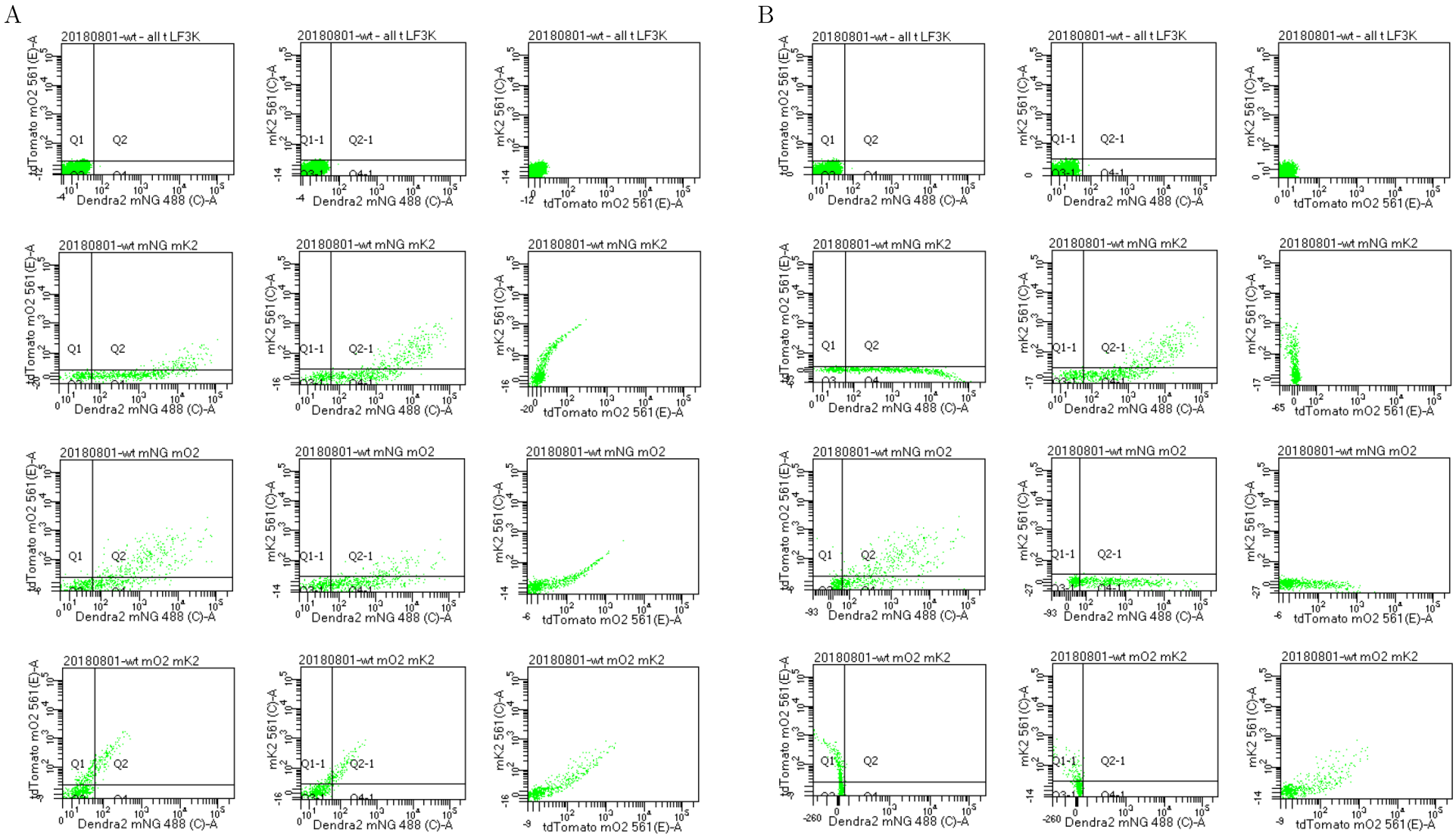
Supplementary Figure 3: Overview of the self-cleaving Palindromic sgRNA (sgPal7).



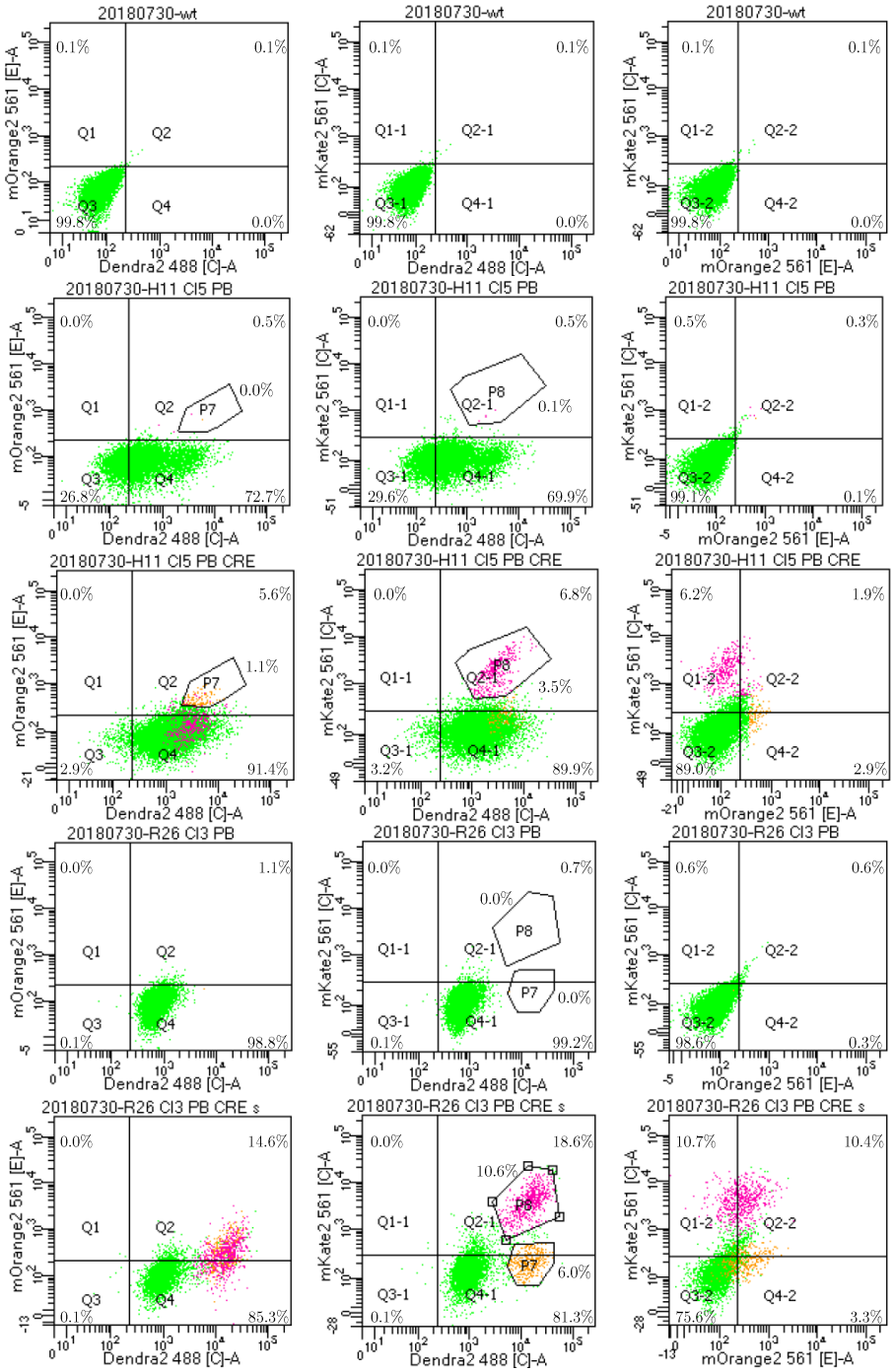
Supplementary Figure 4: Sanger sequencing result of the self-cleaving Palindromic sgRNA plasmid with the sequence for the Rosa26 locus which is partially marked in red (ACTCCAGTCTTTCTAGAAGA).



Supplementary Figure 5: FACS measurements of green fluorescent clones over three months. Passaging the cells over a larger period influences the expression of the PB construct.



Supplementary Figure 6: FACS results of wt cells co-transfected with two different plasmids encoding FPs (mNeonGreen, mOrange2 or mKate2). The plots in column B have color configuration enabled and thus are fluorescence corrected.



Supplementary Figure 7: H11-PB Cl5 and Rosa26-PB Cl3 were FACS analyzed three days after transfection with a CMV-CRE encoding plasmid. wt cells were used as a control. Cells in gate P7 correspond to mOrange2 expressing cells and those in gate P8 to mKate2.

Supplementary Table 1: Primers used for vector cloning. The last two primers were for generating the Cas9-Palindromic plasmid with a sgRNA for the Rosa26 locus. In italic are the base pairs that mark the sgRNA sequence of the Rosa26 locus.

Name	Sequence
pCAGGS Eos V5 forward	GAGATCTTTTTCCCTCTGCC
pCAGGS Eos V5 reverse	TCGACCTCGAGGAATTCTTTG
mKate2 In-Fusion forward	ATTCCTCGAGGTCGAATGGTGAGCGAGCTGATTAAGGAG
mNeonGreen In-Fusion forward	ATTCCTCGAGGTCGATCGCGTACGGCCACCATG
mOrange2 In-Fusion forward	ATTCCTCGAGGTCGATCTAACGTTGCCACCATGGTGA
WPRE In-Fusion reverse	AGGGAAAAAGATCTCGCGGGGAGGCGGCCCAAAG
CRE In-Fusion forward	ATTCCTCGAGGTCGAATGTCCAATTTACTGACCGTACACC
CRE In-Fusion reverse	AGGGAAAAAGATCTCCTAATCGCCATCTTCCAGCAGG
Inv PCR sgPal7 forward	*ACTCCAGTCTTTCTAGAAGA*GTTTAAGAGCTATGCTGGAAAACAGC
Inv PCR sgPal7 reverse	GGTGTTTCGTCCTTTCCACAAG

Supplementary Table 2: Primers used for genotyping.

Name	Sequence
WPRE forward	GCTATTGCTTCCCGTATGGCTT
mOrange2 PB reverse	CCTTGGAGCCGTAGGTGAAAT
Dendra2 PB forward	GCATCGAGATCCTGGGCAAC
Rosa26 reverse	TACTCCGAGGCGGATCACAA
CMV-to-Rosa26 reverse	GGAAAGTCCCTATTGGCGTTAC
Rosa26 fw	CTTGCTCTCCCAAAGTCGCT
Rosa26 fw 1	GGTAGGGGATCGGGACTCTG

Supplementary Table 3: Compensation configuration for the FPs mNeonGreen, mOrange2 and mKate2.

Fluorochrome	- % Fluorochrome	Spectral Overlap [%]
mOrange2	mNeonGreen	0.12
mKate2	mNeonGreen	0
mNeonGreen	mOrange2	46.63
mKate2	mOrange2	19.79
mNeonGreen	mKate2	41.58
mOrange2	mKate2	17.16

Supplementary Table 4: FACS result for wt cells transfected with a plasmid encoding mNeonGreen, mOrange2 or mKate2. Enabling the compensation control reduces the amount of wrongly analyzed cells.

Compensation	Disabled			Enabled		
Channel	mNeonGreen	mOrange2	mKate2	mNeonGreen	mOrange2	mKate2
wt cells	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
wt mNeonGreen	49.9%	0.0%	0.0%	49.8%	0.0%	0.0%
wt mOrange2	14.2%	22.9%	9.9%	2.2%	22.9%	0.1%
wt mKate2	13.4%	9.6%	19.0%	3.9%	0.0%	18.9%

Supplementary Table 5: FACS result for wt cells co-transfected with two different plasmids encoding for one FP (mNeonGreen, mOrange2 or mKate2). Enabling the compensation control reduces the amount of wrongly analyzed cells.

Compensation	Disabled			Enabled		
Channel	mNeonGreen	mOrange2	mKate2	mNeonGreen	mOrange2	mKate2
wt cells	0.0%	0.0%	0.0%	0.0%	0.0%	0.0%
wt mNG + mK2	78.1%	12.2%	27.8%	78.2%	0.0%	28.0%
wt mNG + mO2	70.0%	36.4%	14.6%	68.5%	35.1%	0.1%
wt mO2 + mK2	20.8%	36.0%	25.1%	1.0%	34.6%	16.8%

Supplementary Table 6: Transfection results for increasing the efficiency: (1+2) amount of Lipofectamine should be at least double the volume of the amount of total plasmid used per transfection, (3) co-transfection does not decrease the efficiency, (4) transfect cells after passaging/in suspension, (7) one day after transfection FPs can be detected.

1

Protein	1 µg CMV-CRE + tdT			
Lipofectamine	LF2K		LF3K	
Amount	0.75 µL	1.5 µL	0.75 µL	1.5 µL
Post transfection	3 days			
wt cells	13.6%	21.2%	4.1%	9.5%
H11 Cl5 cells	14.5%	13.2%	4.1%	6.5%

2

Protein	1 µg CMV-CRE + tdT		2 µg CMV-CRE + tdT	
Lipofectamine	LF2K			
Amount	1.5 µL	2.5 µL	1.5 µL	2.5 µL
Post transfection	3 days			
wt cells	26.4%	29.3%	24.4%	28.9%
H11 Cl5 cells	21.0%	25.4%	23.4%	23.9%

3

Protein	2 µg CMV-CRE + tdT	1 µg tdT	2 µg CMV-CRE + tdT	1 µg tdT
Lipofectamine	1.5 µL LF2K			
Post transfection	3 days			
wt cells	26.2%	27.9%	26.3%	29.4%
H11 Cl5 cells	27.3%	28.1%	23.5%	31.0%

4

Protein	0.5 µg tdT	
Lipofectamine	1 µL LF2K	
Technique	adherent	suspension
Post transfection	2 days	
wt cells	29.8%	69.1%
H11 Cl5 cells	-	85.0%
H11 Cl5 R26 Cl41 cells	-	82.5%

5

Protein	0.5 µg mNG		0.5 µg mO2		0.5 µg mK2	
Lipofectamine	1 µL LF3K + 1 µL P3000					
Post transfection	2 days	4 days	2 days	4 days	2 days	4 days
wt cells	77.9%	21.6%	24.9%	1.2%	22.8%	0.2%
H11 Cl2 cells	25.1%	1.5%	21.2%	0.8%	17.9%	0.0%

6

Protein	0.5 µg mNG					
Lipofectamine	1 µL LF2K		1 µL LF3K			
P3000	0 µL		1 µL		0 µL	
Post transfection	2 days	4 days	2 days	4 days	2 days	4 days
wt cells	61.8%	19.5%	67.5%	18.4%	56.5%	14.1%

7

Protein	0.5 µg mNG						1 µg mNG		
Lipofectamine	1 µL LF3K + 1 µL P3000			2 µL LF3K + 2 µL P3000			2 µL LF3K + 2 µL P3000		
Post transfection	1 day	2 days	3 days	1 day	2 days	3 days	1 day	2 days	3 days
wt cells	54.40%	35.20%	21.70%	65%	42.20%	32.90%	73.60%	44.50%	21.60%

Protein	0.5 µg mO2			0.5 µg mK2		
Lipofectamine	1 µL LF3K + 1 µL P3000					
Post transfection	1 day	2 days	3 days	1 day	2 days	3 days
wt cells	24%	20.20%	11.80%	17.50%	18.50%	11.40%

University of Basel

Faculty of Science

Declaration on Scientific Integrity

(including a Declaration on Plagiarism and Fraud)

~~Bachelor's~~ / Master's Thesis *(Please cross out what does not apply)*

Title of Thesis *(Please print in capital letters)*:

The generation of transgenic PhOTO-Bow embryonic stem cells for direct multicolor lineage tracing

First Name, Surname *(Please print in capital letters)*: Scherrer, Dan-Felix

Matriculation No.: 14-053-052

I hereby declare that this submission is my own work and that I have fully acknowledged the assistance received in completing this work and that it contains no material that has not been formally acknowledged.

I have mentioned all source materials used and have cited these in accordance with recognised scientific rules.

In addition to this declaration, I am submitting a separate agreement regarding the publication of or public access to this work.

☒ Yes ☐ No

Place, Date: Basel, 23.07.2018

Signature: DFScherrer

Please enclose a completed and signed copy of this declaration in your Bachelor's or Master's thesis .