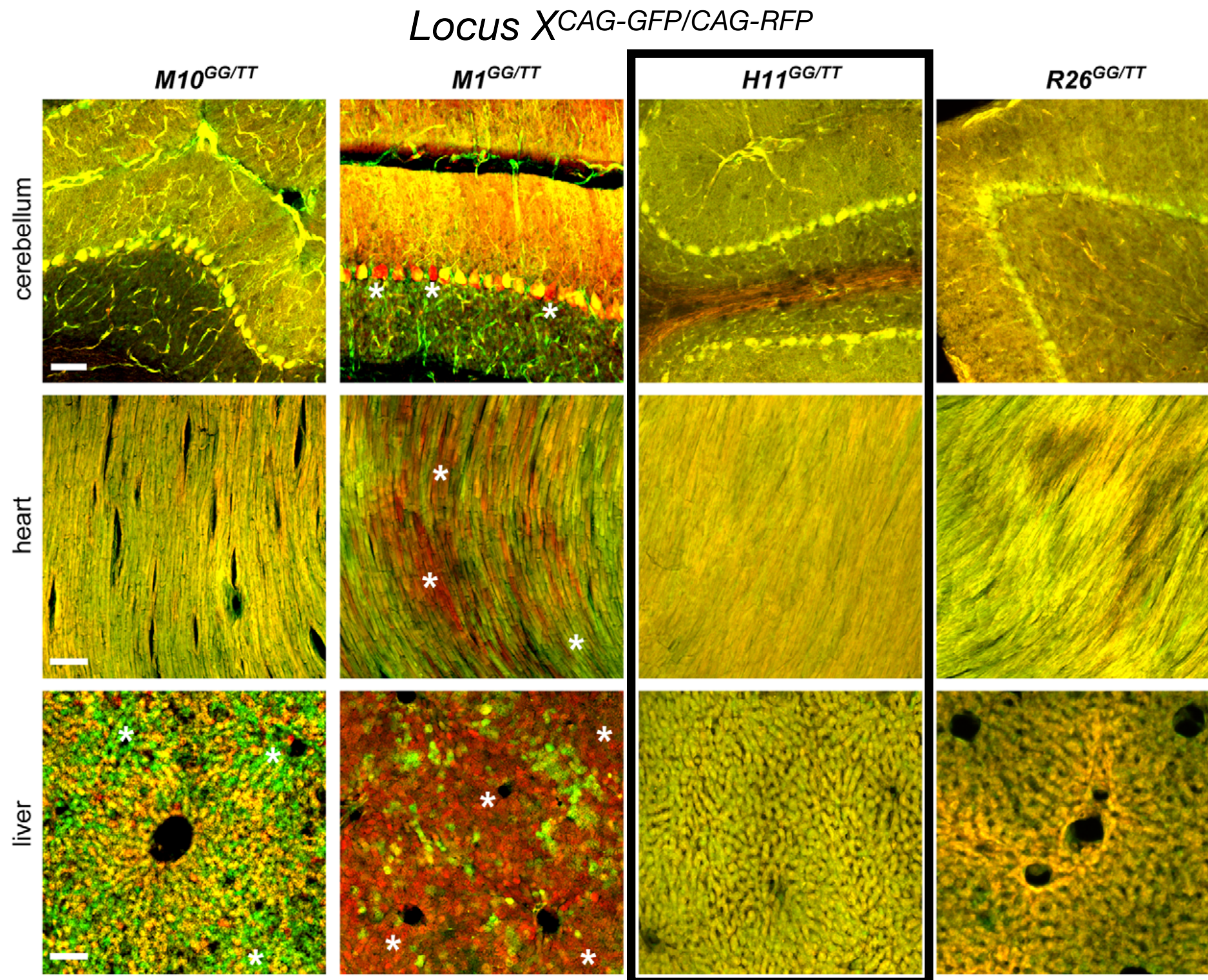


Safe harbor loci for ubiquitous reporter expression



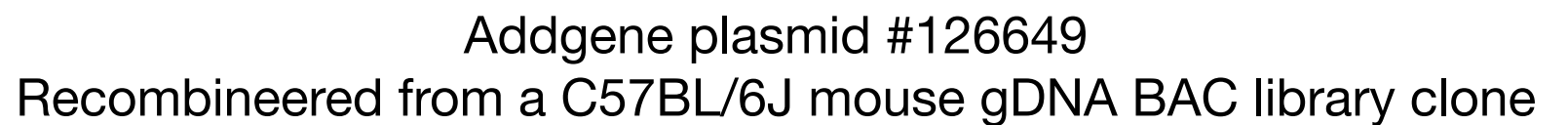
11:3150893-3218423

Eif4enif1

Hipp11

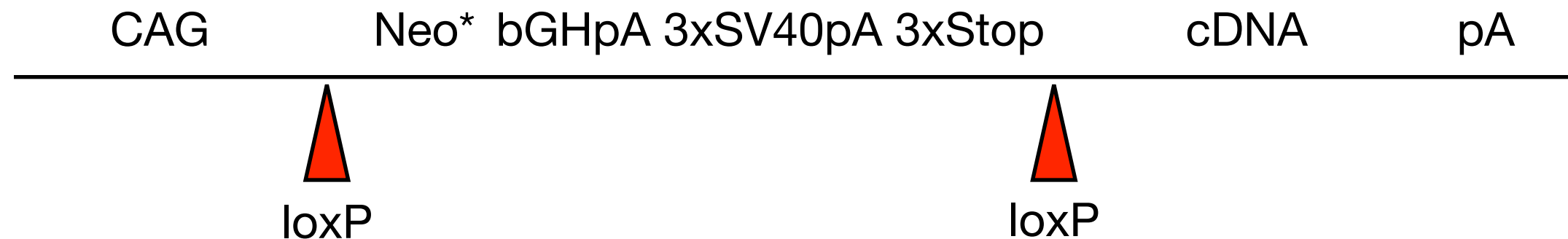
Drg1

Hippenmeyer et al., 2010

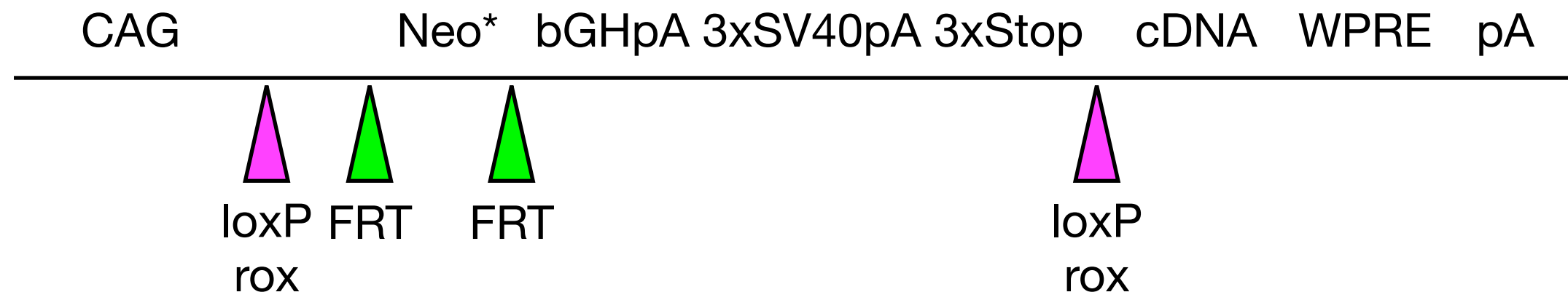


cre- or dre-inducible expression vectors 1.1

CAG-LSL vector 1.0 (Addgene #182363)

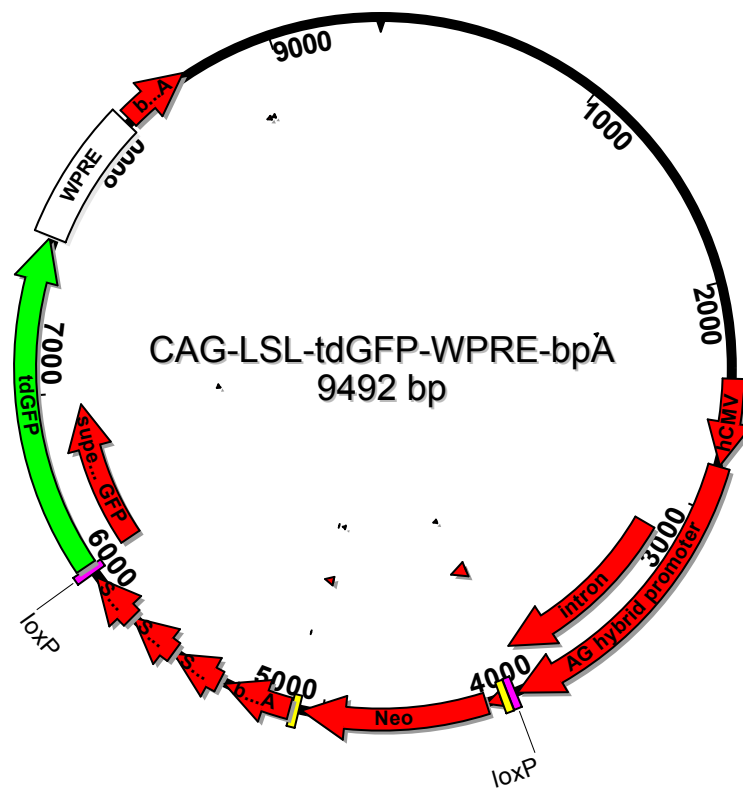


Minimizes Neo cassette into the same expression cassette → works
Constitutive Neo expression could theoretically be problematic → add FRT sites
Also, add a WPRE to increase expression

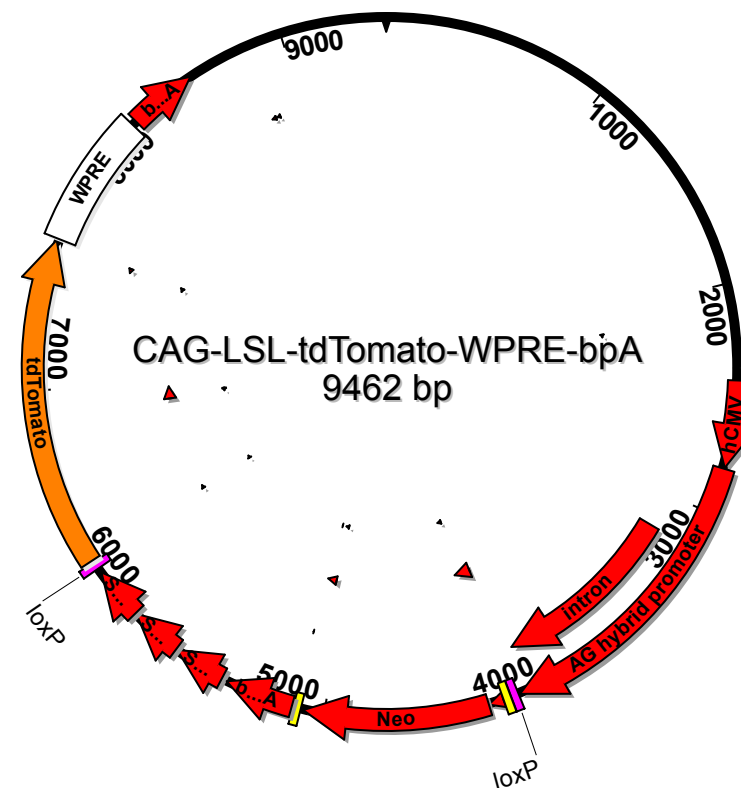


The ~1 kb distance of the stop cassette might decrease the recombination efficiency. At the time 1.0 was generated, >1 kb stop cassettes were the norm but not after Ai9/Ai14.

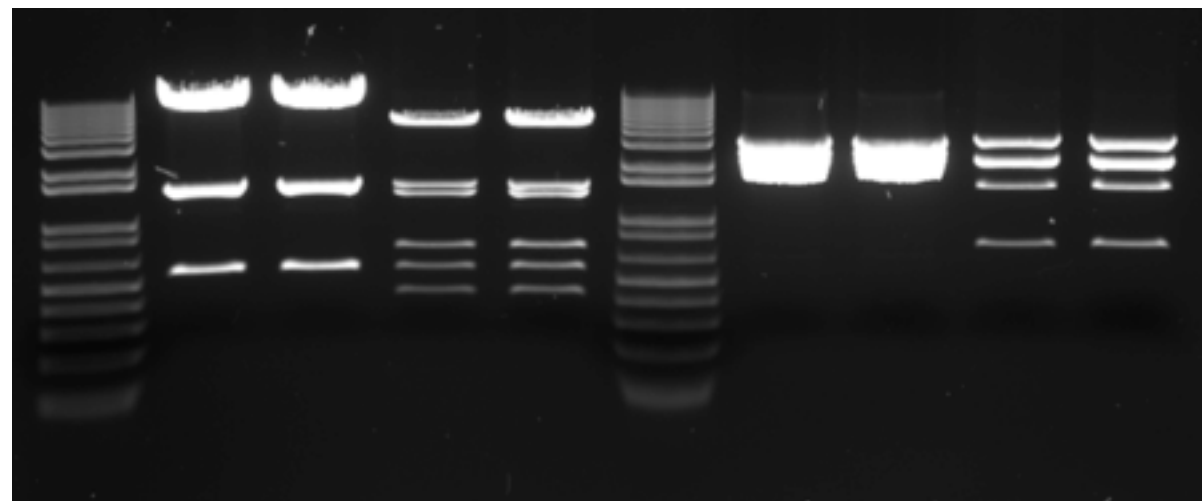
cre reporters 1.1



cre reporter GFP 1.1
Addgene #180151

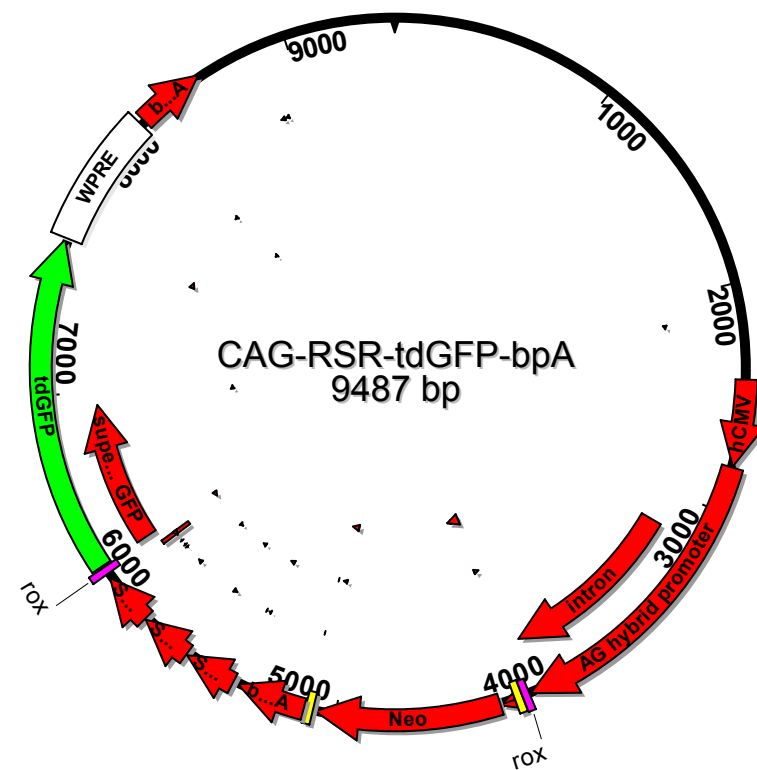


cre reporter RFP 1.1
Addgene #180152

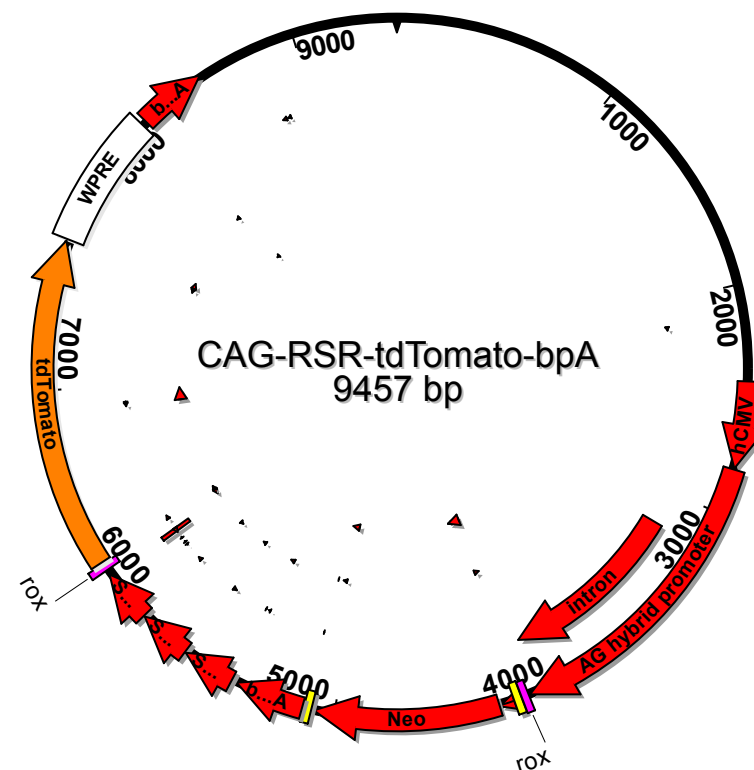


Sequenced

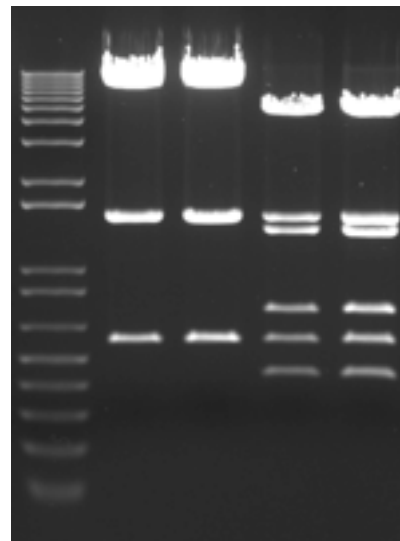
dre reporters 1.1



dre reporter GFP 1.1
Addgene #180153



dre reporter RFP 1.1
Addgene #180154



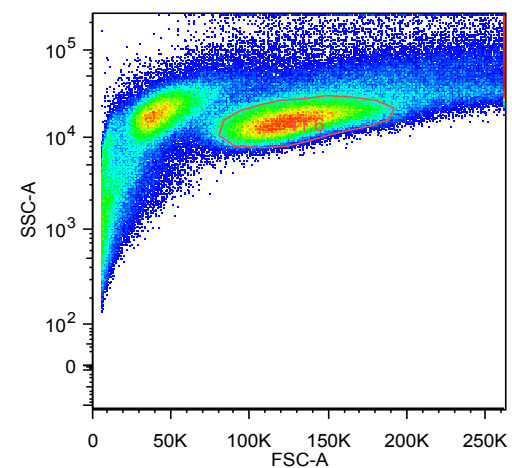
cre or dre expression vectors

- pCAGGS-pac-T2A-iCre, Addgene #125821
- pCAGGS-pac-T2A-dre, Addgene #124833

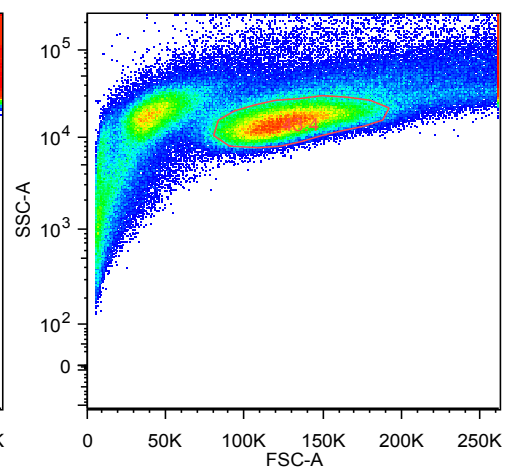
cre reporters 1.1 test

- G4 ES cells
- Electroporated
 - pFRT-pac-FRT
 - cre reporter GFP (td-sfGFP) or RFP (tdTomato) -/+ pac-T2A-iCre
- Analyses at day 1 and day 2 post-electroporation

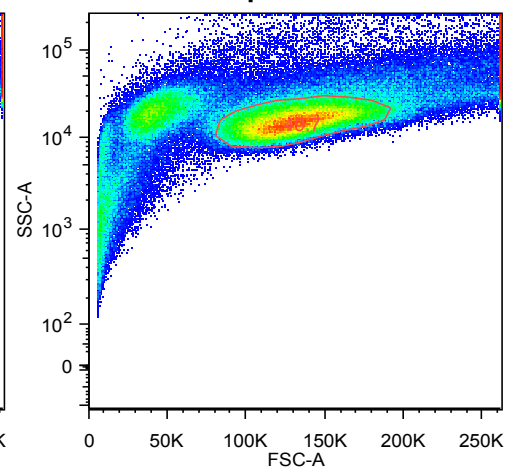
CAG-LSL-RFP +iCre



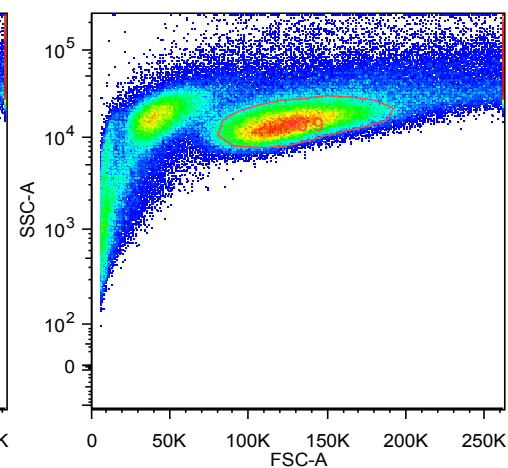
CAG-LSL-RFP -iCre



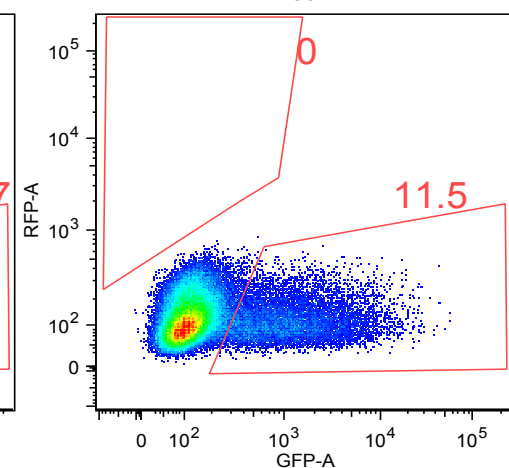
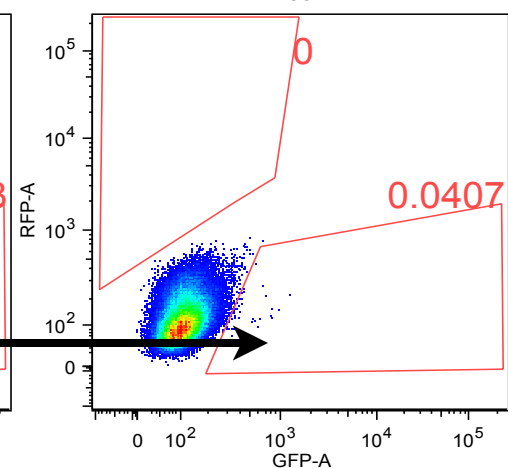
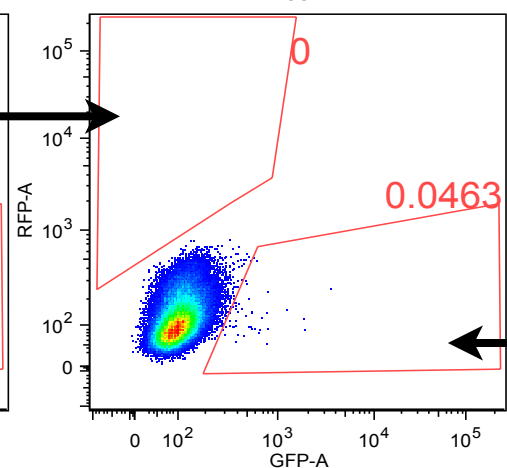
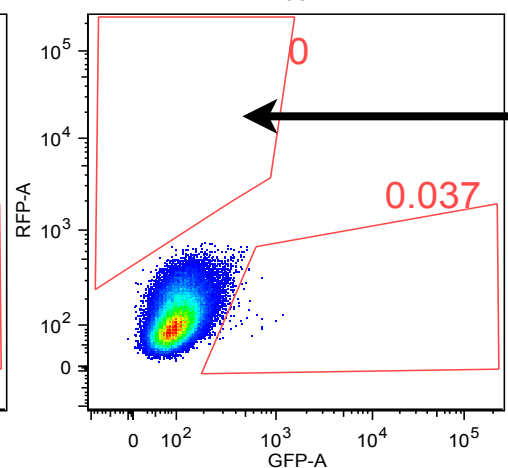
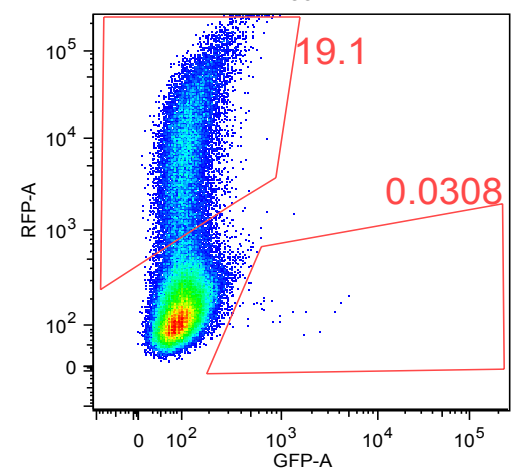
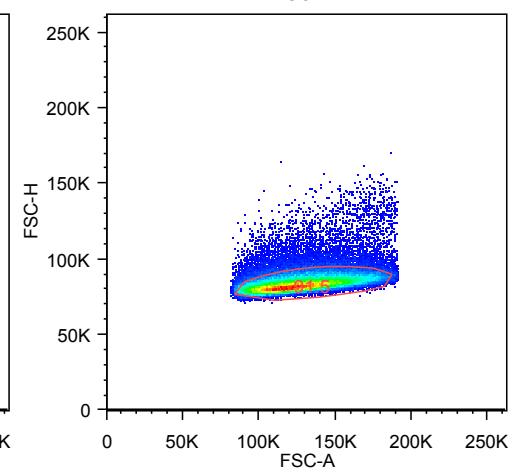
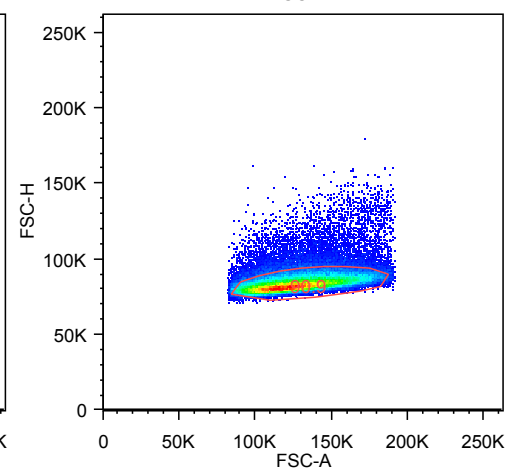
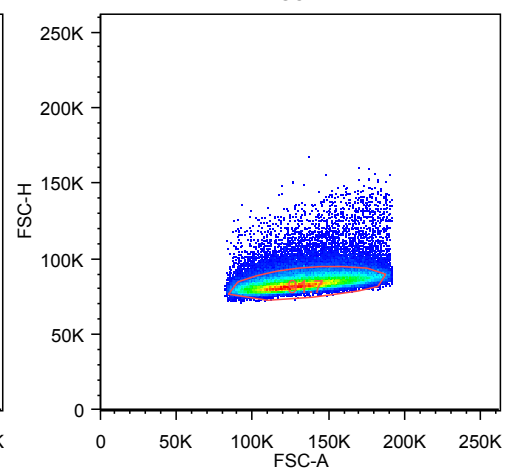
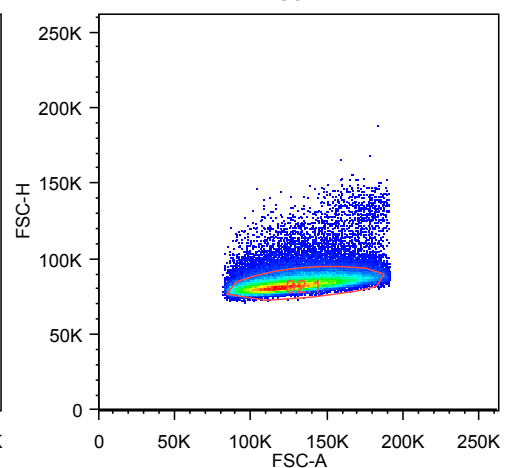
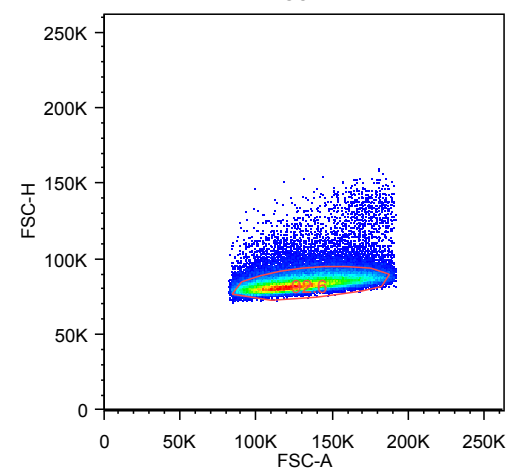
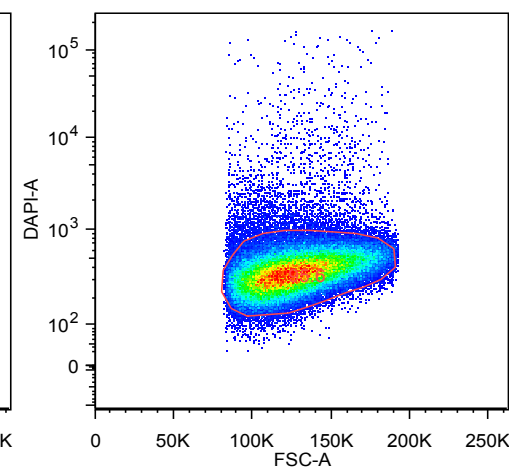
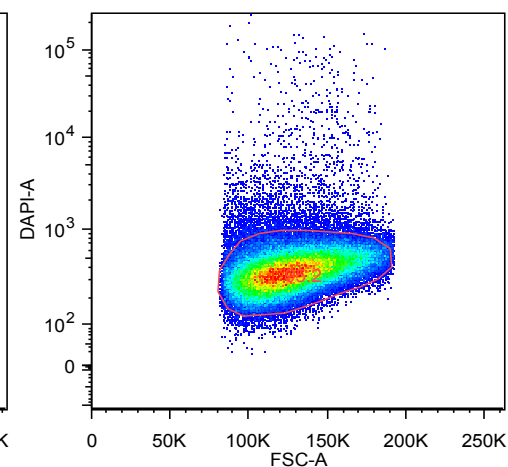
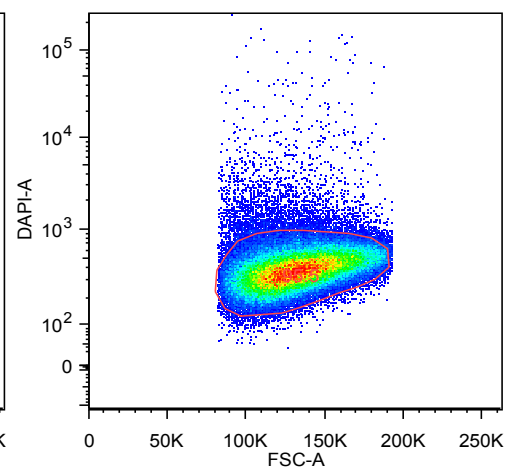
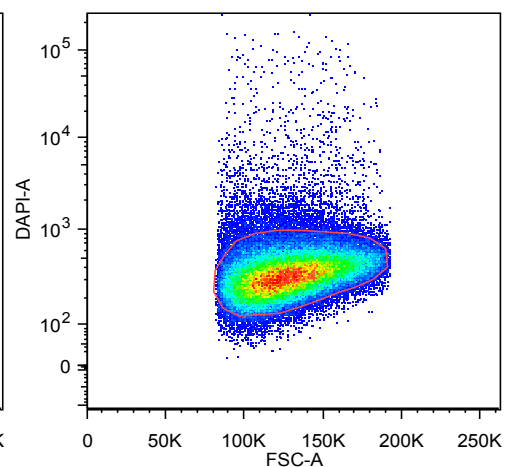
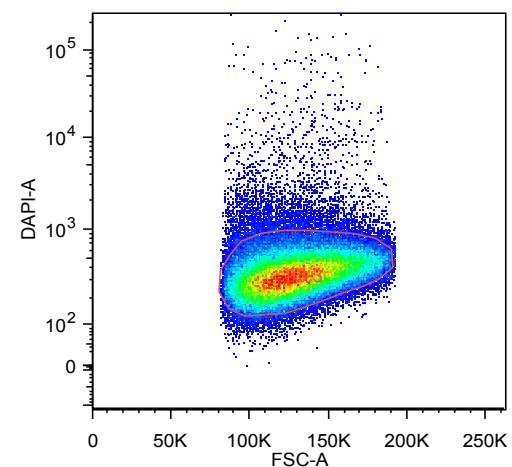
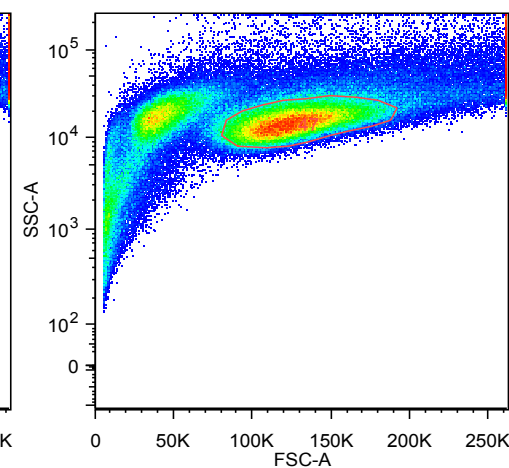
pFPF



CAG-LSL-GFP -iCre

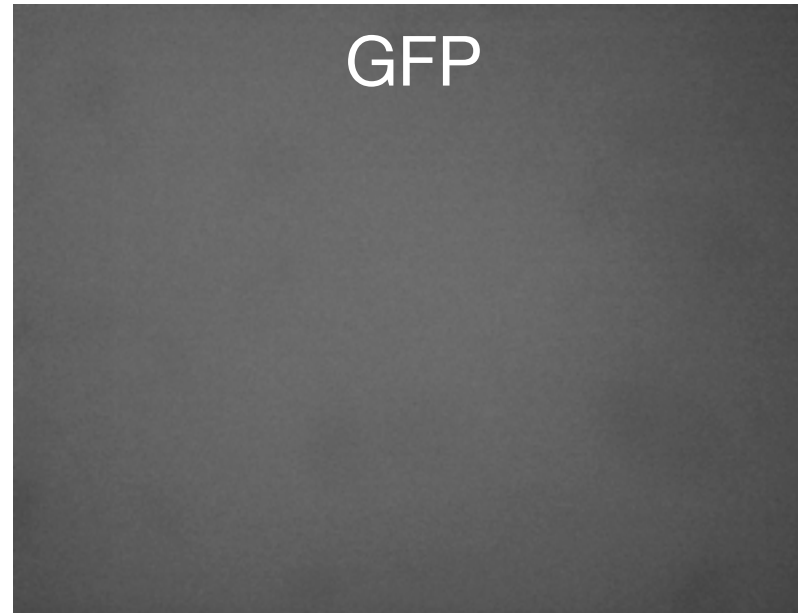


CAG-LSL-GFP +iCre

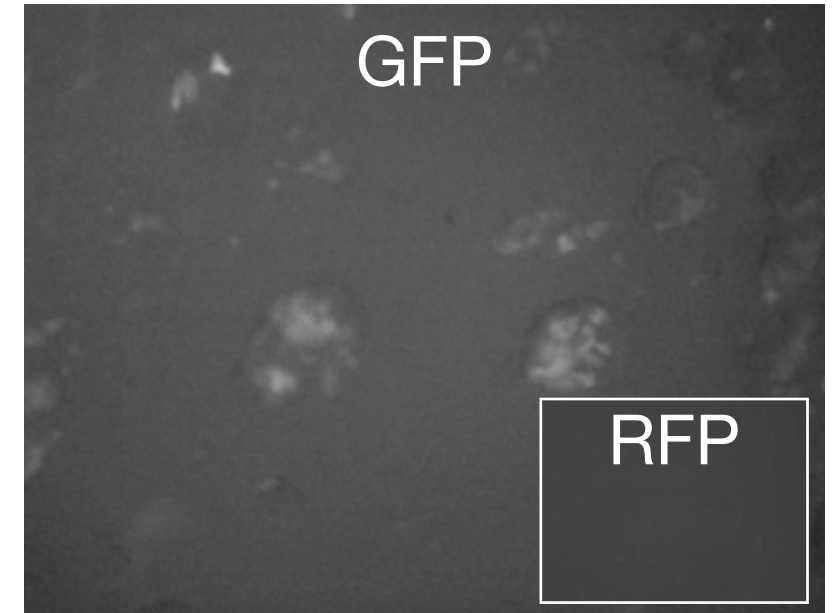


Live cell fluorescence microscopy

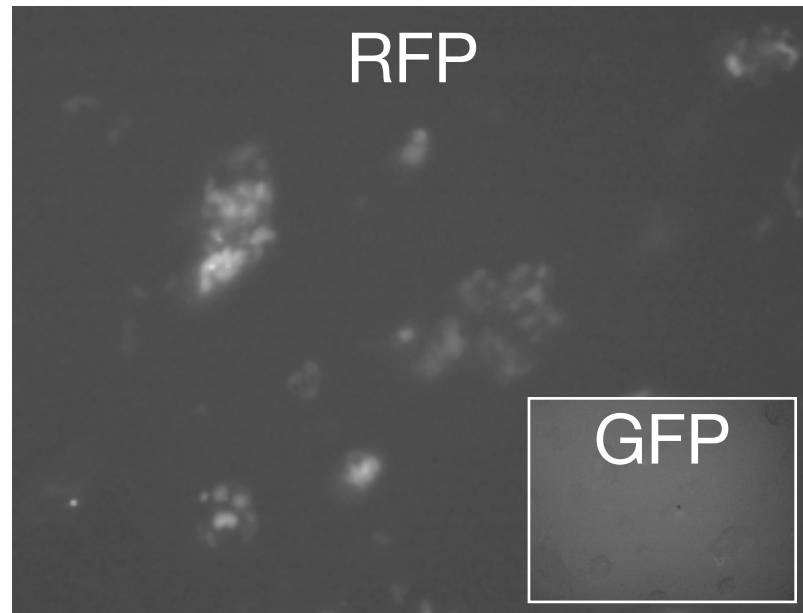
CAG-LSL-GFP -iCre



CAG-LSL-GFP +iCre



CAG-LSL-RFP +iCre



CAG-LSL-RFP -iCre



dre reporters 1.1 test

- G4 ES cells
- Electroporated
 - pFRT-pac-FRT
 - dre reporter GFP (td-sfGFP) or RFP (tdTomato) +/- pac-T2A-dre
- Analyses at day 1 and day 2 post-electroporation

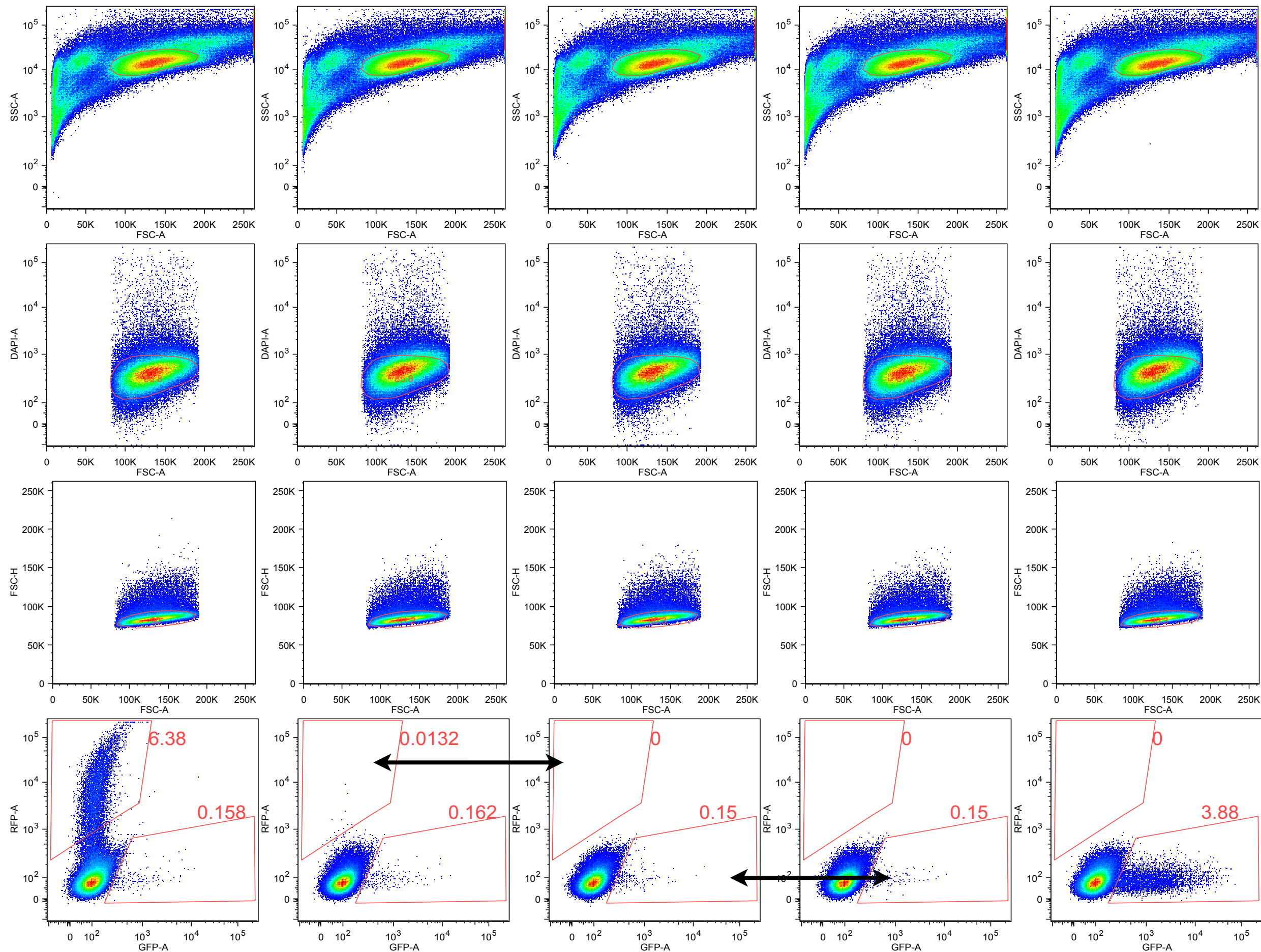
CAG-RSR-RFP +dre

CAG-RSR-RFP -dre

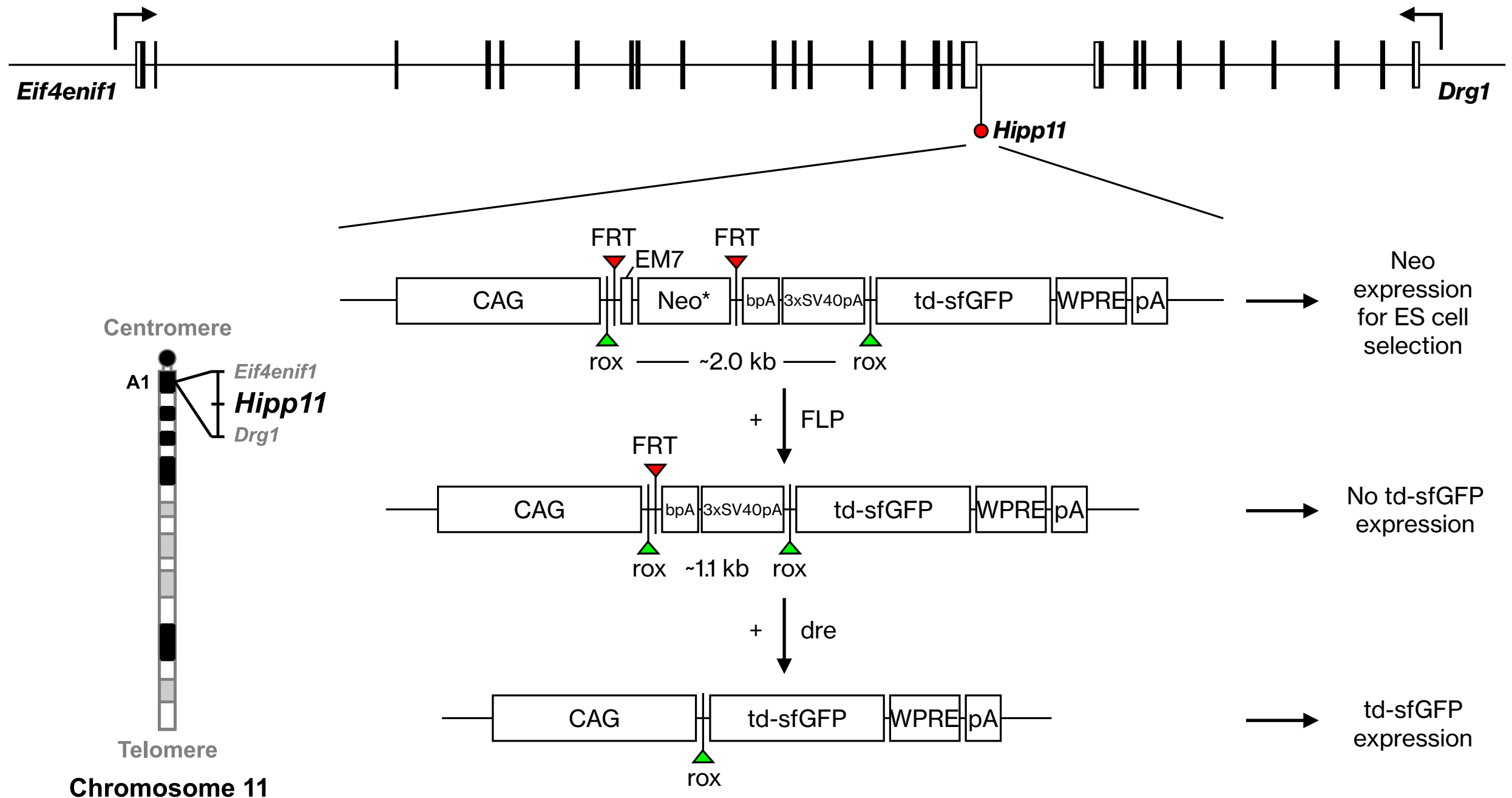
pFPF

CAG-RSR-GFP -dre

CAG-RSR-GFP +dre

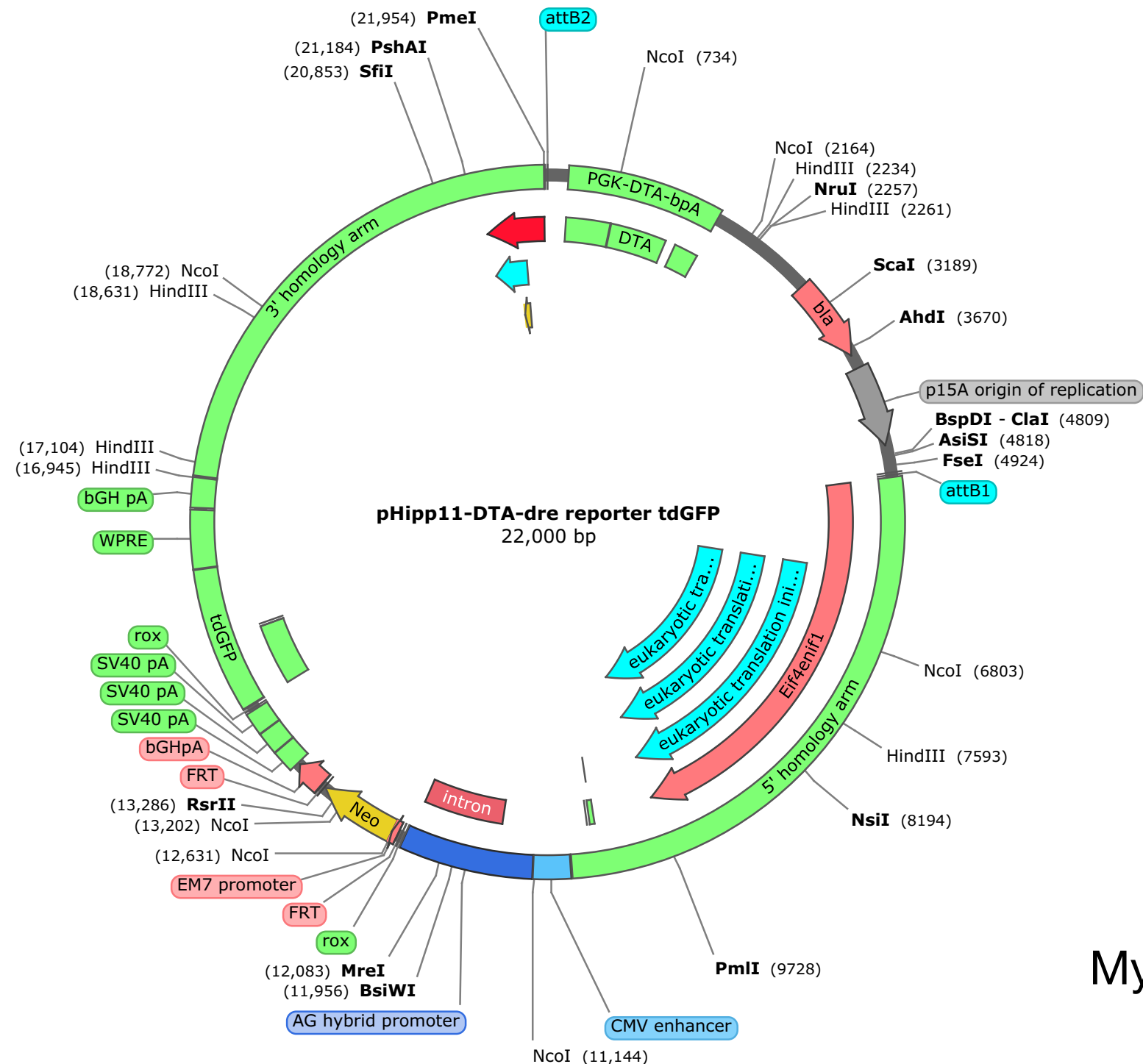


Hipp11-dre reporter td-sfGFP allele concept



Hipp11-dre reporter td-sfGFP allele targeting vector design

Created with SnapGene®



My sequence

11:3150893-3218423

Eif4enif1

Hipp11

Drg1

Hippenmeyer et al., 2010



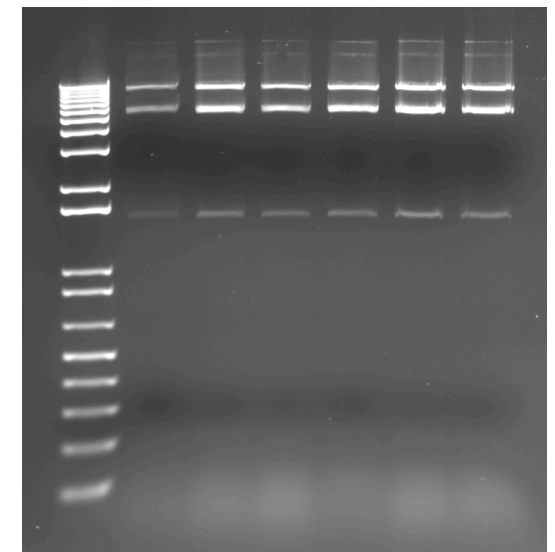
Shuttle vectors for *Hipp11* targeting vector recombineering

- Hipp11-CAG-loxP-FNF-bpA-3xSV40pA-loxP-td-sfGFP recombineering shuttle vector, Addgene #180155
- Hipp11-CAG-loxP-FNF-bpA-3xSV40pA-loxP-tdTomato recombineering shuttle vector, Addgene #180156
- Hipp11-CAG-rox-FNF-bpA-3xSV40pA-rox-td-sfGFP recombineering shuttle vector, Addgene #180157

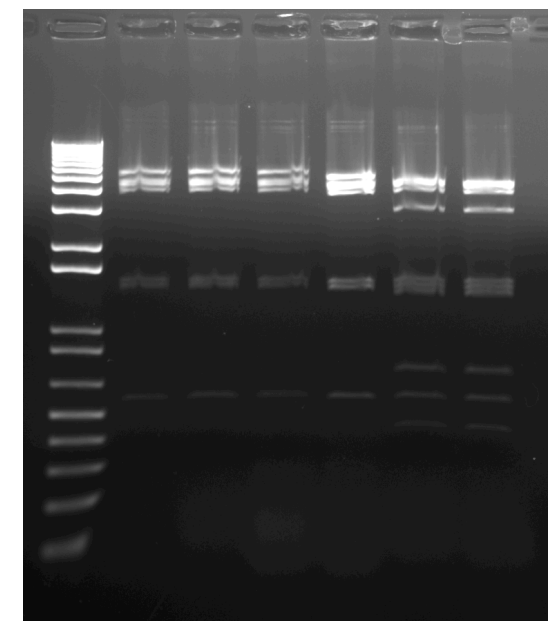
Hipp11-dre reporter *td-sfGFP* allele targeting vector constructed

Crude plasmid DNA prep
(no column)

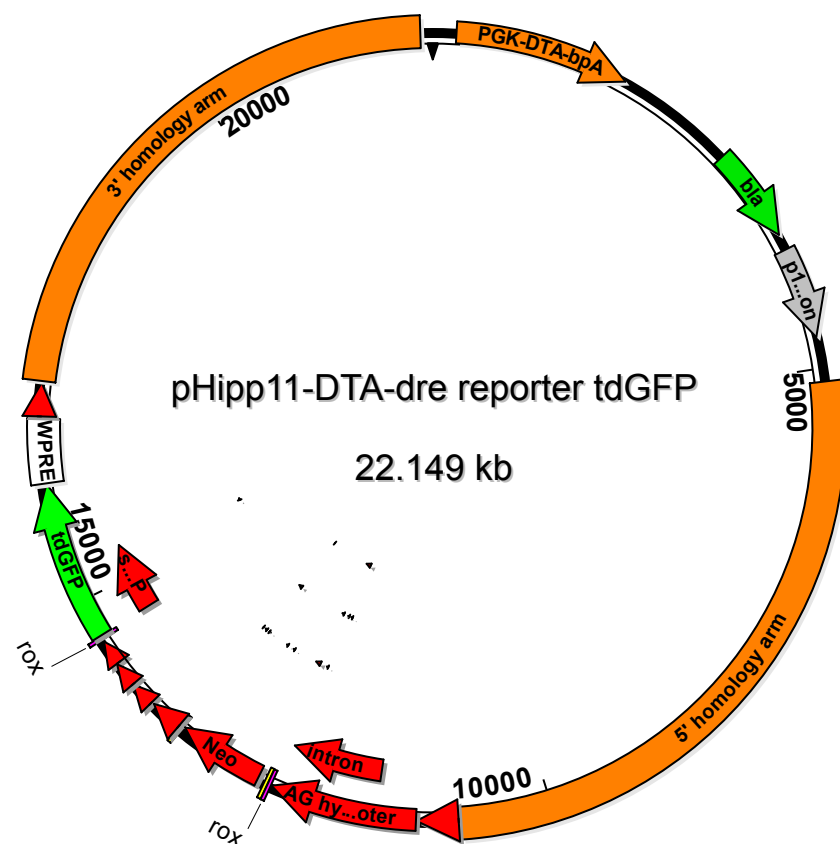
HindIII digests



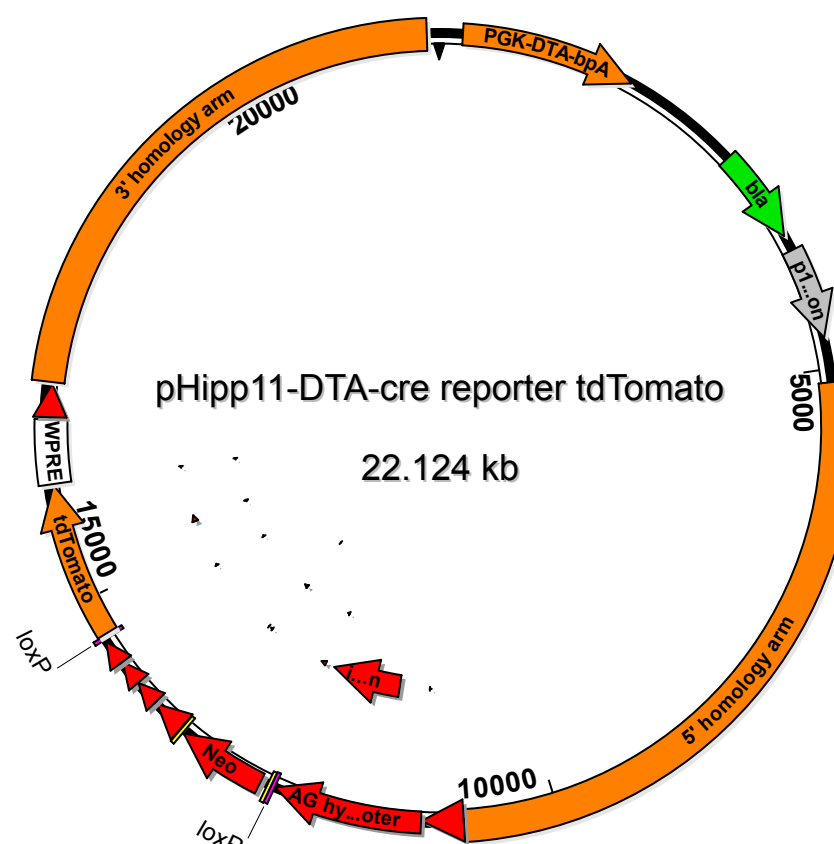
NcoI digests



Addgene #139513



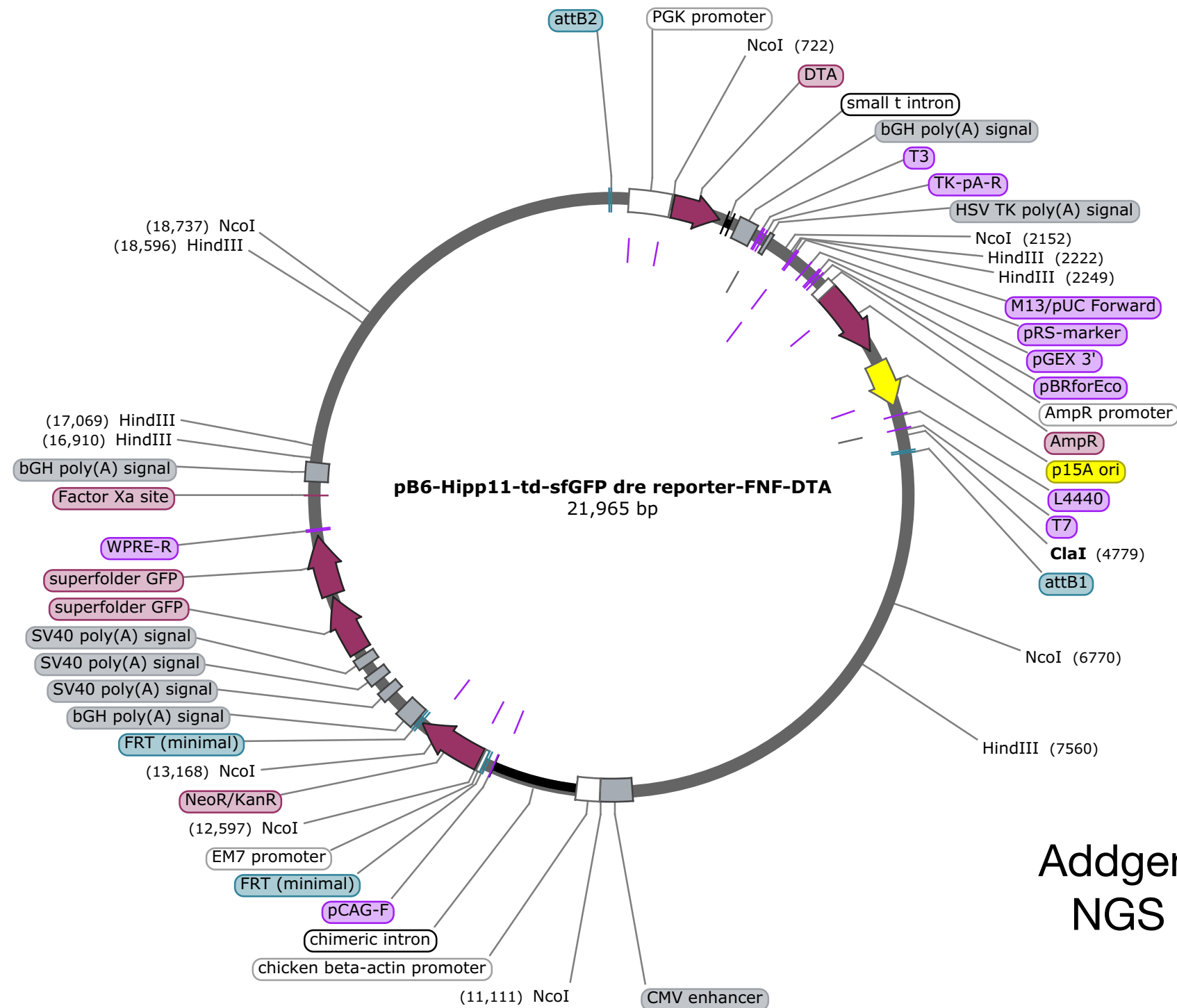
Addgene #183024



Sequenced

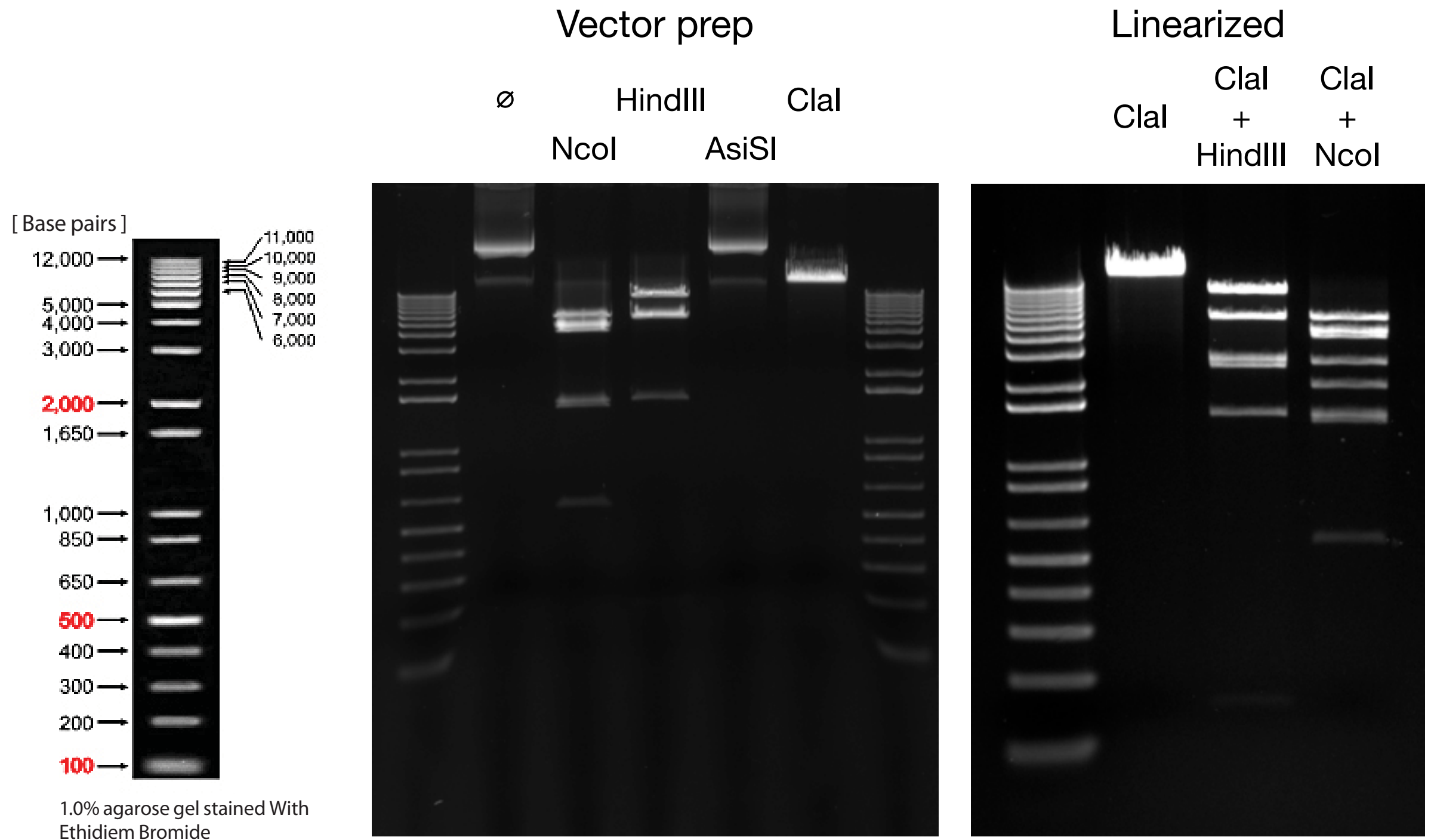
Hipp11-dre reporter td-sfGFP allele targeting vector NGS sequence

Created with SnapGene®



Addgene #139513
NGS sequence

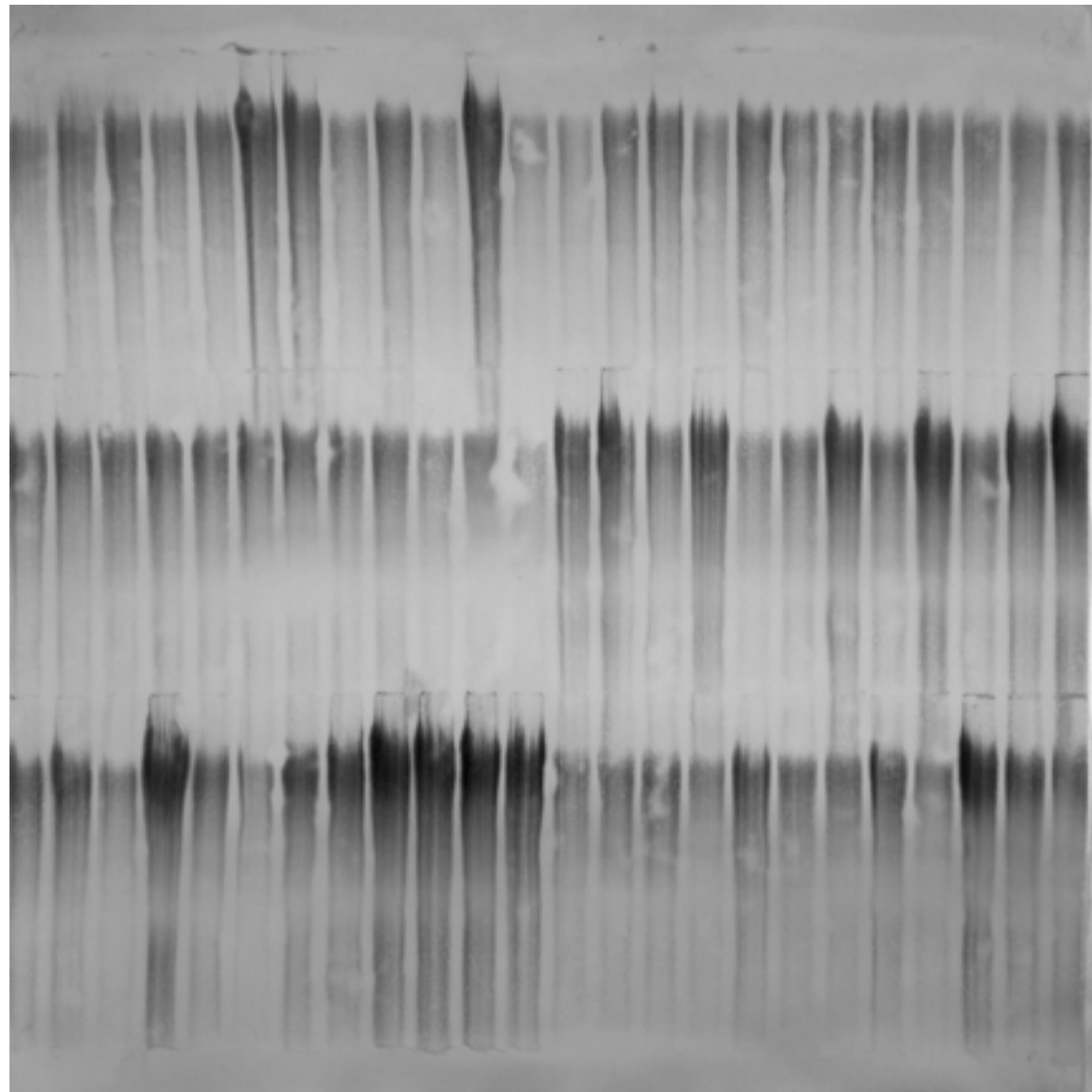
Hipp1 1-dre reporter td-sfGFP allele targeting vector prep for electroporation



AsiSI didn't cut - the Addgene NGS sequence indeed showed no AsiSI site

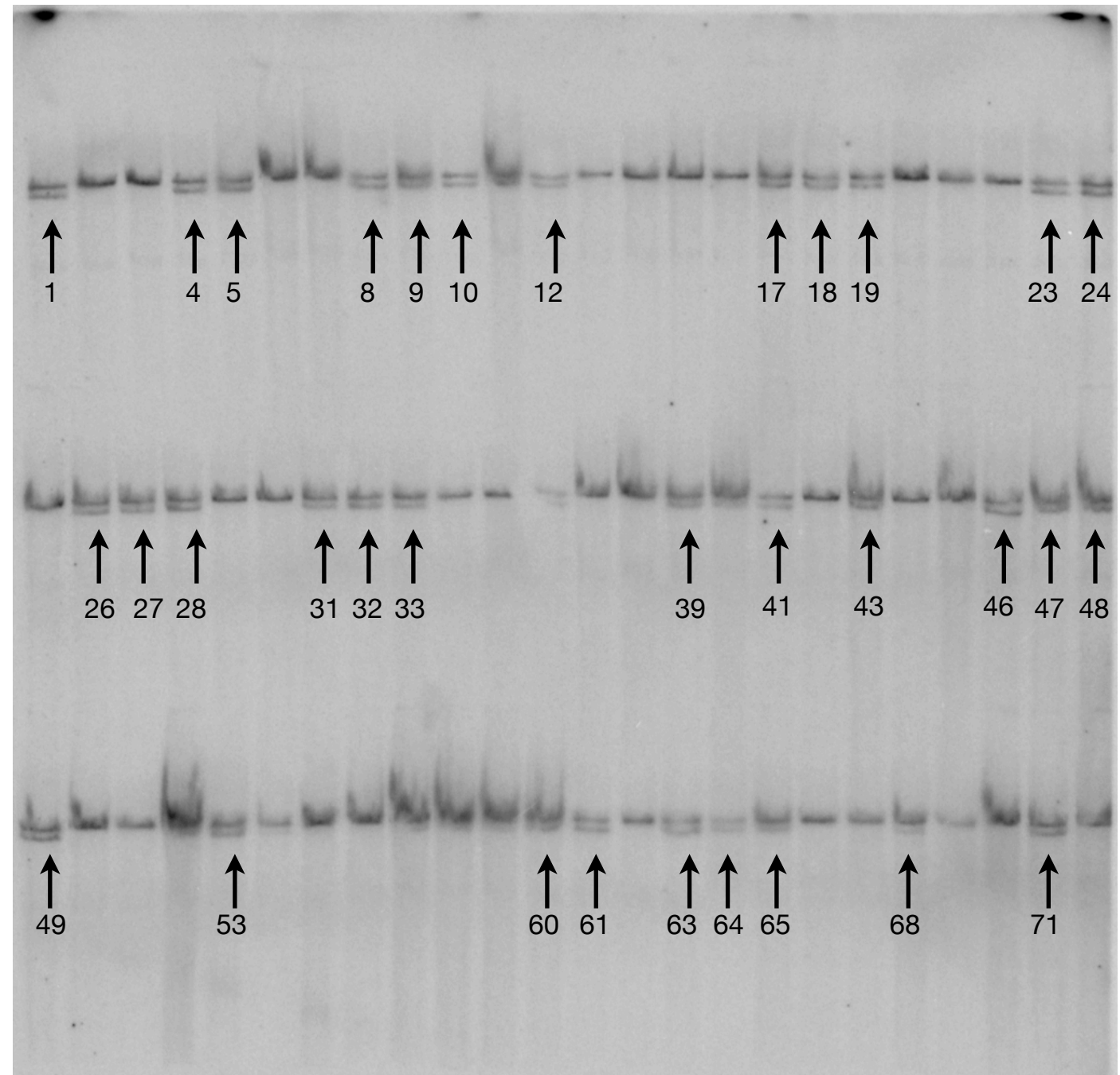
Hipp1 1-dre reporter *td-sfGFP* allele EP Southern blots

Methylene blue stained membrane



3' blot

BamHI
9.0 kb wt
8.0 kb knock-in

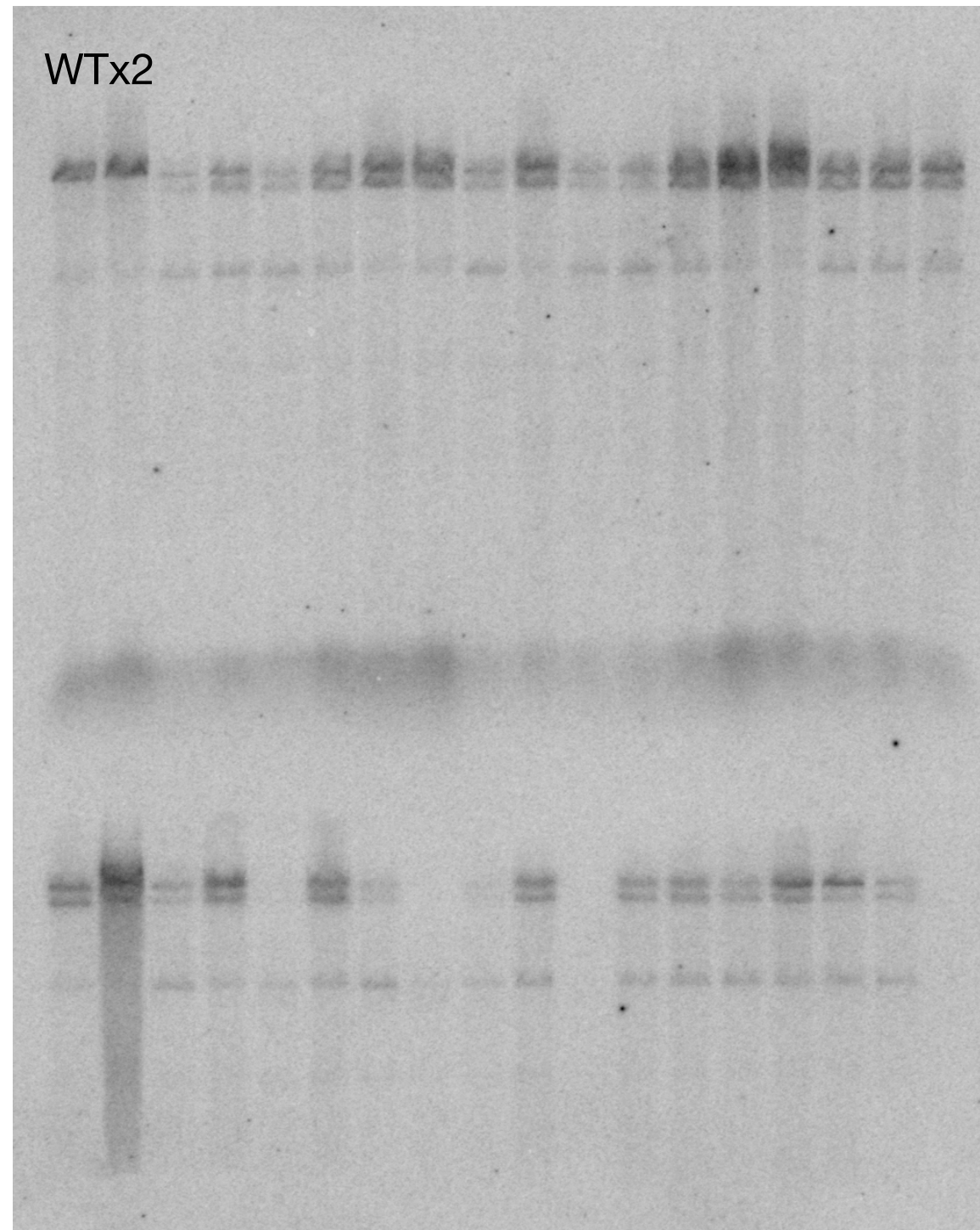


46%

Hipp1 1-dre reporter *td-sfGFP* allele EP Southern blots

5' blot

KpnI-HF + BstZ17I
16 kb wt
13 kb knock-in



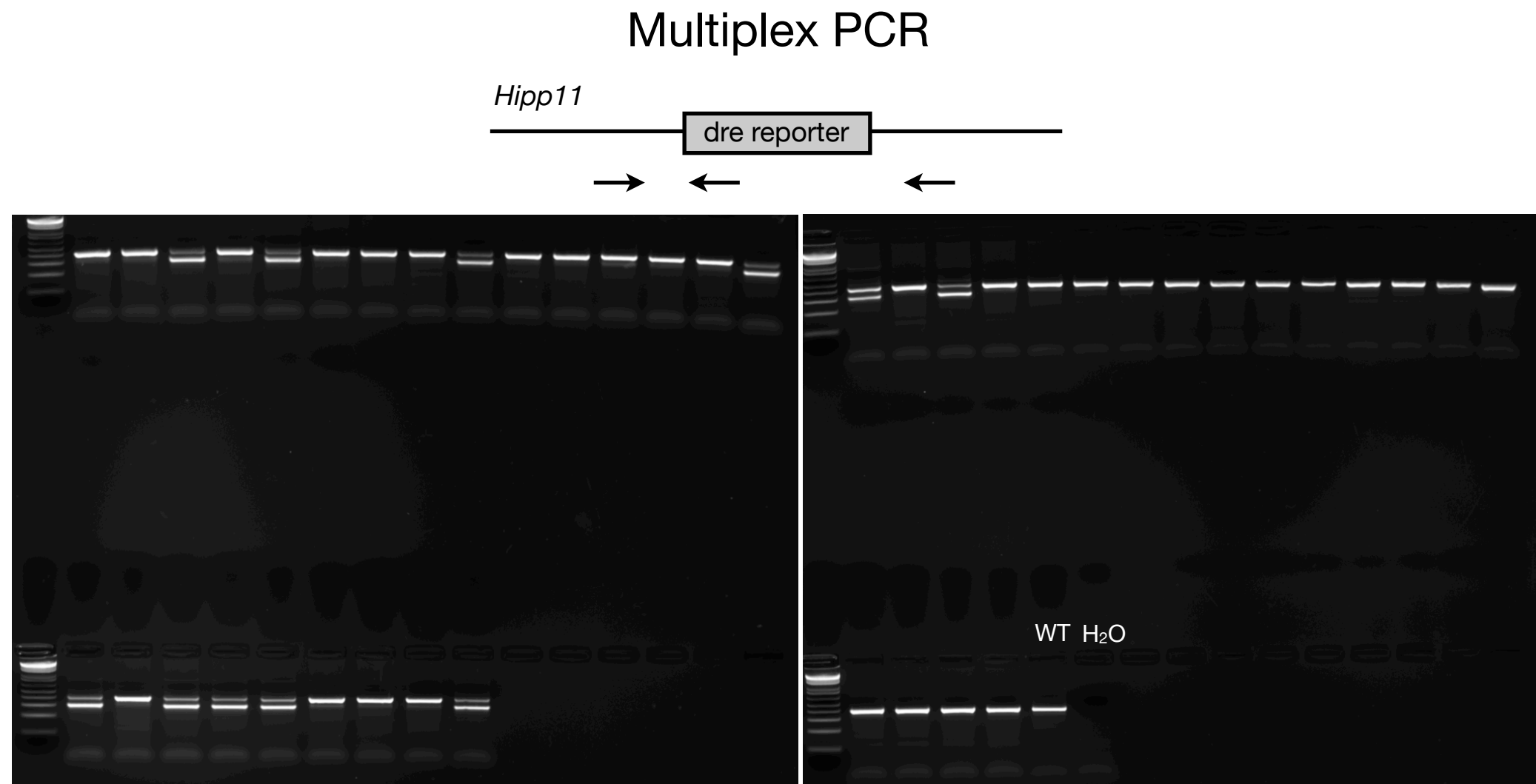
Hipp1 allele EP

- ~50% recombination efficiency
 - Highly recombinogenic locus
- pCAG-FRT-Neo*-FRT-bpA positive selection and pPGK-DTA-bpA negative selection worked well in mouse ES cells

Hipp1 1-dre reporter td-sfGFP allele chimeras

- EP 1023 clone #53
 - Males
 - 2 – 10%
 - Females
 - 1 – 50% runt
 - 1 – 30%
 - 1 – 15%
 - Black pups only
- EP 1023 clone #61
 - Males
 - 1 – 90%
 - 1 – 70%
 - 1 – 60%
 - 1 – 50%
 - 2 – 40%
 - 1 – 2%
 - Female
 - 1 – 20%
 - Agouti pups

Hipp1 1-dre reporter td-sfGFP allele germline transmission



Pups from crosses of different clone #61 chimeras x B6J females

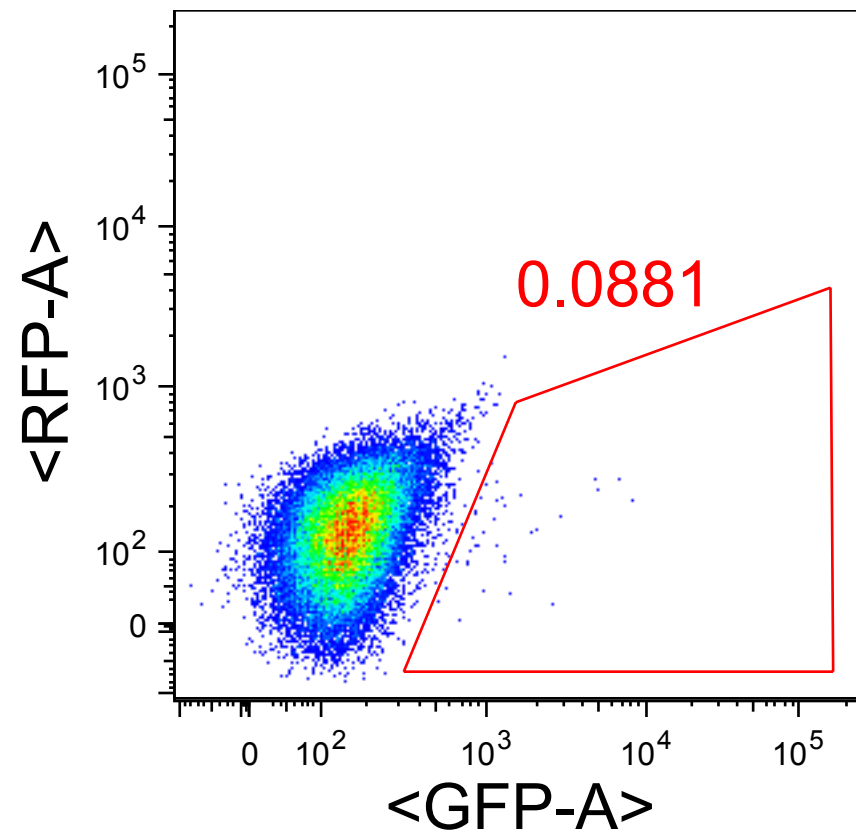
Germline transmission

Bred to mixed background ACTB-FLPe transgenic mice

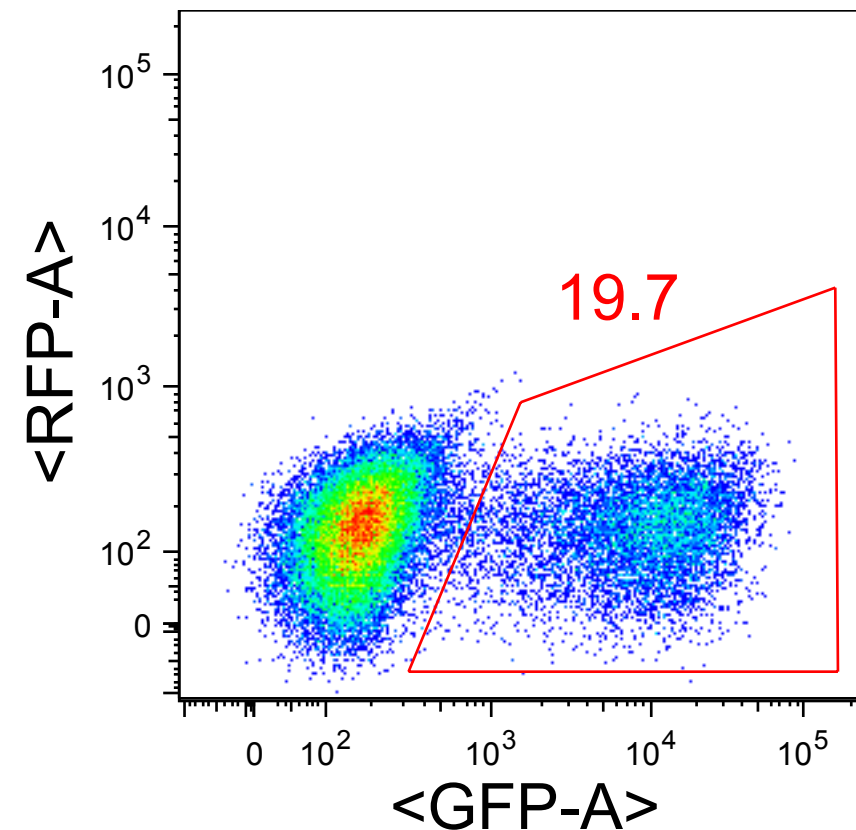
Hipp11-dre/rox reporter *td-sfGFP* allele characterization in cells

Day 1 after electroporation of clone #61 ES cells
Flow cytometry of live cell fluorescence

+pBS2SK-

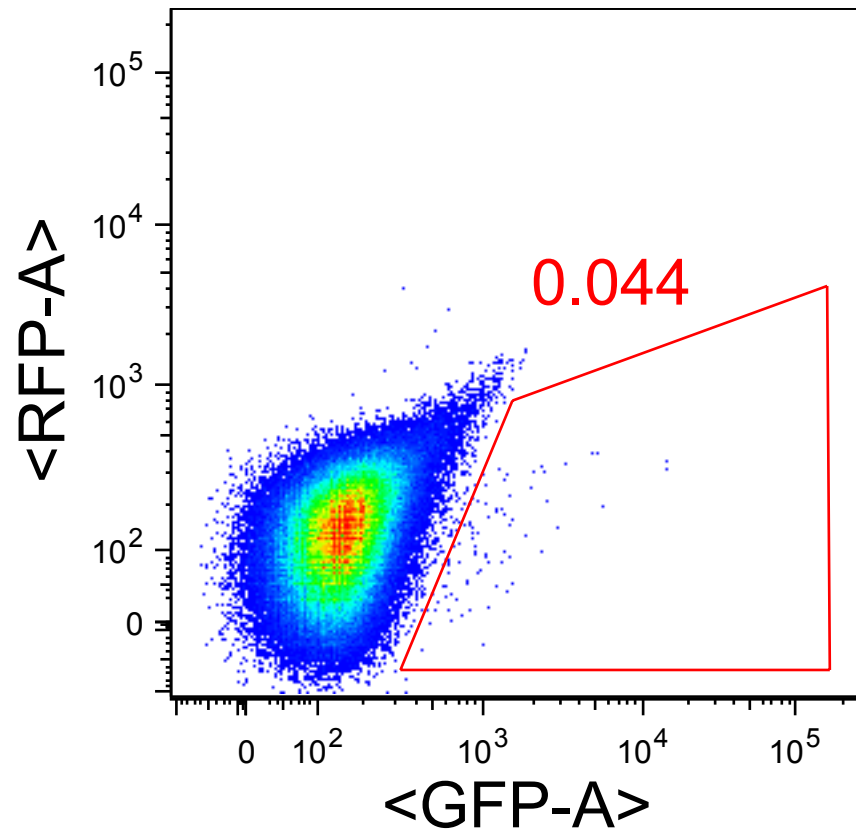


+pCAGGS-pac-T2A-dre

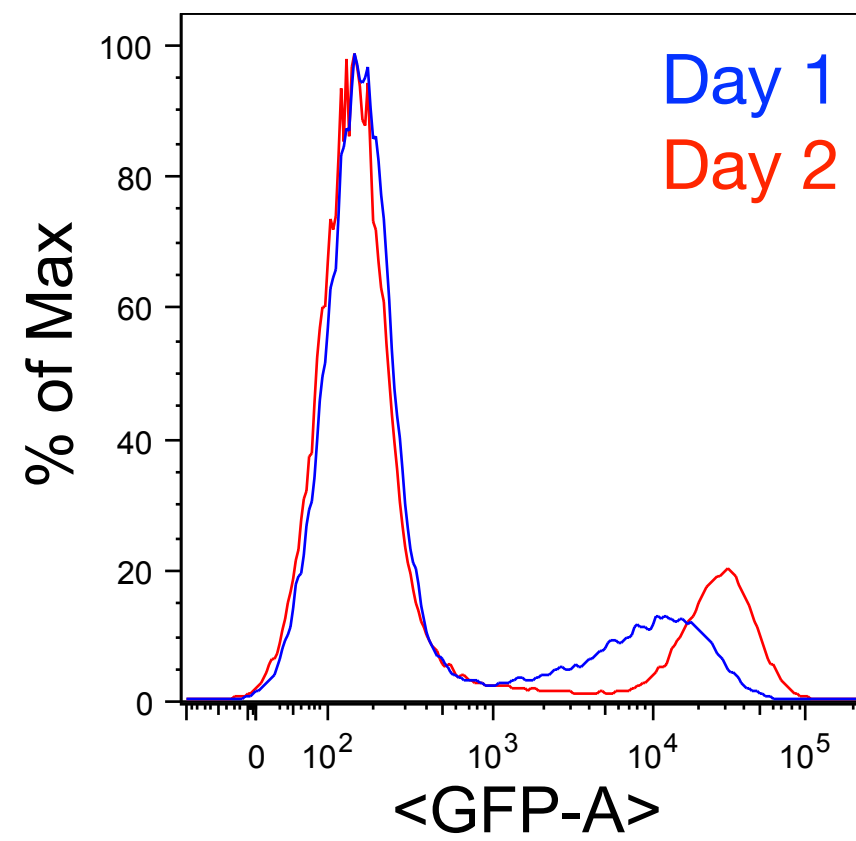
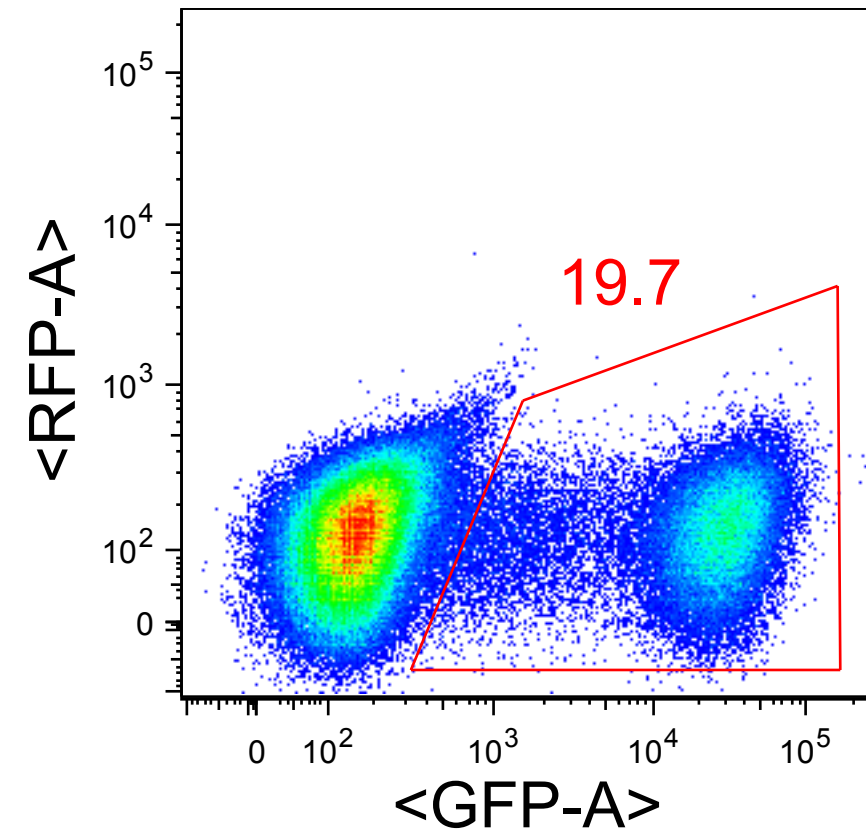


Hipp11-dre/rox reporter *td-sfGFP* allele characterization in cells
Day 2 after electroporation

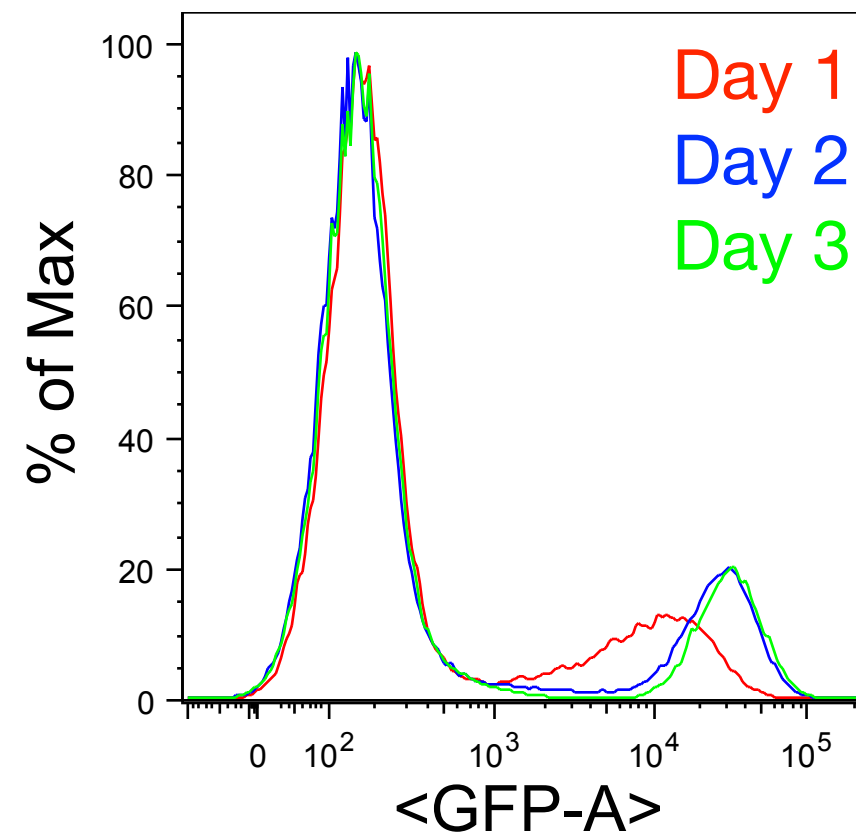
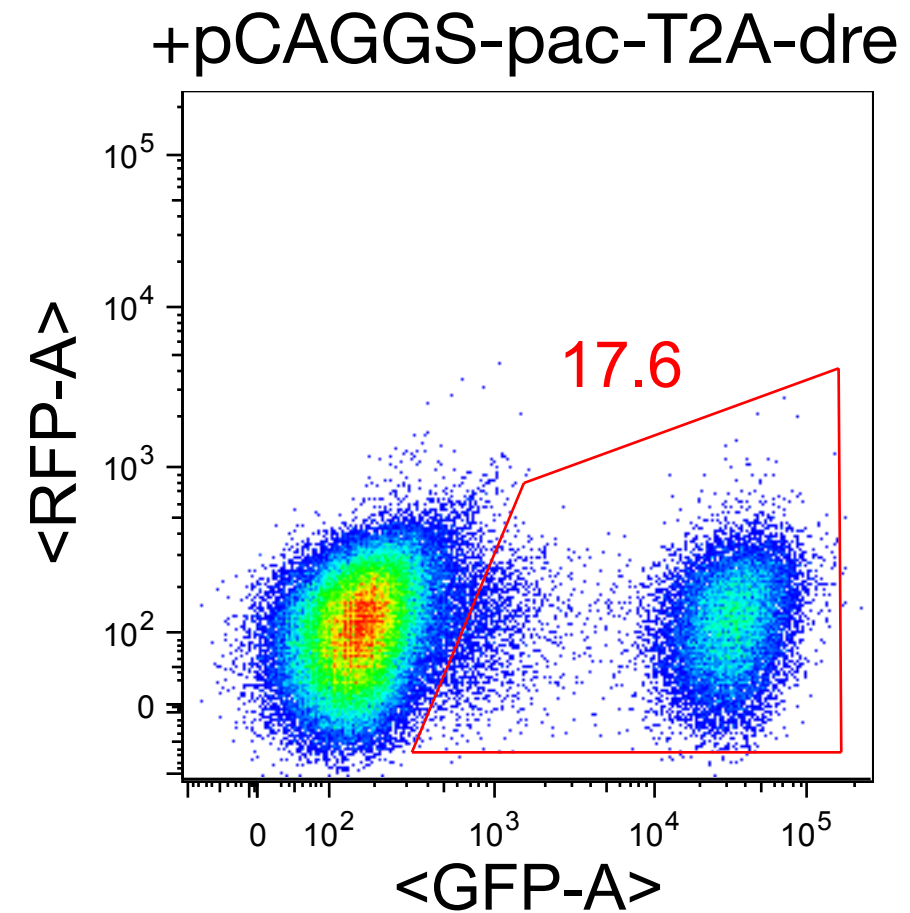
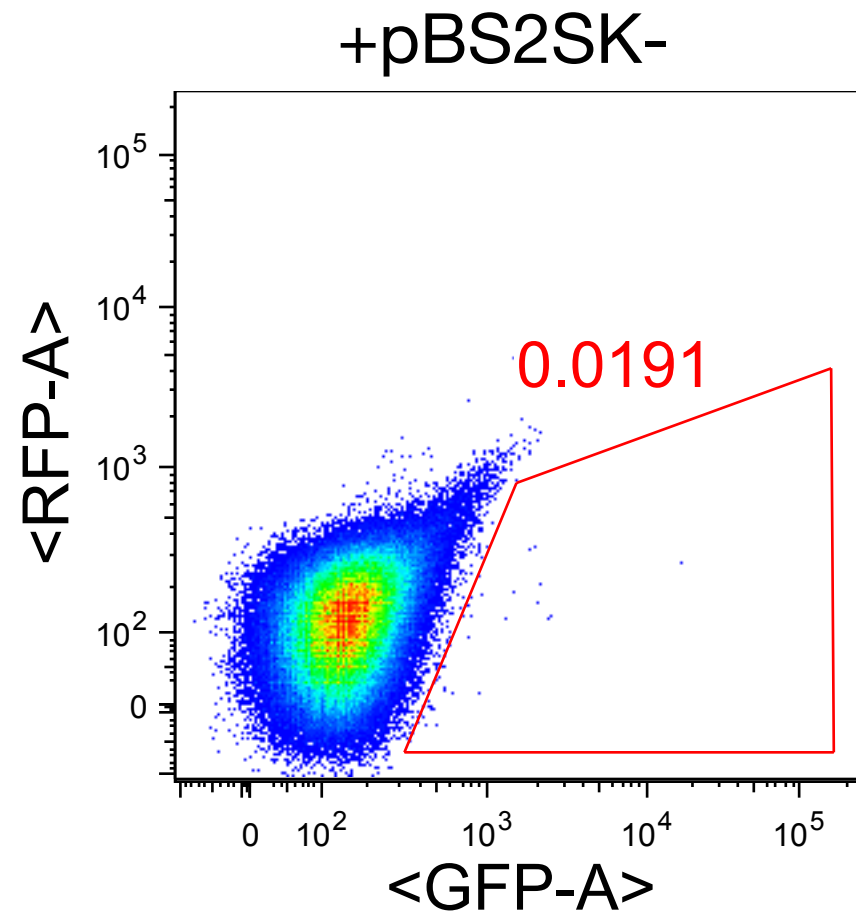
+pBS2SK-



+pCAGGS-pac-T2A-dre



Hipp11-dre/rox reporter *td-sfGFP* allele characterization in cells
Day 3 after electroporation



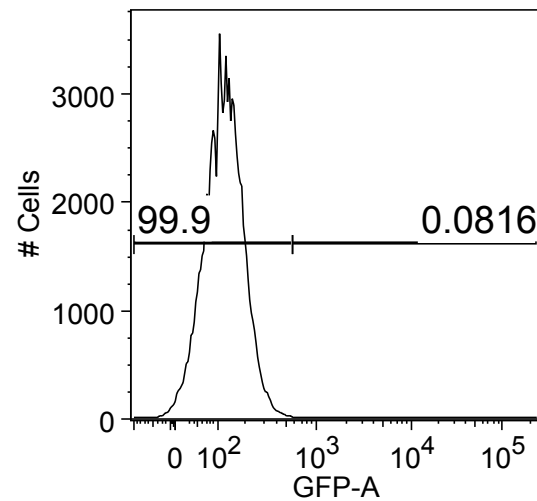
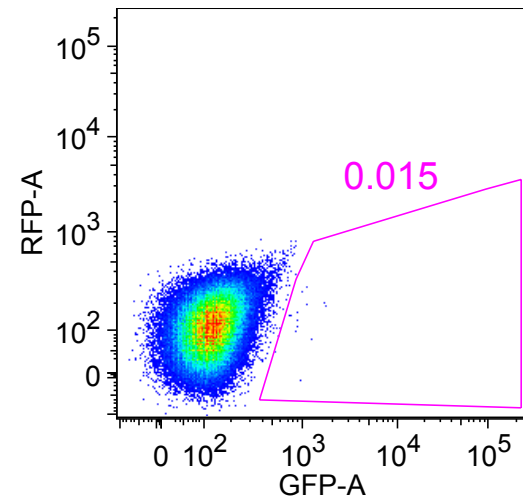
Exceptionally bright
green fluorescence
(see next page)

Also, iCre plasmid
electroporation did not
recombine the rox
sequences (not shown)

Comparison of *Rosa26* and *Hipp11* in ES cells

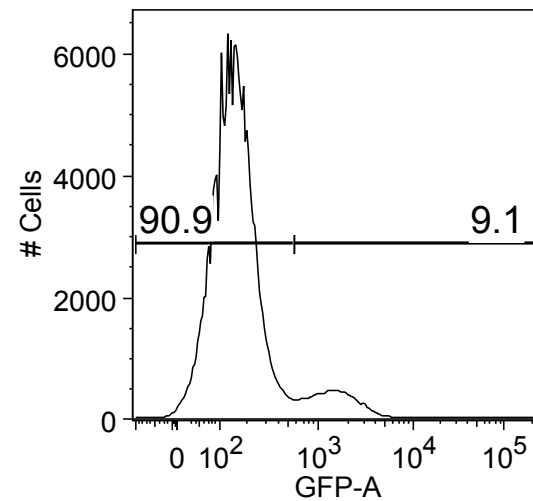
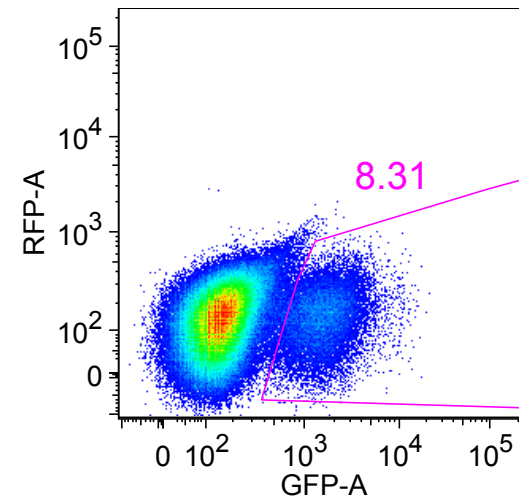
Day 2 after electroporation

No recombinase plasmid
control



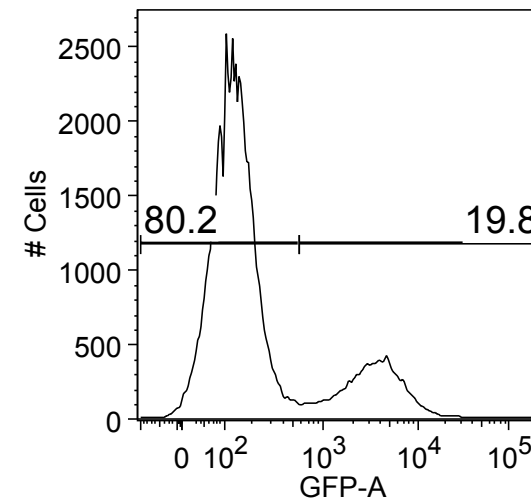
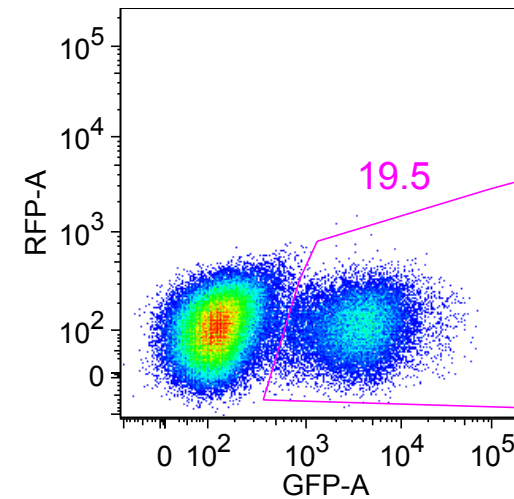
(GFP-A): Geom. Mean = 697
(GFP-A): Median = 655

Rosa26-CAG-EGFP
(Addgene #185963)
+ pCAGGS-pac-T2A-iCre



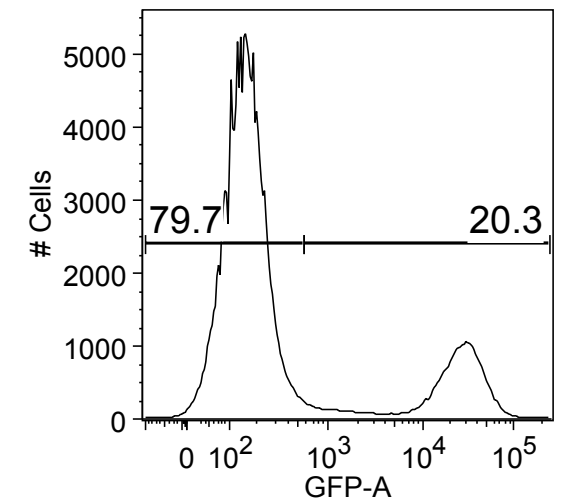
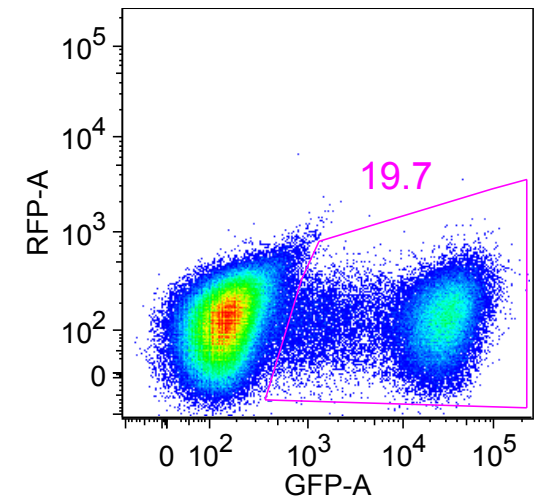
(GFP-A): Geom. Mean = 1459
(GFP-A): Median = 1418

Rosa26-CAG-sfGFP
(Addgene #185965)
+ pCAGGS-pac-T2A-iCre



(GFP-A): Geom. Mean = 3248
(GFP-A): Median = 3432

Hipp11-CAG-td-sfGFP
(Addgene #139513)
+ pCAGGS-pac-T2A-dre



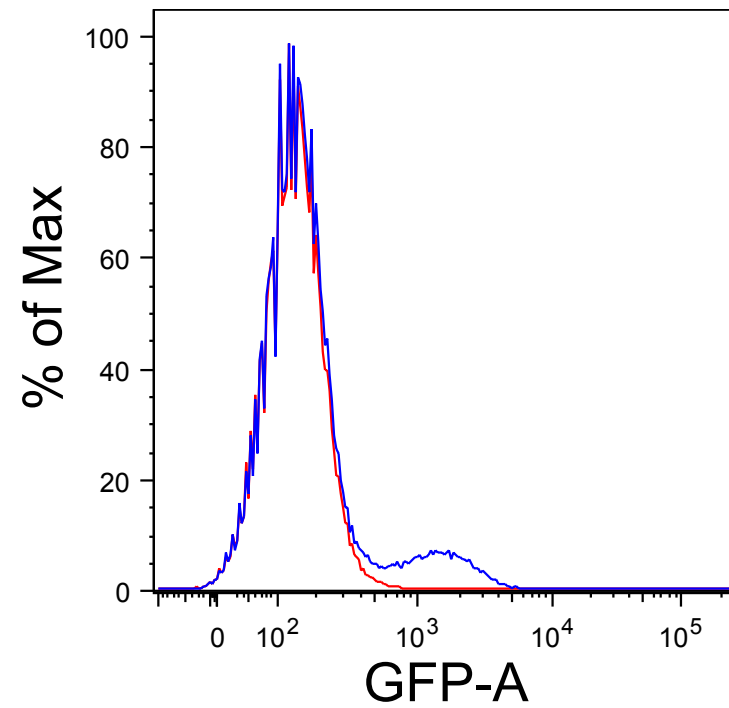
(GFP-A): Geom. Mean = 17380
(GFP-A): Median = 25293

Comparison of *Rosa26* and *Hipp11* in ES cells

Day 2 after electroporation

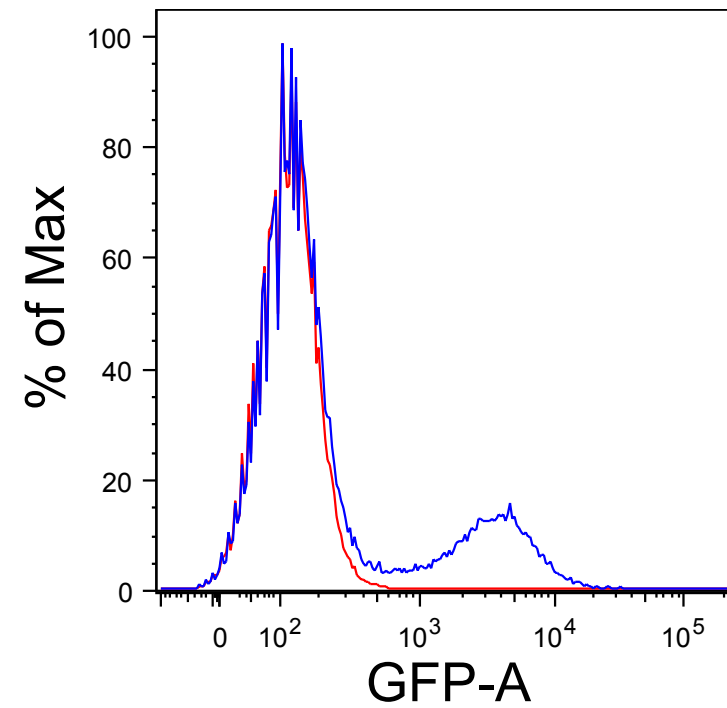
Rosa26-CAG-EGFP (#185963)

Control



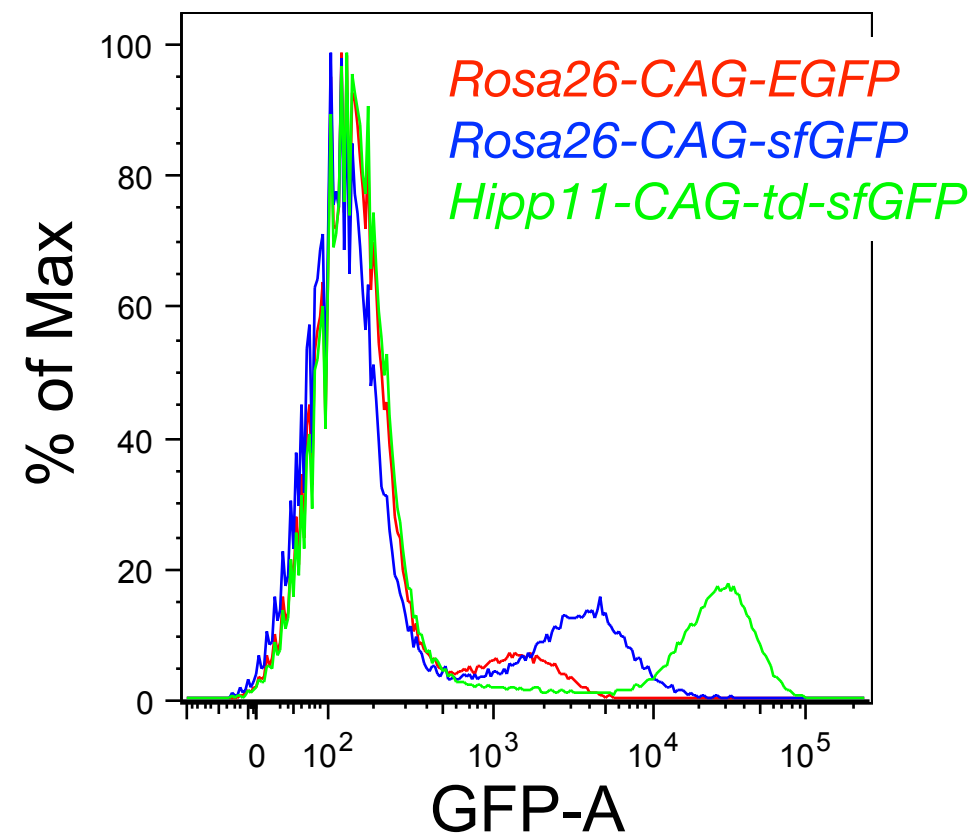
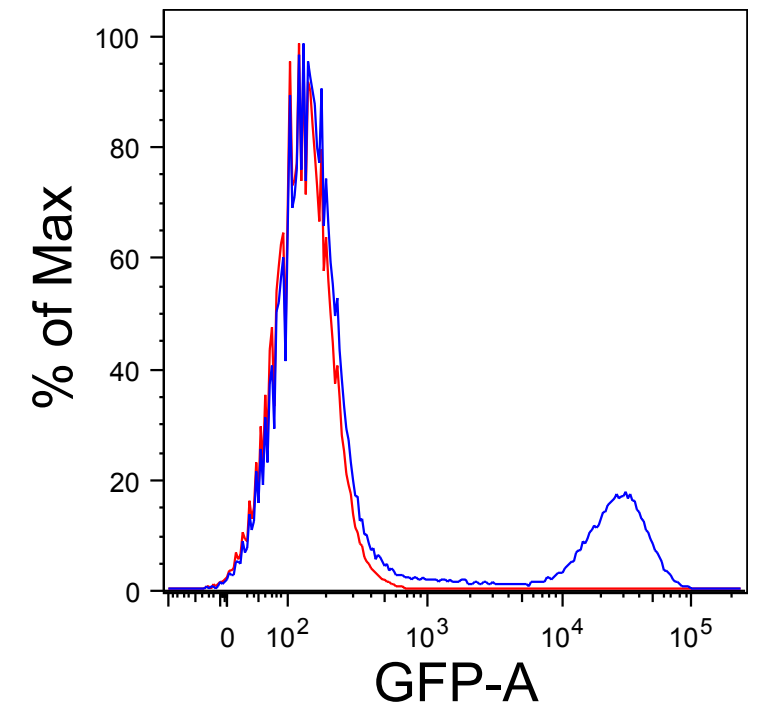
Rosa26-CAG-sfGFP (#185965)

Control



Hipp11-CAG-td-sfGFP (#139513)

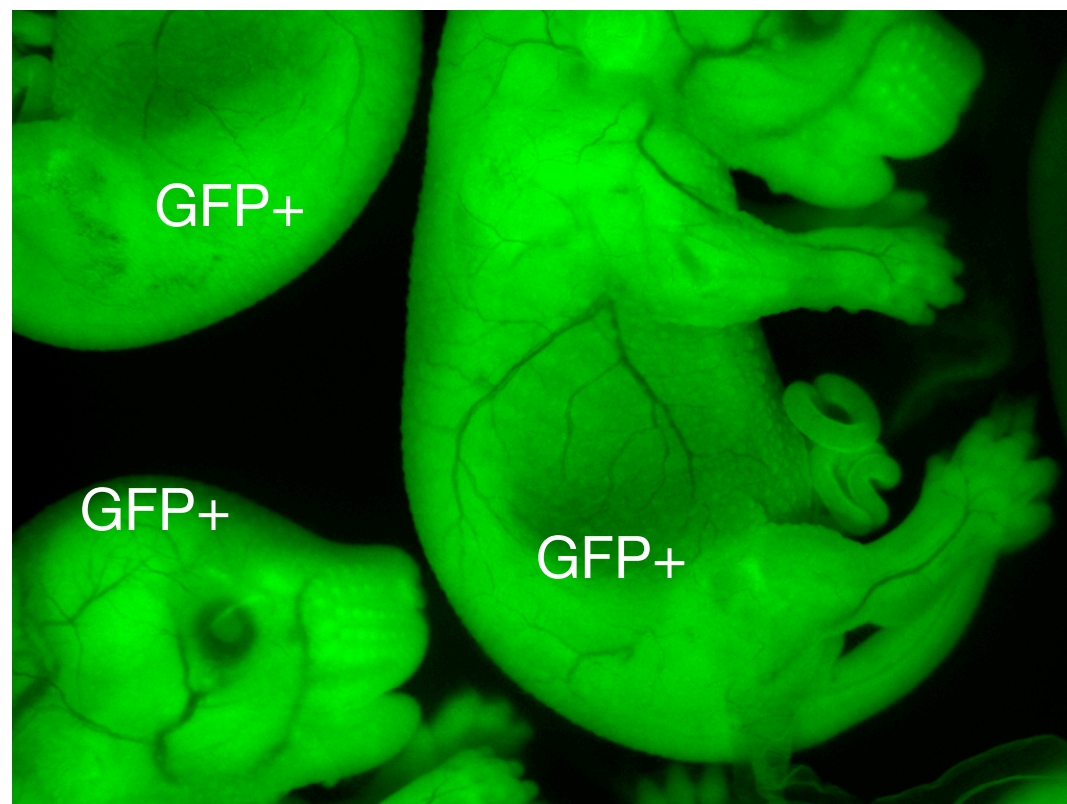
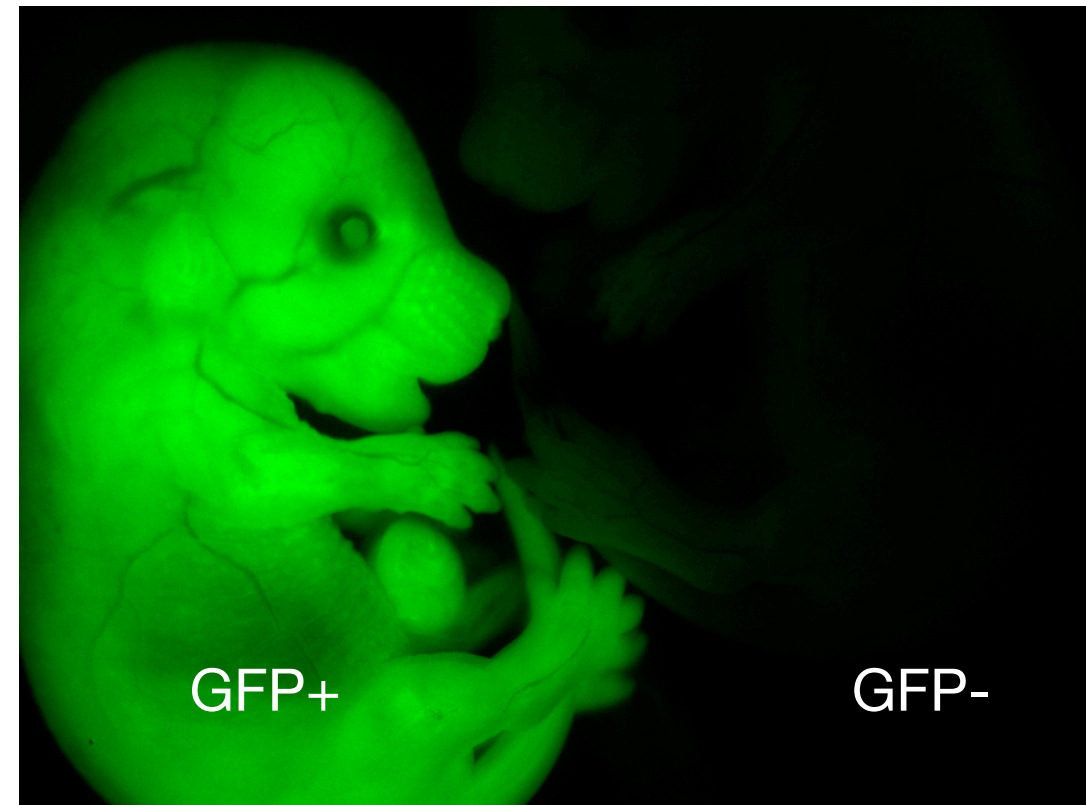
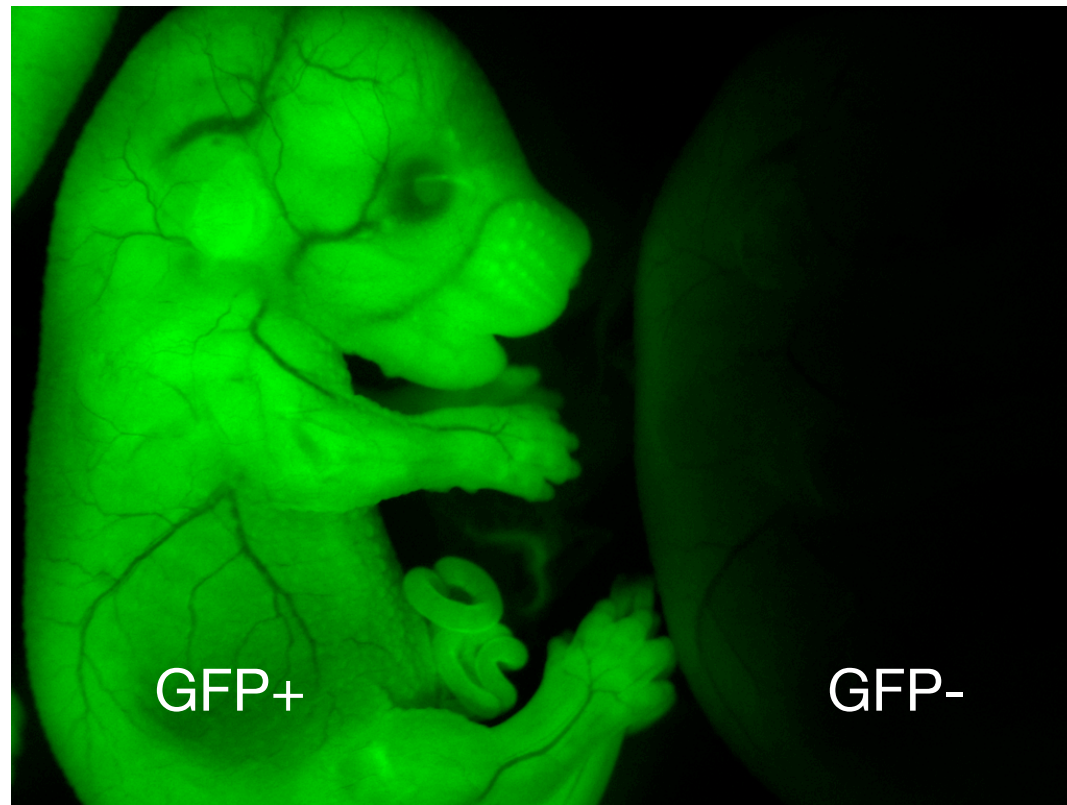
Control



Reporter allele functionality in vivo

Embryos from cross of *Hipp11-dre reporter td-sfGFP* het male x *Hprt-CAG-dre* hom female

Fluorescence from live embryos

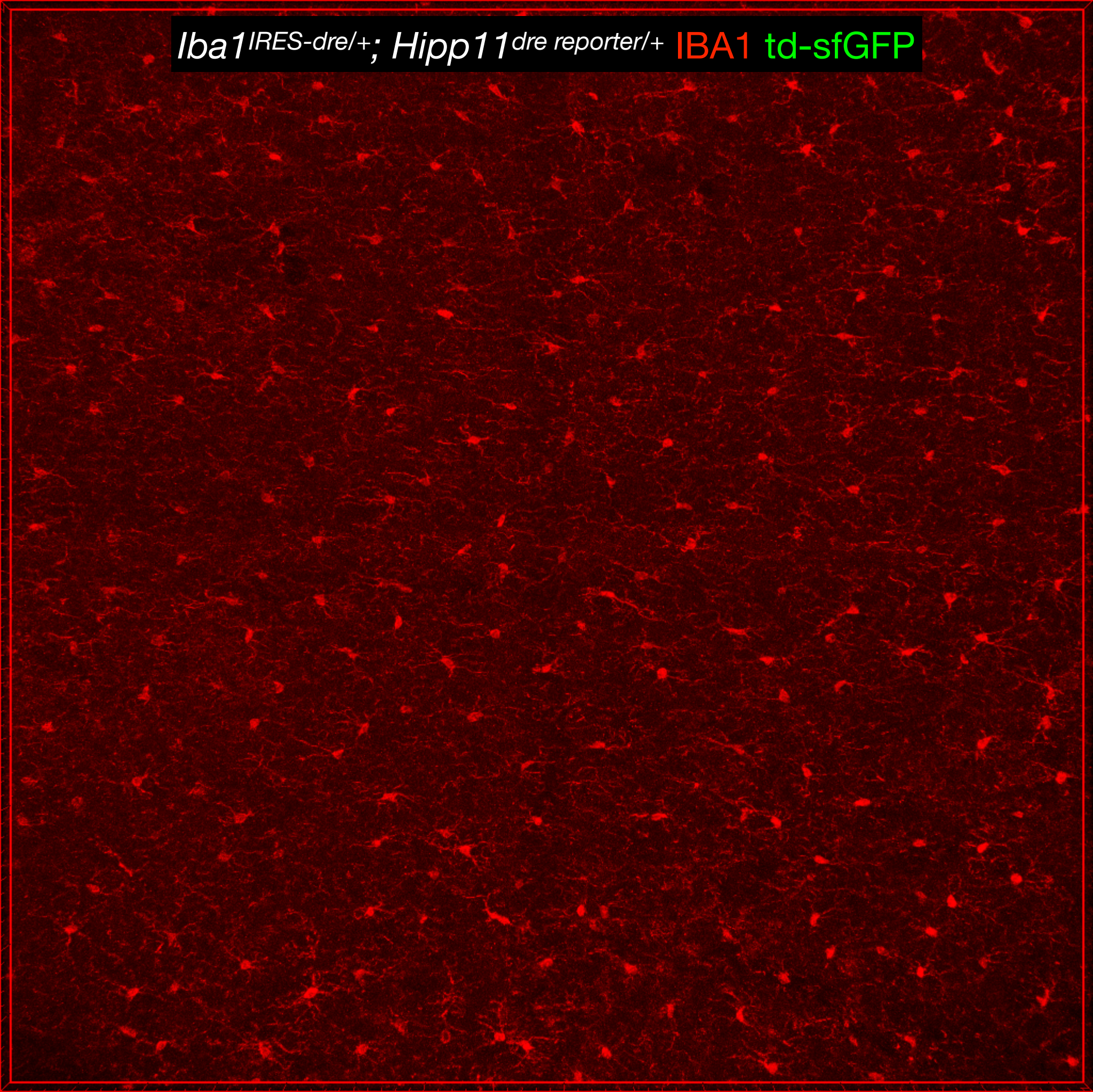


Green fluorescent embryos →
the rox sites and the reporter
construct are intact

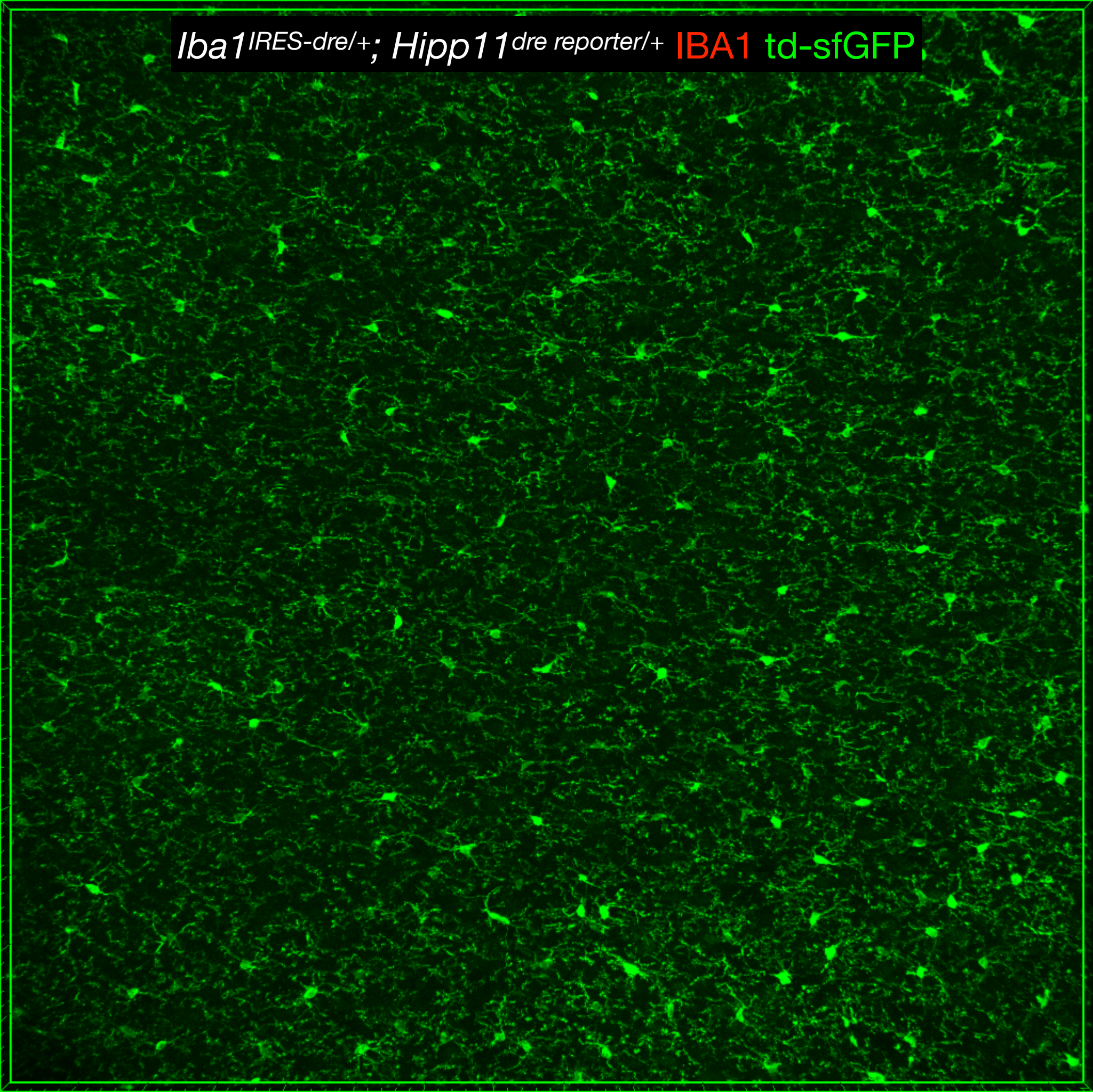
The *Hprt-CAG-dre* mice from
Dr. Simon Titen via Dr. Ben Xu

- *Hipp11-dre reporter* tested with *Iba1-IRES-dre* by Dr. Ben Xu
- *Iba1^{IRES-dre/+}; Hipp11^{dre reporter/+}* mouse
- IBA1 protein immunoreactivity in red, td-sfGFP protein immunoreactivity (chicken polyclonal anti-GFP antibody) in green

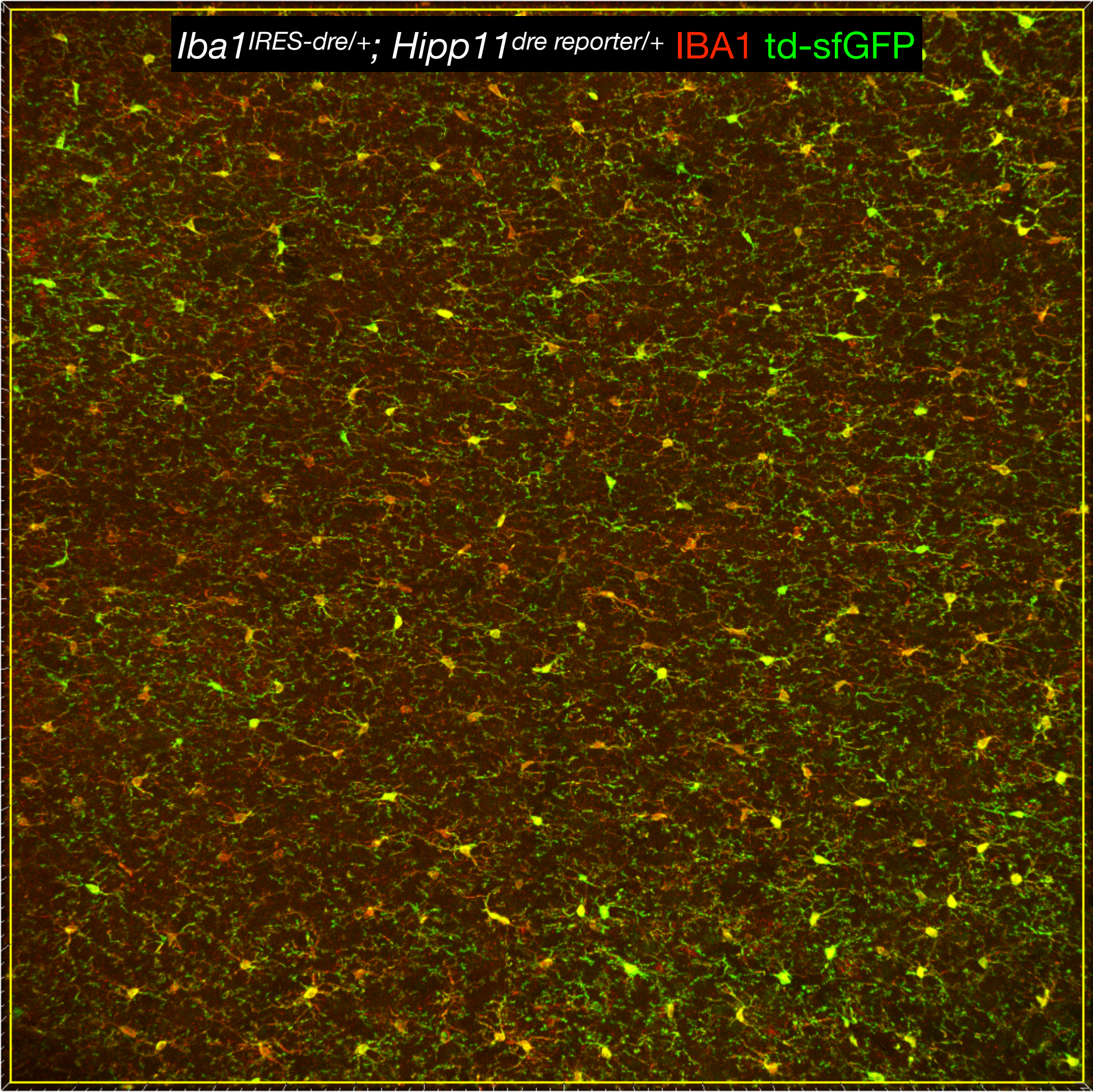
Iba1^{IRES-dre/+}; *Hipp1*^{1^{dre reporter}/+} IBA1 td-sfGFP



Iba1^{IRES-dre/+}; *Hipp1*^{dre reporter/+} IBA1 td-sfGFP



Iba1^{IRES-dre/+}; *Hipp1*^{dre reporter/+} IBA1 td-sfGFP



- Some spots are dimmer for IBA1, and some spots are dimmer for td-sfGFP (I don't know why, Dr. Ben Xu did the staining and imaging), but the green and red channels seem to overlap fairly well
- The anti-GFP antibody is compatible with the sfGFP variant used in this mouse line

Dre specificity in vivo

Tg(Nestin-cre)/+; *Rosa26^{HN21/+}*

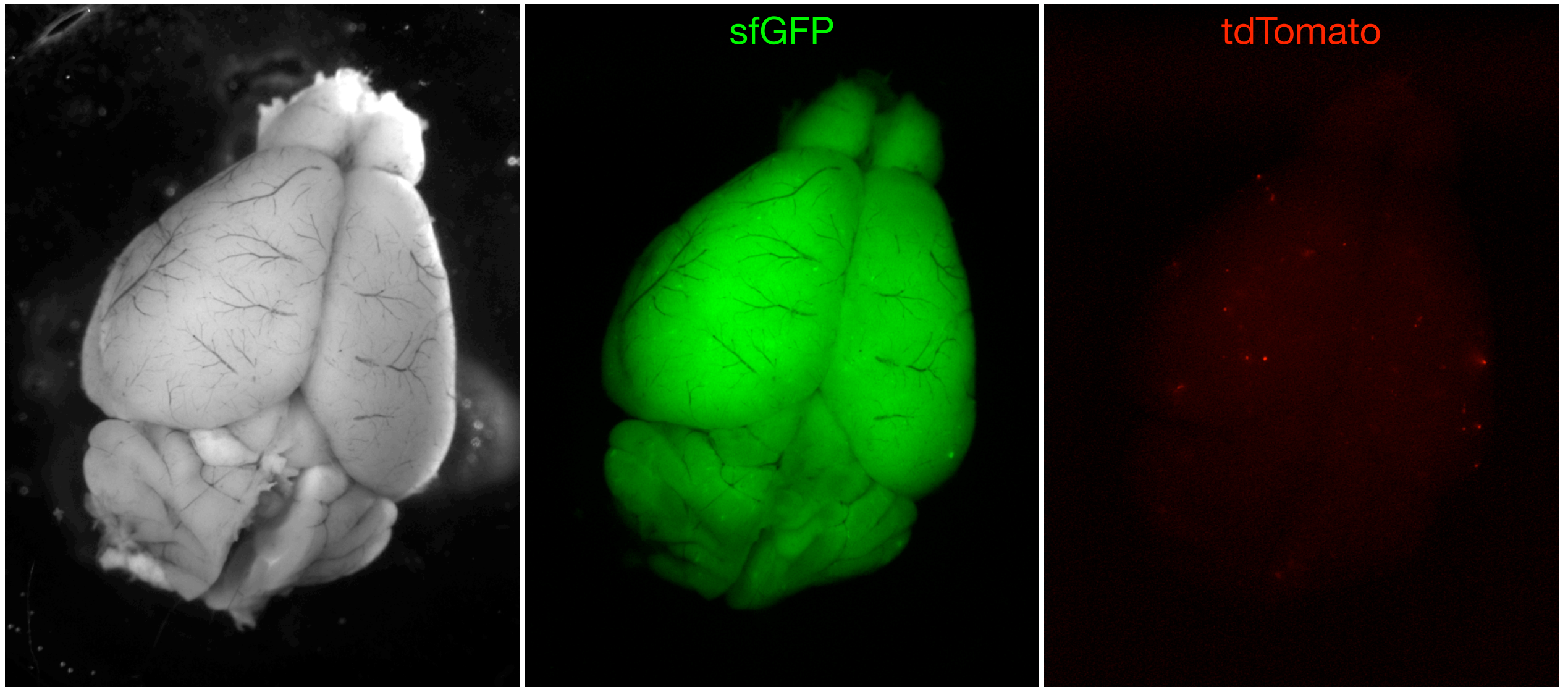
Rosa26^{HN21} is a reporter allele with both lox and rox sites (Addgene #185965)

designed to express after

cre recombination → green fluorescence

cre and dre recombination → green and red fluorescence

Live brain fluorescence from sfGFP and tdTomato

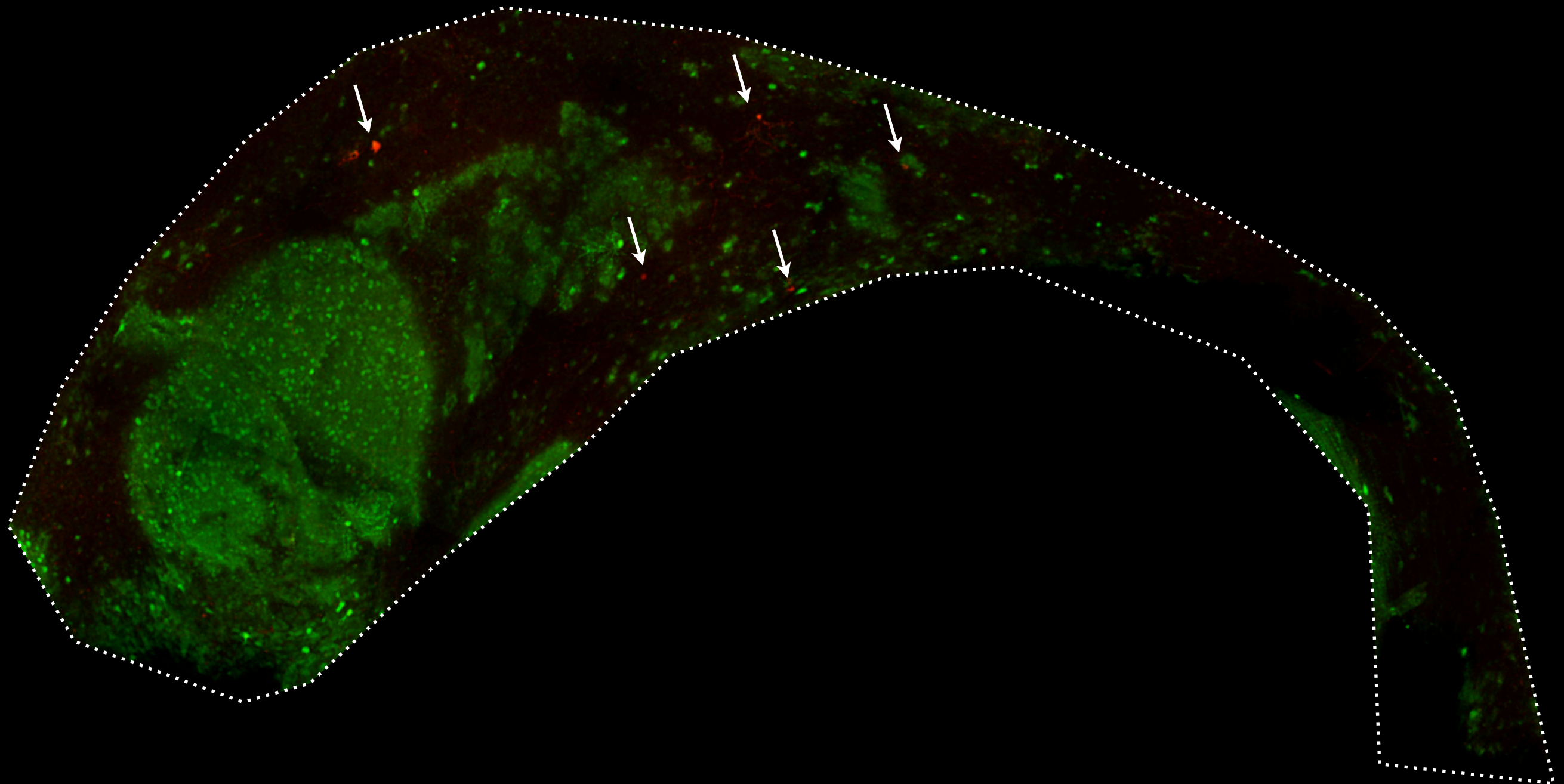


Can see rare RFP+ cells

Nestin-cre transgene but no dre did result in rare red cells with this reporter

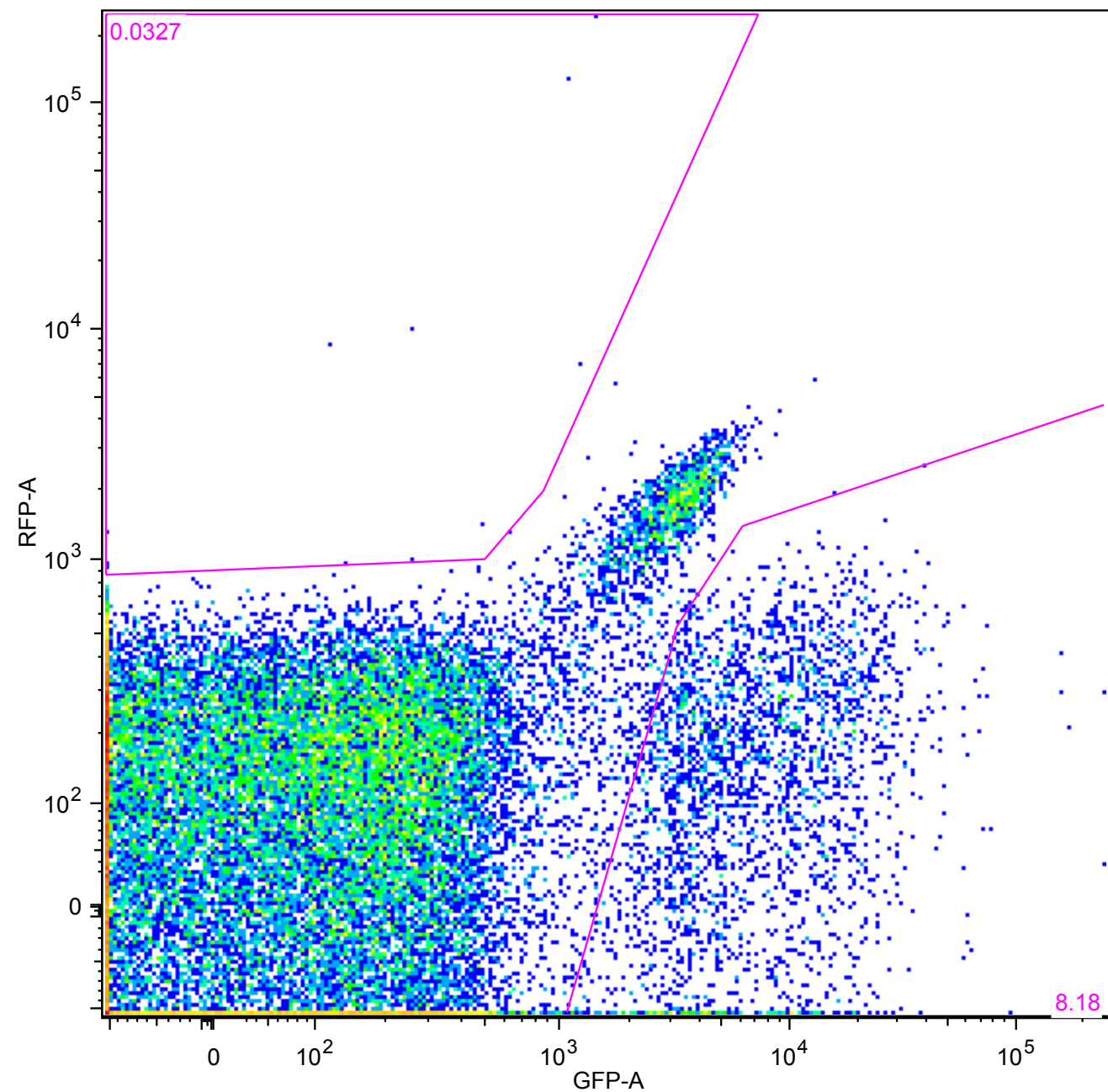
Dre specificity in vivo
Tg(Nestin-cre)/+; *Rosa26^{HN21}*/+
~6 months old

sfGFP tdTomato whole mount immunofluorescence



Nestin-cre transgene but no dre did result in rare
red cells with this reporter (both lox and rox sites)

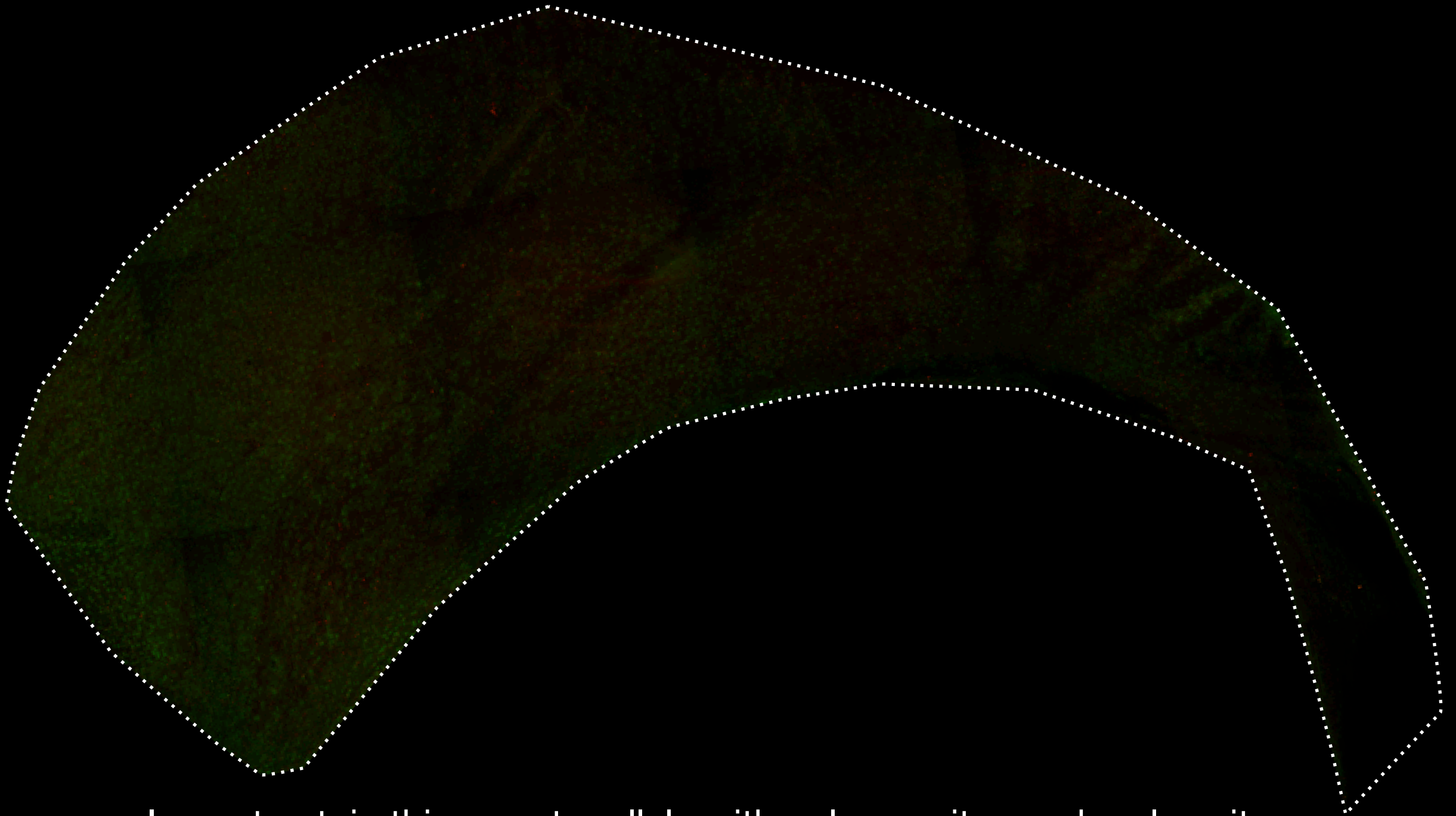
Dre specificity in vivo
Tg(Nestin-cre)/+; *Rosa26^{HN21/+}*
Flow cytometry of live dissociated lateral ventricular wall cells



Nestin-cre transgene but no dre did result in rare red cells with this reporter (both lox and rox sites)

Dre specificity in vivo
Tg(Nestin-cre)/+; *Hipp1*^{dre reporter/+}
~6 months old

td-sfGFP tdTomato whole mount immunofluorescence

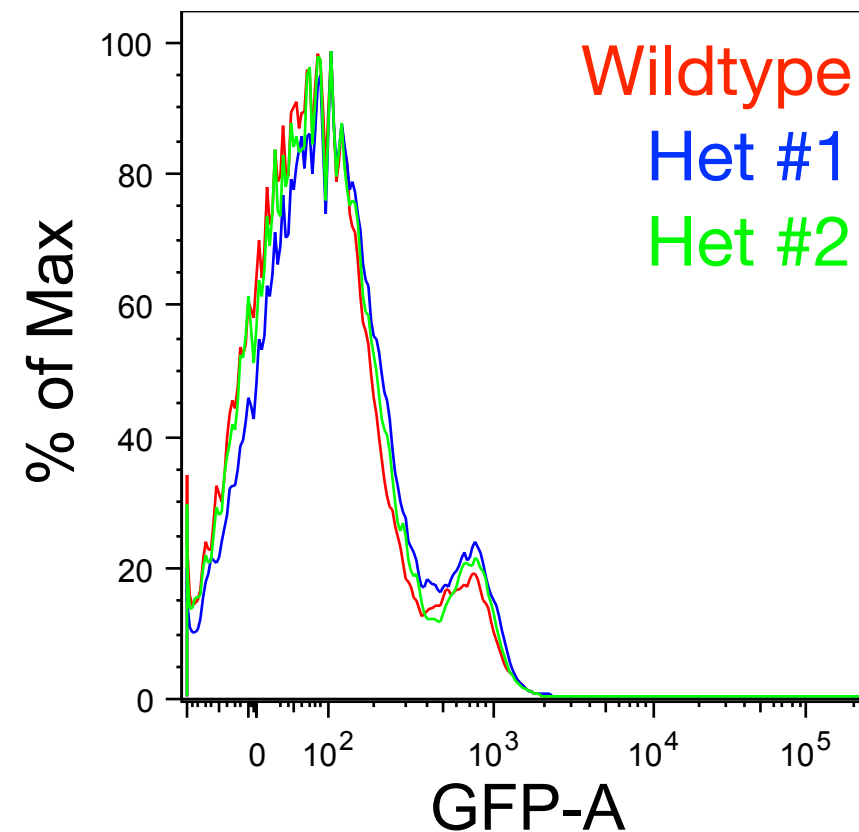
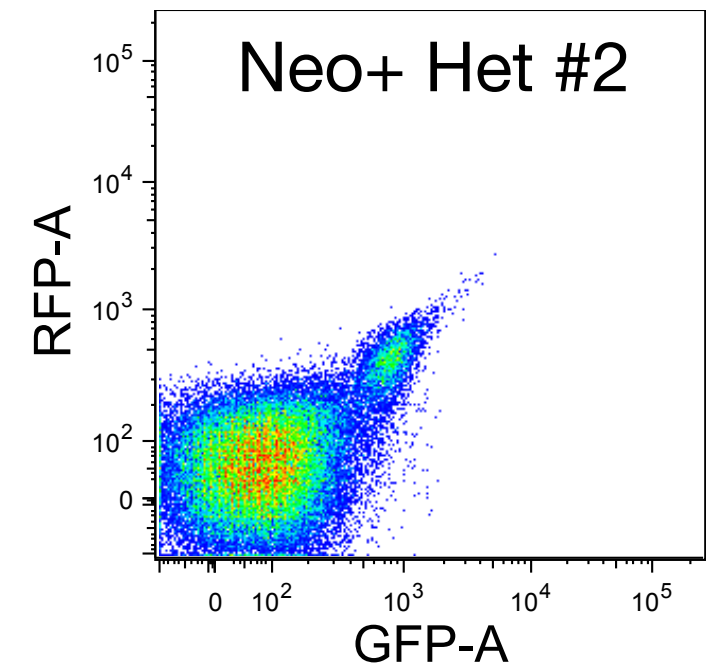
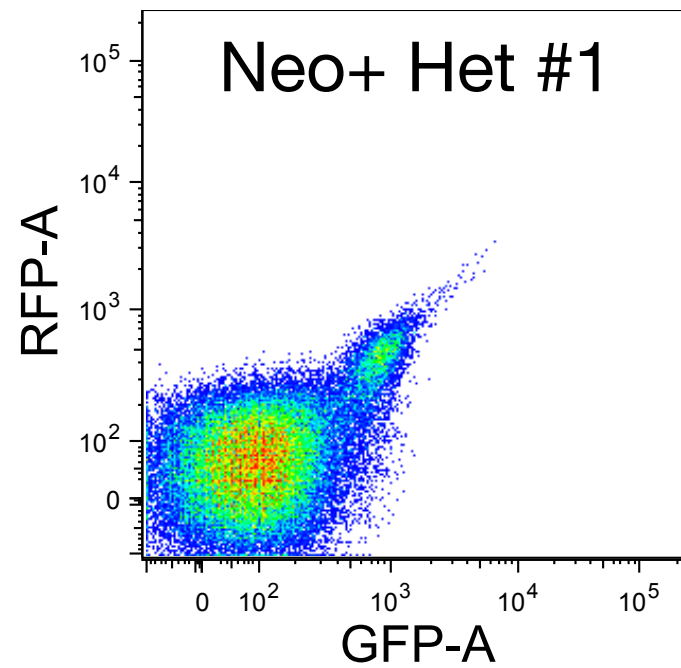
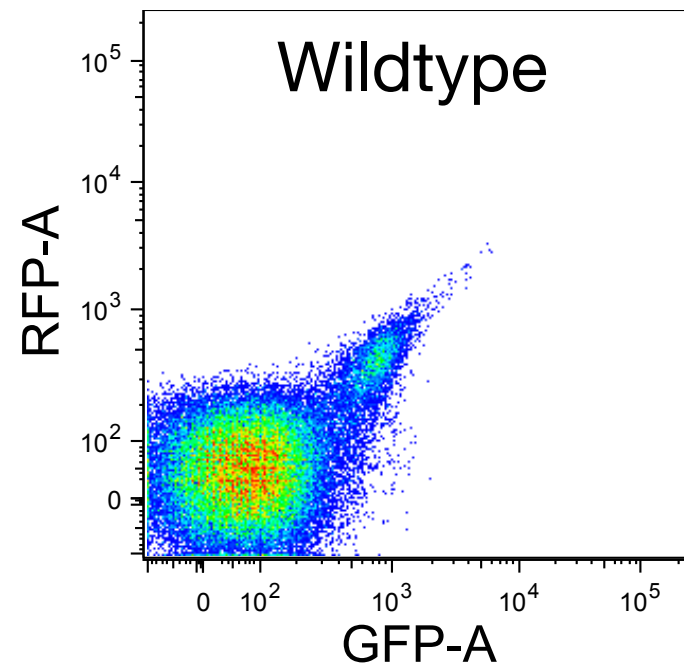
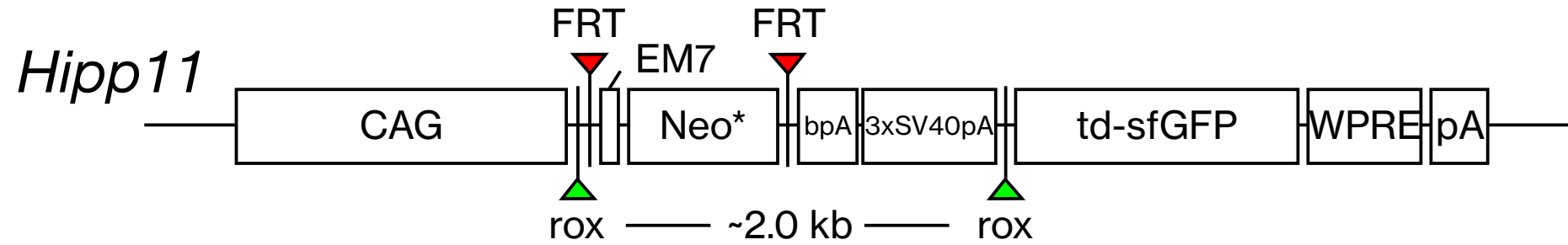


In contrast, in this reporter allele with only rox sites and no lox sites,
no GFP+ cells at all in three mice

The Nestin-cre transgene didn't recombine when rox only

Leakage in vivo

Adult mouse brain lateral ventricular wall cells from Neo+ *Hipp11-dre reporter td-sfGFP*

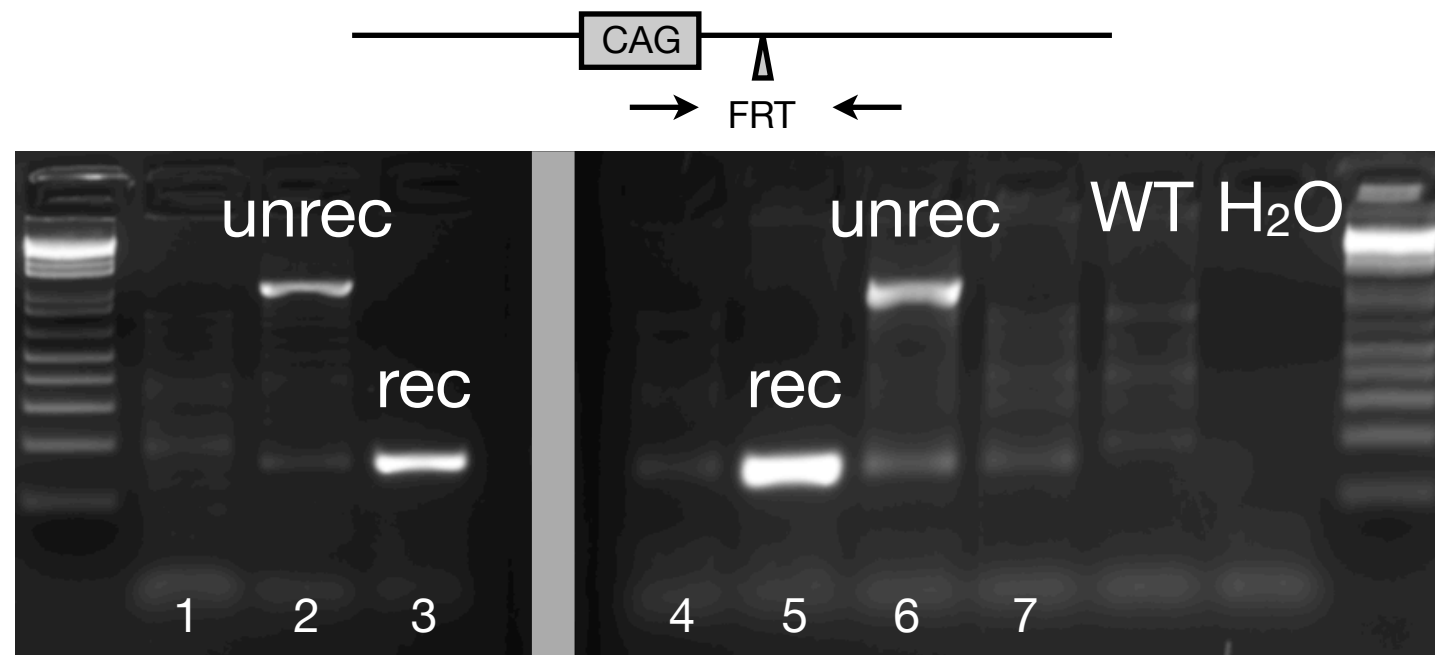


Dissociated lateral ventricular wall cells

FRT-Neo-FRT-
bGHpA-3xSV40pA as
transcriptional stop
cassette

Doesn't leak

Hipp1 1-dre reporter td-sfGFP FRT-Neo*-FRT loop out



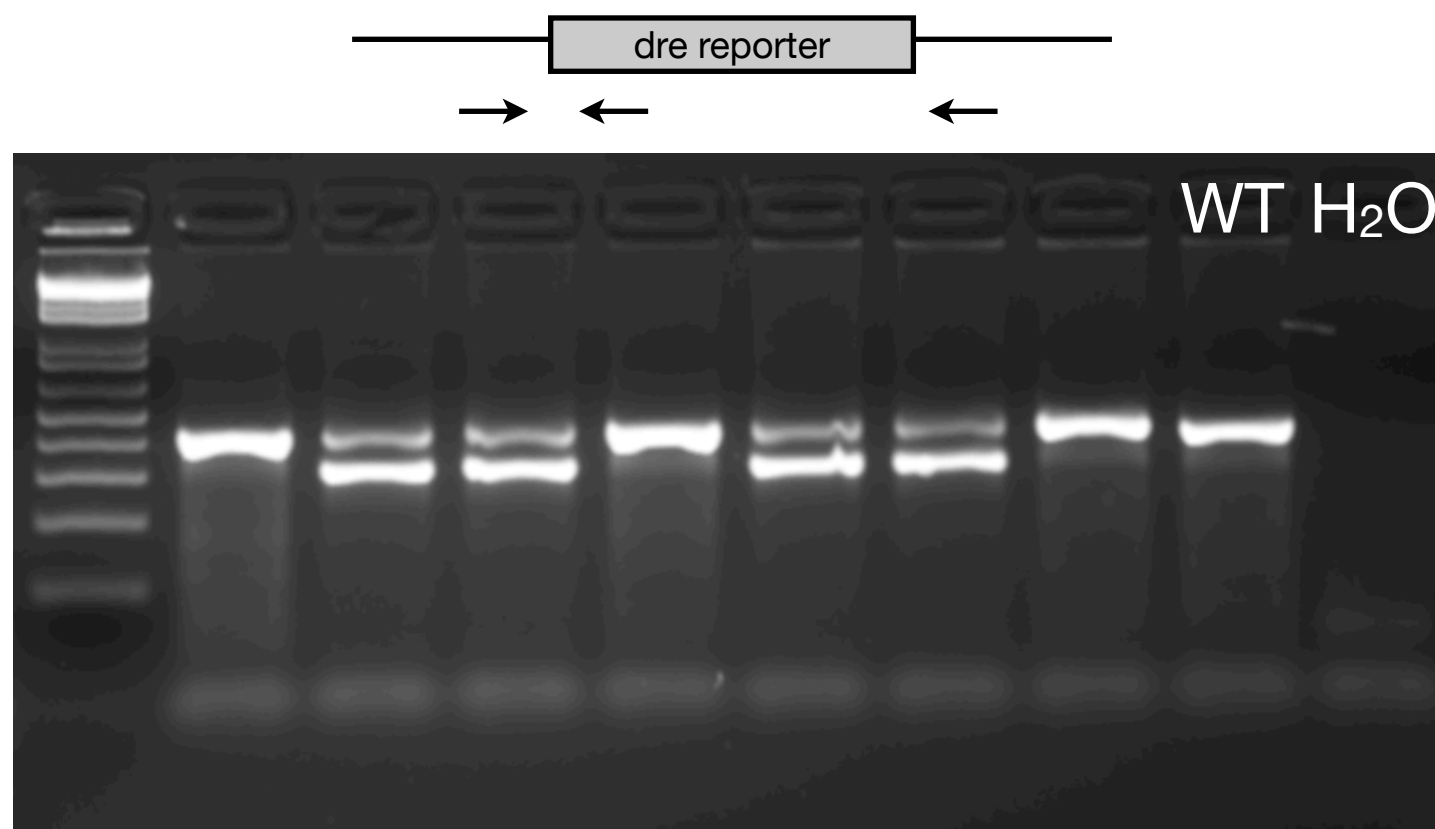
Pups from cross of FLPe+ male x Neo+ HN22 clone #61 female #8303-5

PCR amplified across unrecombined and recombined FRT sites

In some mice, the FRT sites were recombined → the sites were intact

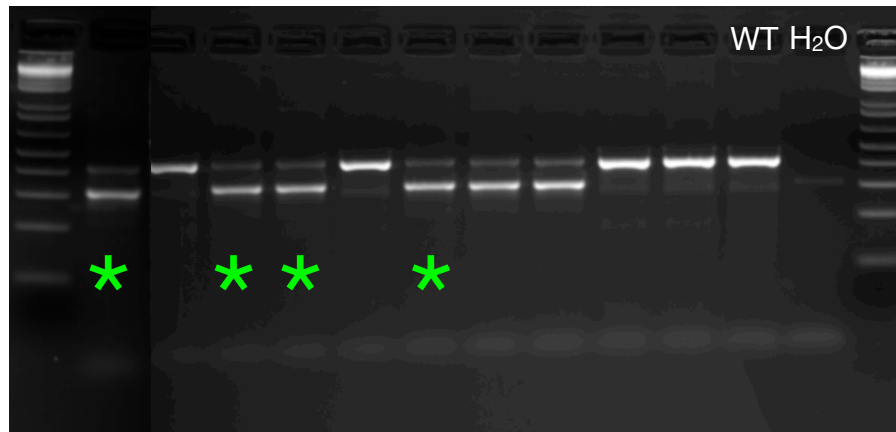
The FLPe+ mouse was probably a het

Hprt-CAG-cre in mother → recombination in all progeny even without the transgene, but not the ACTB-FLPe, especially from the father



Hipp1 1-dre reporter *td-sfGFP* FRT-Neo*-FRT loop out

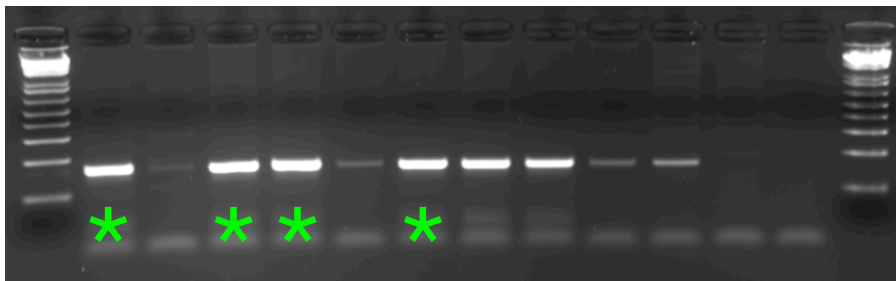
Hipp1 1-dre reporter



F1 pups from cross of B6J male x FLPe+ve Δ Neo HN22 clone #61 G0 female #3

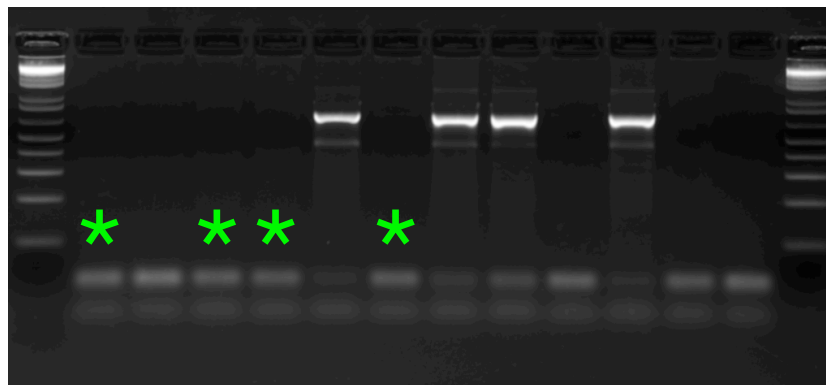
The recombined FRT passed through the germline to the pups

Across the FRT



Selected pups without the FLPe transgene (the mother was indeed FLPe+)

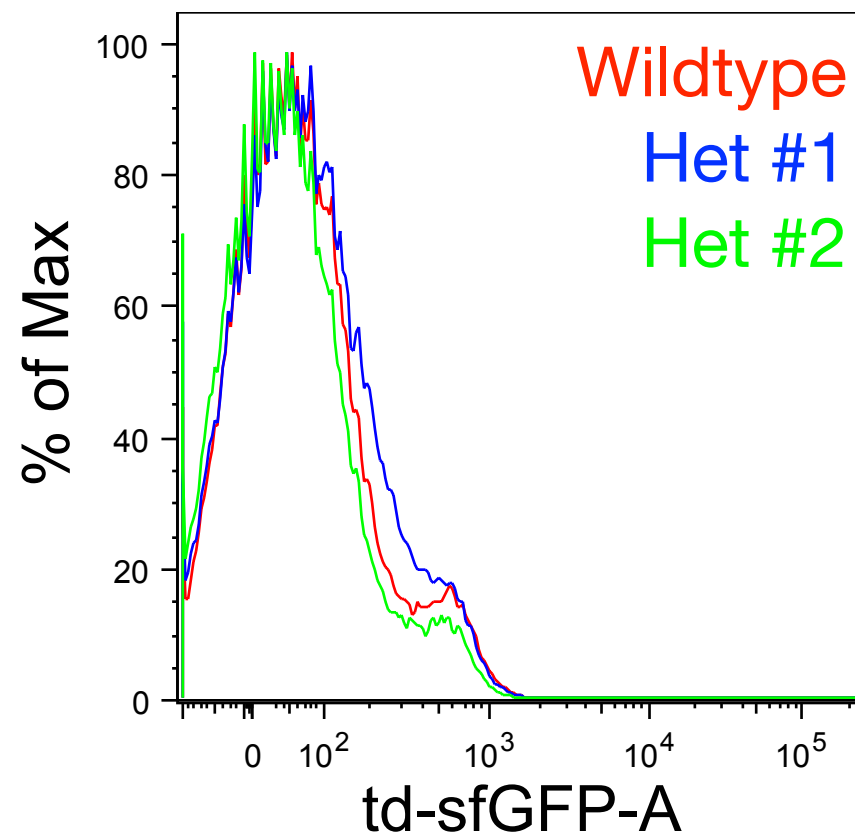
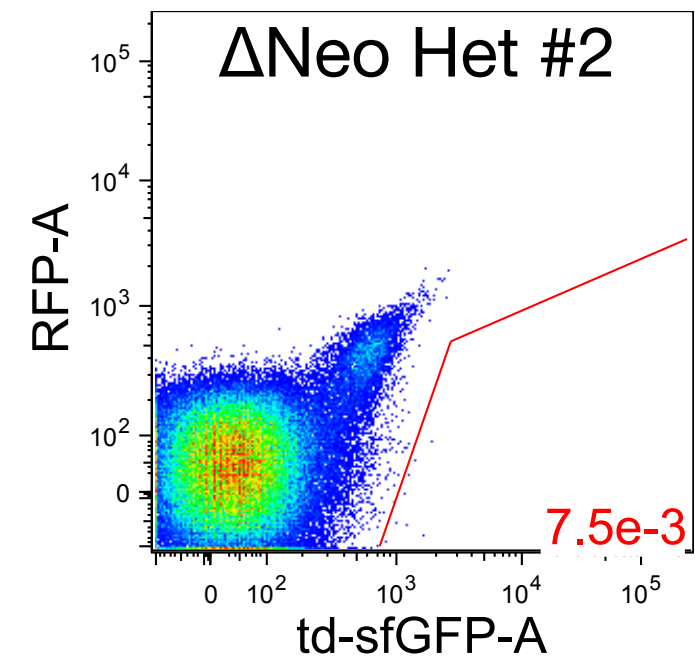
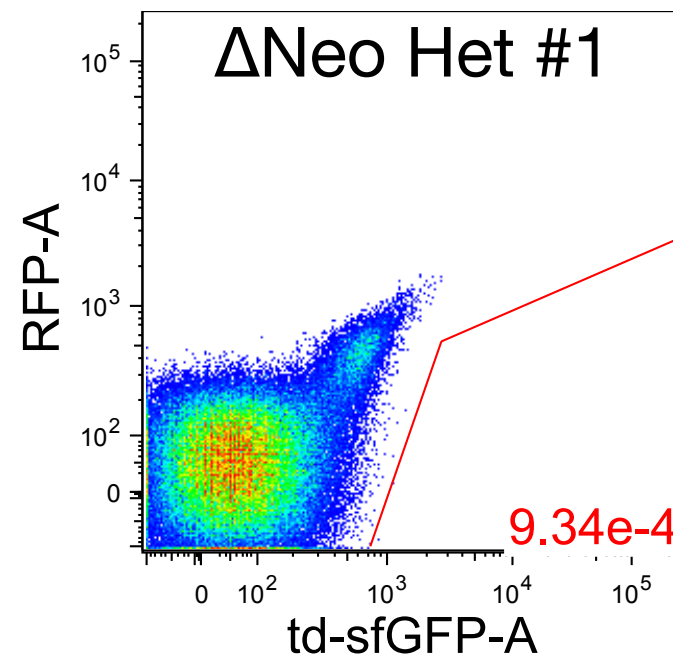
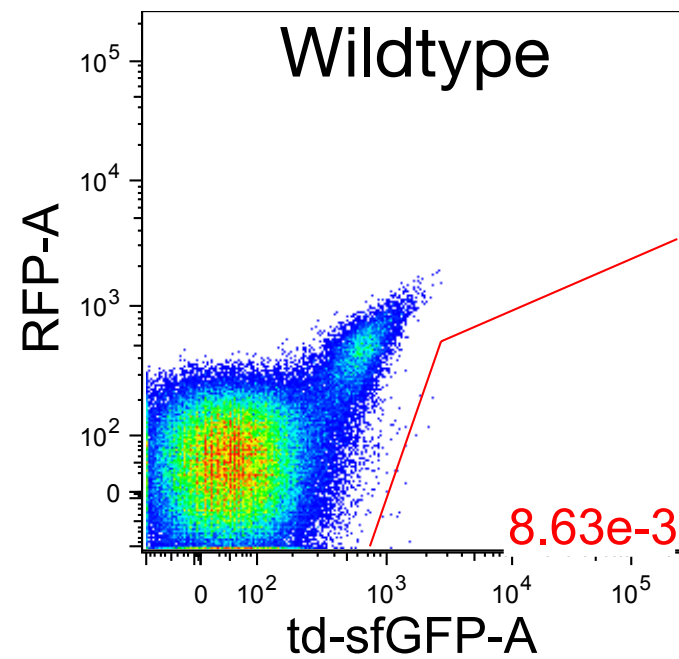
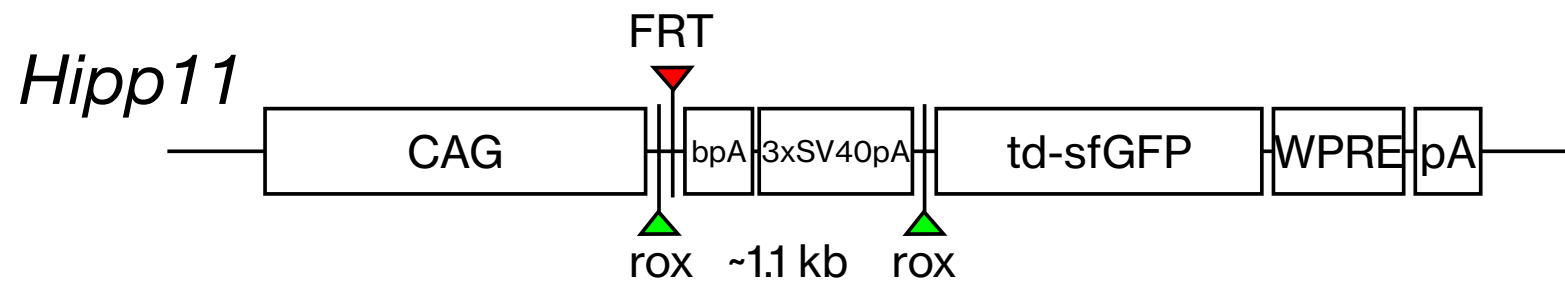
Tg(ACTB-FLPe)



Bred those FLPe-ve F1 males to B6J females → in the N2 male progeny, the X and Y chromosomes were fixed to the C57BL/6J background

Leakage in vivo

Adult mouse brain lateral ventricular wall cells from Δ Neo *Hipp11-dre reporter td-sfGFP*



Dissociated lateral ventricular wall cells

Neo cDNA looped out and only bGHPA-3xSV40pA remain

Still doesn't leak

- Test the *Hipp1* ^{*dre reporter*} allele mice with inducible dre recombinase mice
 - *Cdk6*^{T2A-drePBD*}
 - *Gfap*^{dreERT2}

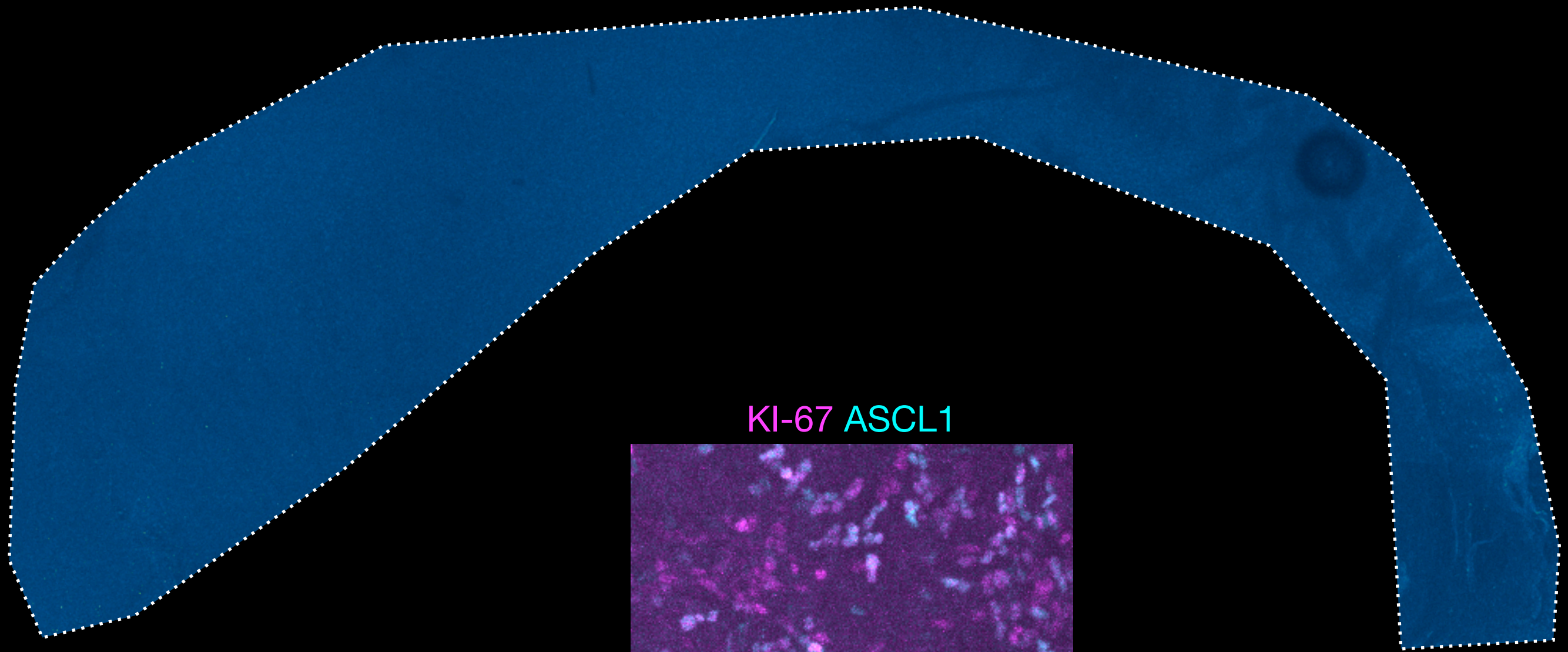
- Bred *Cdk6-T2A-TagRFP-T-drePBD⁺/+; Hipp11-dre reporter td-sfGFP/+*
- Both lines backcrossed separately to the B6J background
- RU-486 - didn't solubilize at 40 mg/ml in corn oil/EtOH, solubilized at 20 mg/ml, less soluble than tamoxifen in the same vehicle
- Injected intraperitoneally 70 μ l of the vehicle or the 20 mg/ml formulation (~24 g male mouse \rightarrow ~58.3 mg/kg - a low dose)
- Perfused at day 3 after the injection

Cdk6^{T2A-drePBD⁺/+}; *Hipp1*^{dre reporter/+}, 10 week old mouse

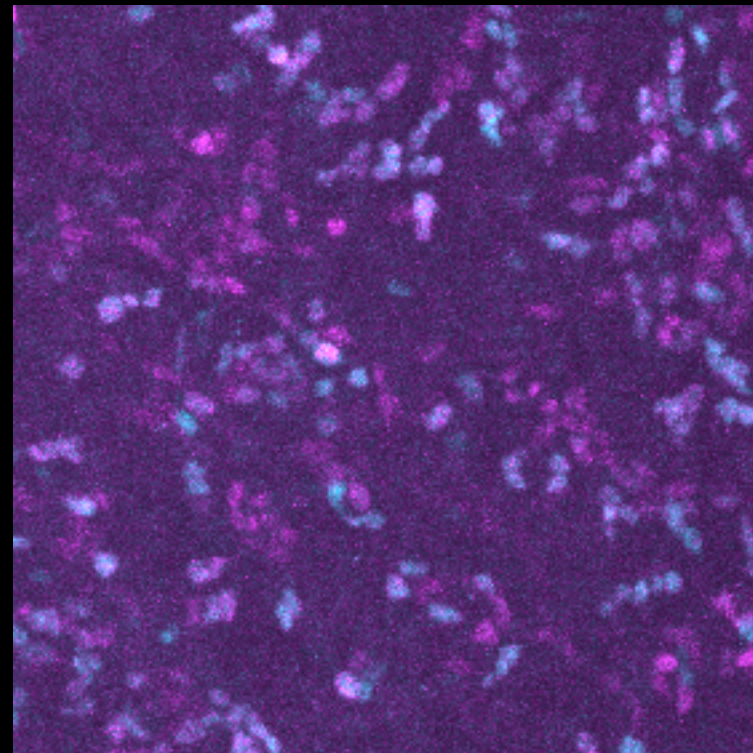
Vehicle 1x i.p. injection

Whole mount immunofluorescence 3 days after

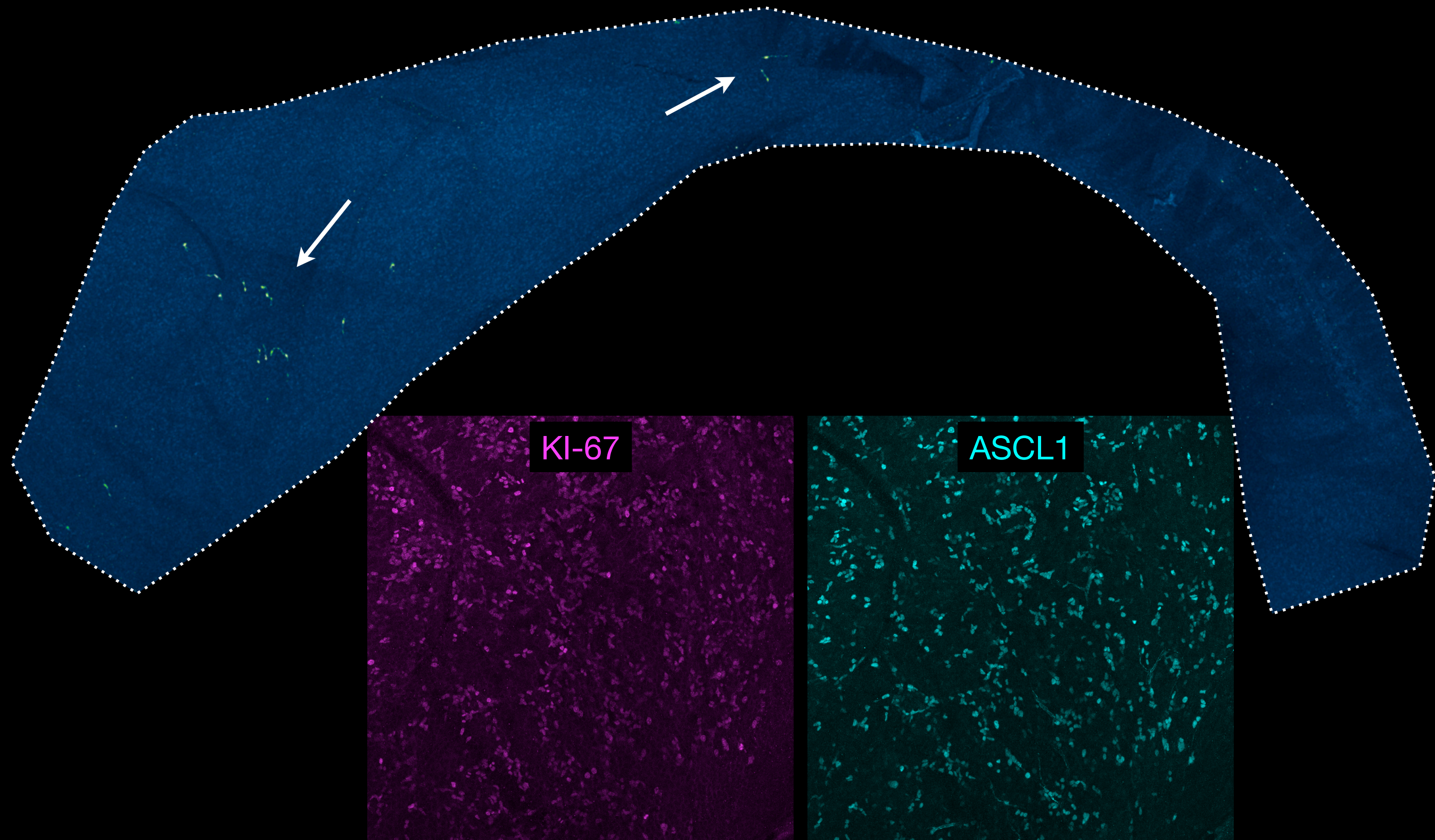
td-sfGFP



KI-67 ASCL1



Cdk6^{T2A-drePBD⁺/+}; *Hipp1*^{dre reporter/+}, 10 week old mouse
RU-486 1x i.p. injection ~58.3 mg/kg in ~70 ul volume (low dose)
Whole mount immunofluorescence 3 days after
td-sfGFP



td-sfGFP

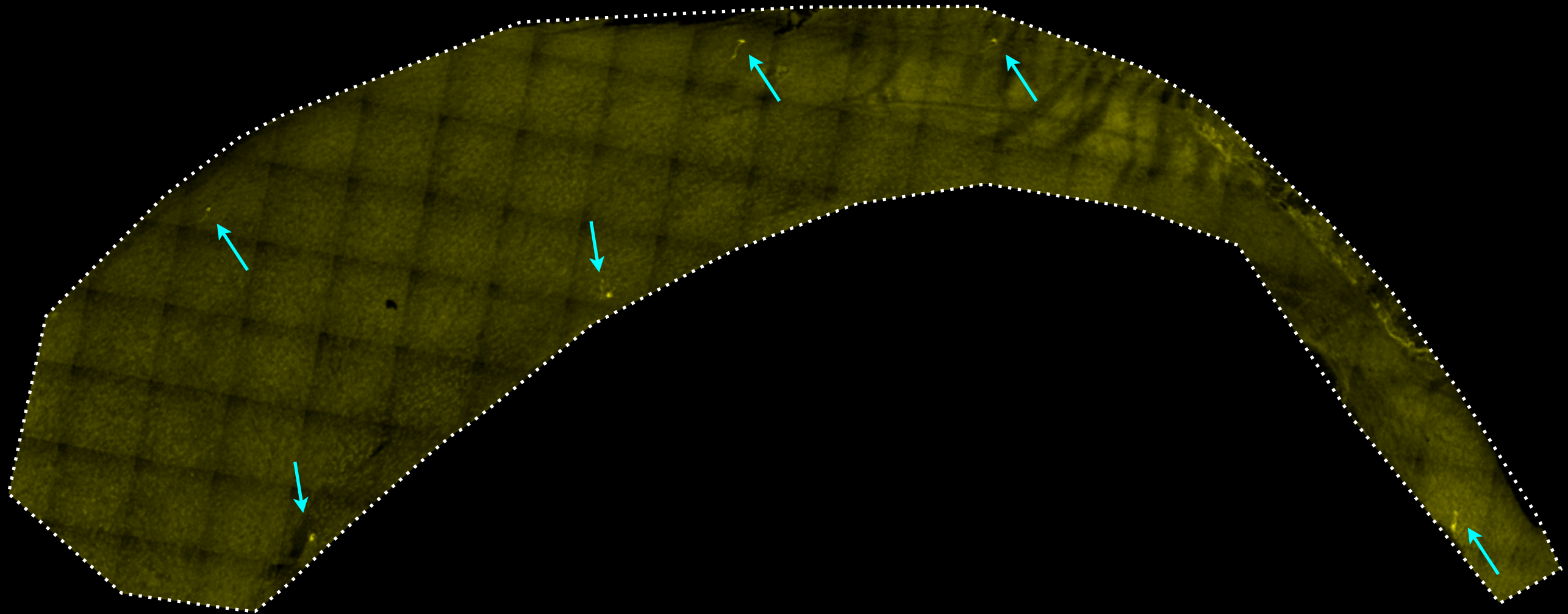
GFP+ cells shown here are KI-67- and ASCL1- → probably neuroblasts not C cells
The anti-GFP antibody is compatible with the sfGFP variant used in this mouse line

td-sfGFP

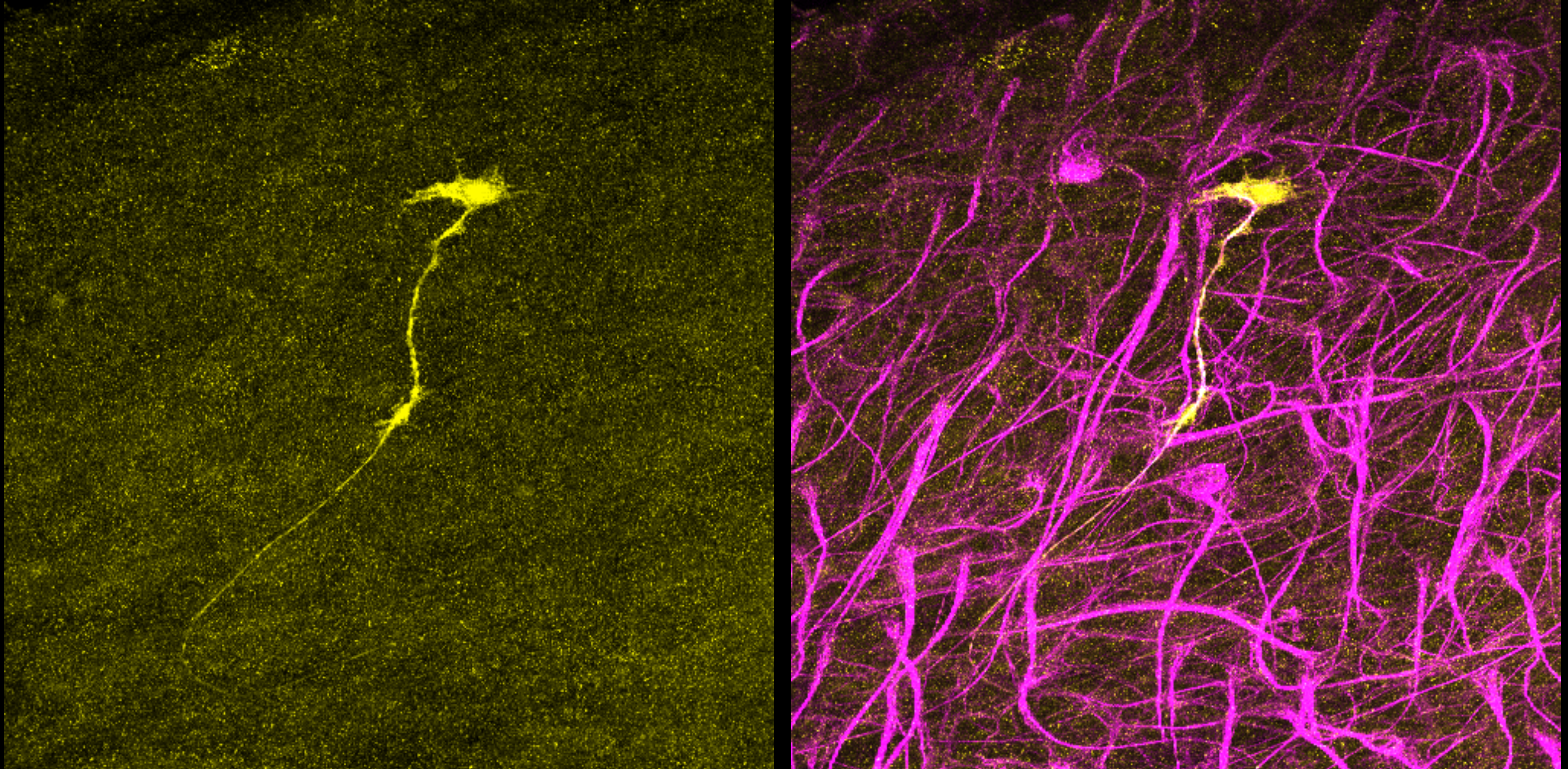
GFP+ cells shown here are KI-67- and ASCL1- → probably neuroblasts not C cells
The anti-GFP antibody is compatible with the sfGFP variant used in this mouse line

- Bred *Gfap-dreERT2/+; Hipp11-dre reporter td-sfGFP/+*
- In the *Gfap-dreERT2* allele, the amino acid sequence that was supposed to be a P2A had 2 conserved amino acids mutated to Alanine
- This "P2A" is not expected to skip
- Thus, a fusion protein of GFAP-dreERT2 or a minimal level of skipping
- Some cells labeled nevertheless after tamoxifen induction

Gfap^{dreERT2/+}; *Hipp11*^{dre reporter/+}
1x i.p. injection of 80 mg/kg TMX, perfused 1 week after
td-sfGFP

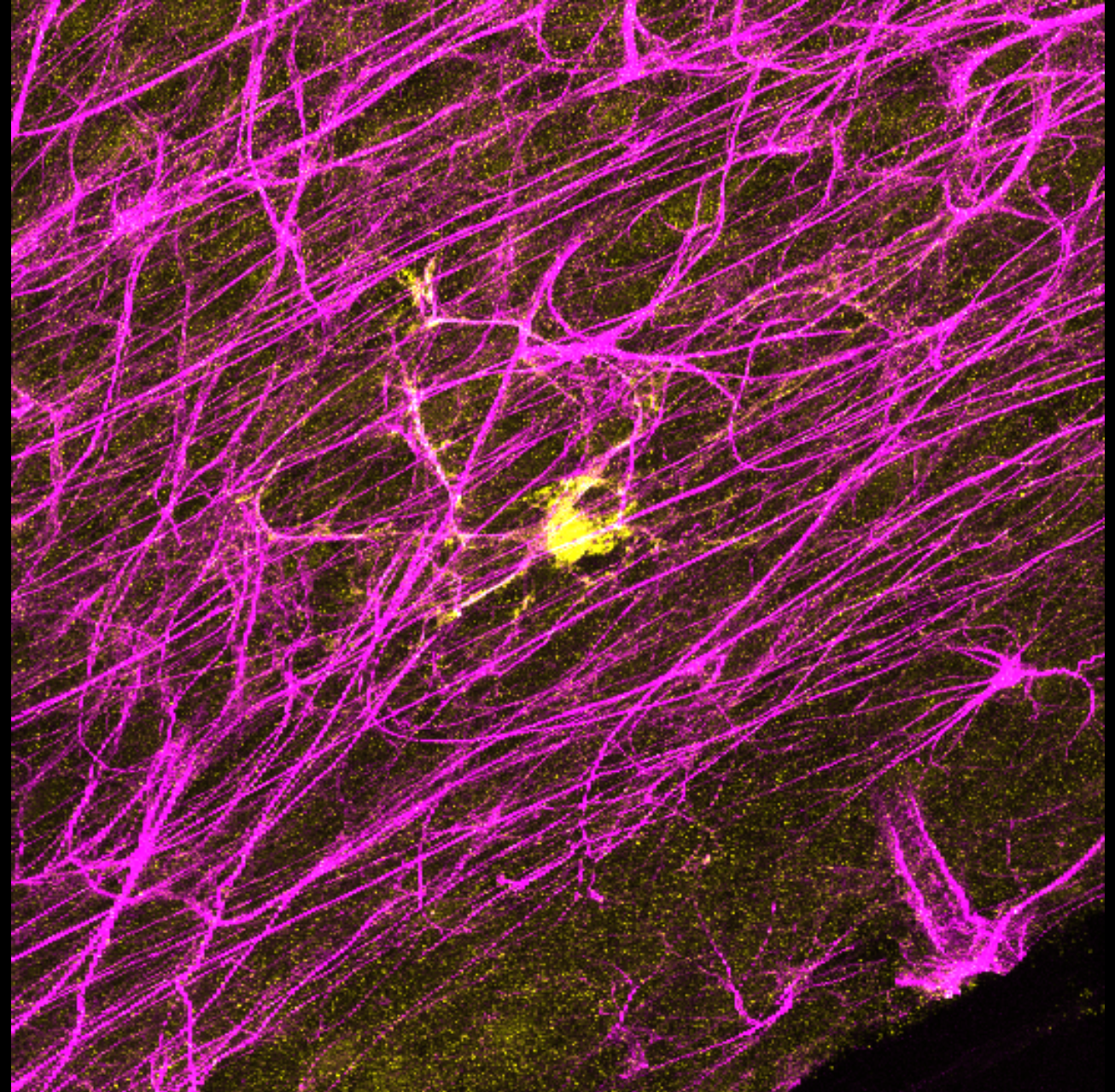
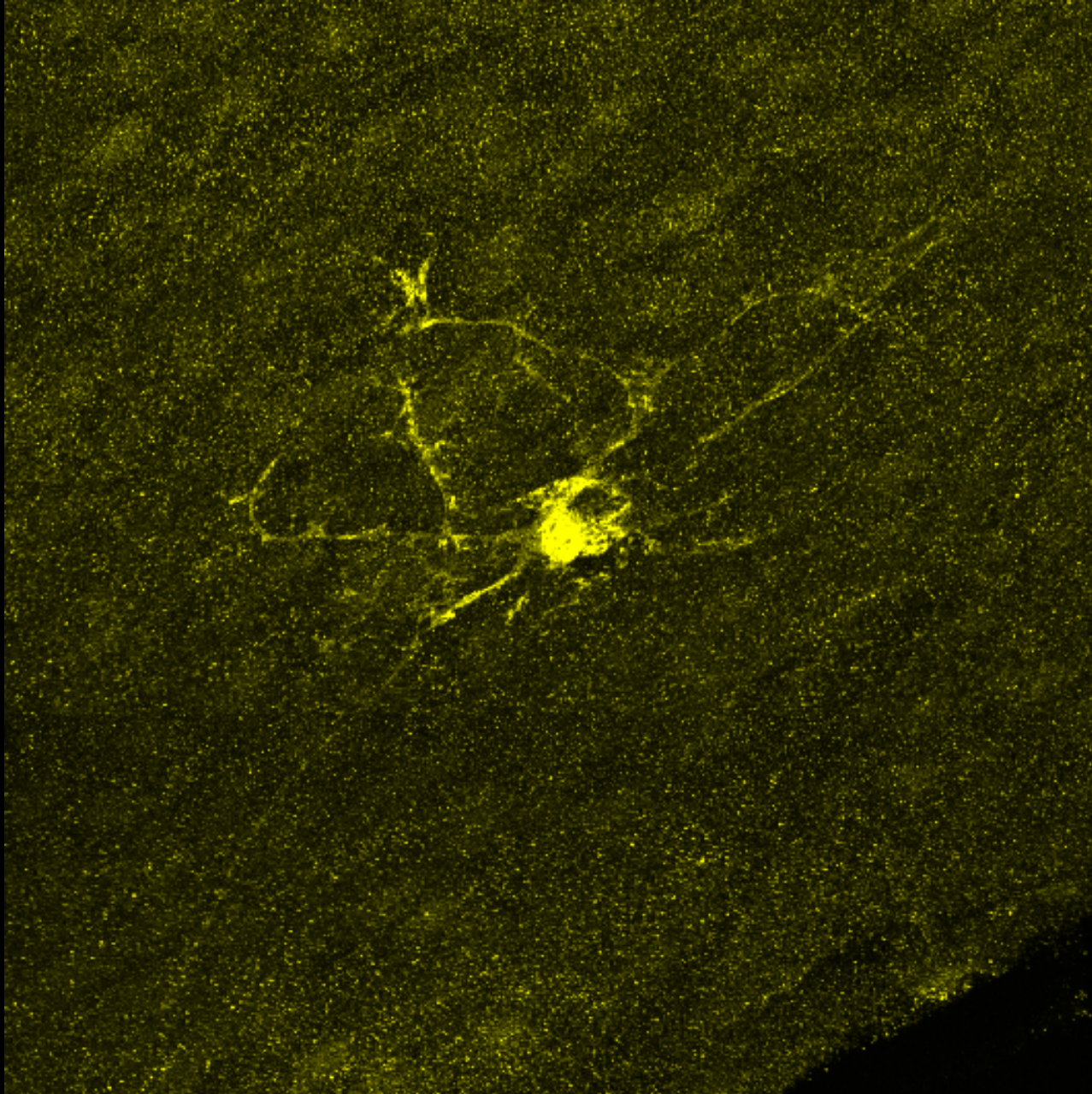


td-sfGFP GFAP



GFP+ cells are GFAP+

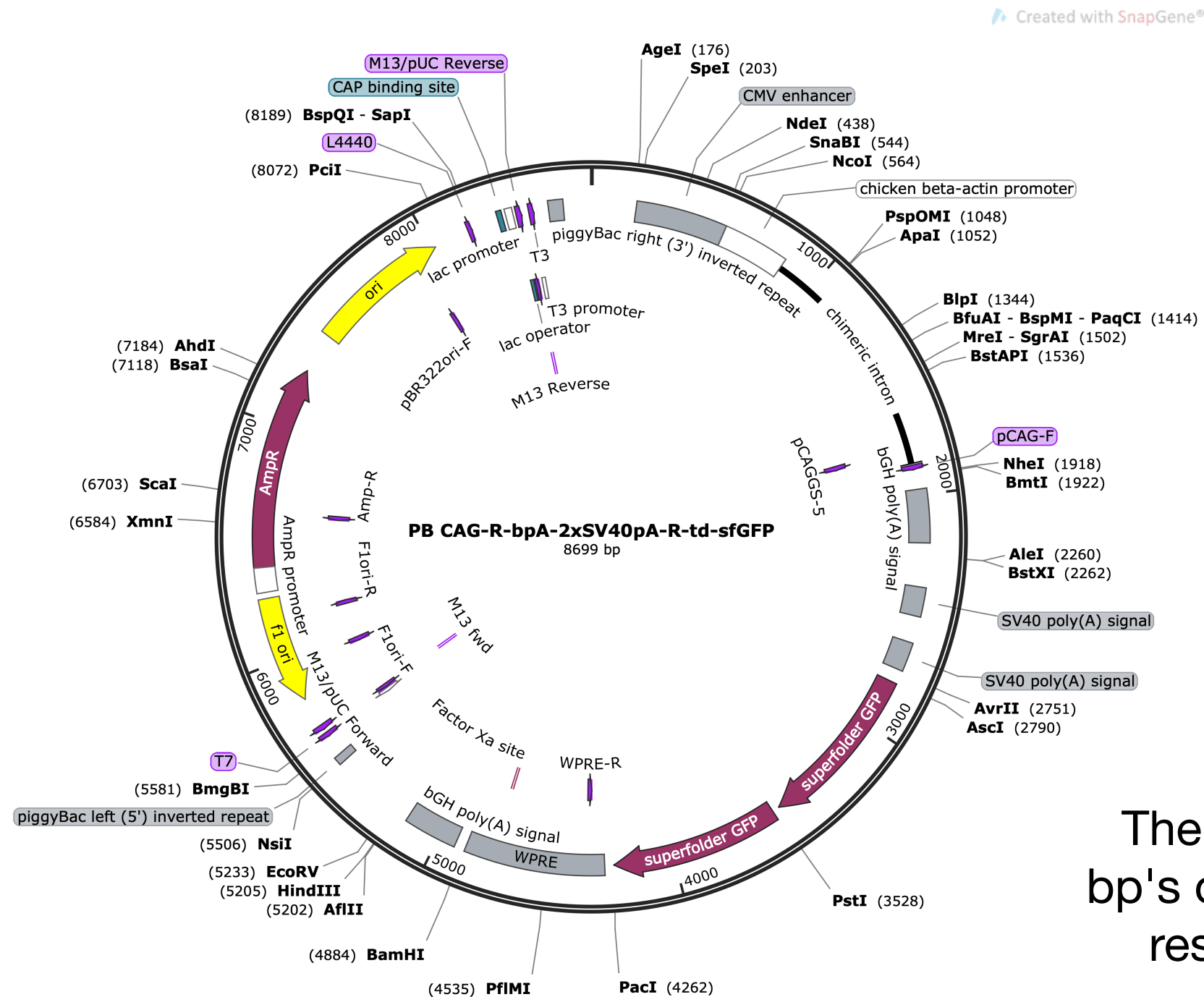
td-sfGFP GFAP



GFP+ cells are GFAP+

- Neither the *Cdk6* nor the *Gfap* allele were efficient drivers
- Nevertheless, the combination of bGH pA and 3x SV40 pA seemed to work well
- However, this could be too long for efficient Cre / lox recombination
- Can I truncate one SV40 pA and still retain similar functionality?
- Constructed bGH pA and 2x SV40 pA stop cassette

Inducible vector 1.3



The missing first 4 bp's of the WPRE are restored as well

Addgene #133578

- Additional plasmids with different stop cassettes available at

<https://www.addgene.org/browse/article/28206910/>

<https://www.addgene.org/browse/article/28215683/>

Hipp1 1-dre reporter td-sfGFP backcross and intercross

- Continued the backcross to the C57BL/6J mice
- At N5 of the backcross, intercross of the N5 het male x N5 het female → First litter = 3 pups, 2 het males, 1 hom female. Second large litter = 3 hom's etc

Pups from N5
male x N5 female



Hipp1 1-dre reporter td-sfGFP backcross and intercross

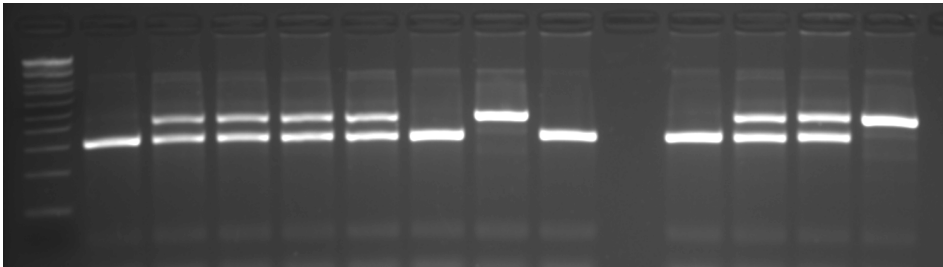
- Bred the hom male and hom females from the N5 intercross
- Weaned 2 large litters of hom males and females
- No sudden deaths or any other obvious abnormalities

Hipp1 1-dre reporter td-sfGFP additional backcrosses

- Continued the backcross of heterozygous males to the C57BL/6J mice for a total of 16 generations
- Intercrossed the N16 het males and females

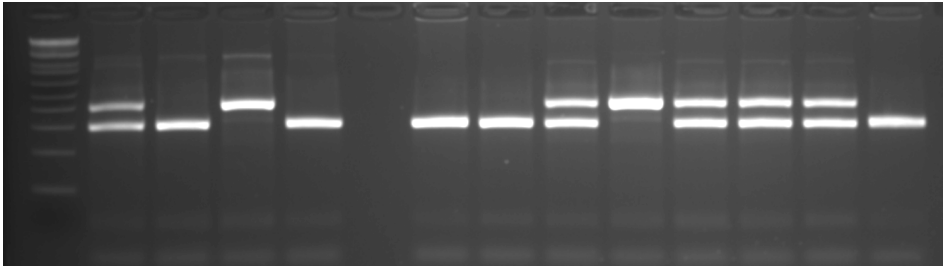
FLPe-ve ΔNeo HN22-61 N16 male #1R x N16 female #10L
HN22

1	male	hom
2	female	het
3		het
4		het
5	female	het
6		hom
7		wt
8		hom



FLPe-ve ΔNeo HN22-61 N16 male #5R x N16 female #3R

9	male	hom
10		het
11	runt	het
12	female	wt
13		het



Neo+ HN22-61 N14 male #8R x N14 female #6RL

14	male	hom
15		wt
16		hom
17		hom
18	female	hom
19		het

Neo+ HN22-61 N14 male #3RL x N14 female #13RL

20	male	wt
21	runt	het
22	female	het
23		het
24		hom

- N16 het intercrosses generated normal homozygous F1 males and F1 females
- Bred the homozygous F1 males and F1 females
- Generated and weaned normal size F2 litters
 - The F1 female nursed the pups
 - The F2 pups grew normally to weaning (see videos of the pups)