A non-invasive photoactivatable split-Cre recombinase system for genome engineering in zebrafish.

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Short running title: Photoactivatable split-Cre recombinase in zebrafish.

Highlights

- zPA-Cre is a novel zebrafish transgenic optogenetic split-Cre tool
- zPA-Cre allows loxP-mediated DNA recombination in various tissues and cell types
- It enables light-induced DNA recombination at different developmental stages.
- It allows spatio-temporal DNA recombination in a specific tissue region with high resolution

Abstract

The cyclic recombinase (Cre)/*loxP* recombination system is a powerful technique for *in vivo* cell labeling and tracking. However, achieving high spatiotemporal precision in cell tracking using this system is challenging due to the requirement for reliable tissue-specific promoters. In contrast, light-inducible systems offer superior regional confinement, tunability and non-invasiveness compared to conventional lineage tracing methods. Here, we took advantage of the unique strengths of the zebrafish to develop an easy-to-use highly efficient, genetically encoded, Magnets-based, light-inducible transgenic Cre/*loxP* system. Our system relies on the reassembly of split Cre fragments driven by the affinity of the Magnets and is controlled by the zebrafish *ubiquitin* promoter. We demonstrate that our system does not exhibit phototoxicity or leakiness in the dark, and it enables efficient and robust Cre/*loxP* recombination in various tissues and cell types at different developmental stages through noninvasive illumination with blue light. Our newly developed tool is expected to open novel opportunities for light-controlled tracking of cell fate and migration in vivo.

Keywords

Lineage tracing; Zebrafish; Optogenetics; Split-Cre recombinase; Magnets.

Introduction

Cell marking by genetic recombination is a powerful technique for noninvasively tracing cell fate and migration in rapidly evolving contexts such as embryonic development[1,2]. Various recombinases have been used in zebrafish studies[3,4], however, the Cre/*loxP* system remains the primary method for genetic lineage tracing in this species[2]. The Cre recombinase, derived from the bacteriophage P1, specifically targets two pairs of 34-bp sequences known as *loxP* sites[5]. Due to its simplicity and efficiency, the Cre/*loxP* system has been widely used in applications such as lineage tracing and genome manipulation in transgenic animals[1]. Typically, the Cre recombinase is expressed under a tissue- or cell-specific promoter and can be combined with chemical-inducible systems such as tamoxifen to achieve temporally controlled genetic recombination[1,6,7]. By crossing transgenic lines containing both Cre and *loxP-STOP-loxP* constructs, Cre specifically activates the reporter in tissues or cells expressing the promoter by permanently removing the STOP cassette, thereby enabling the tracing of their progeny[1,2]. Nonetheless, these conventional Cre/*loxP* recombination approaches have some limitations. For instance, it can be challenging to target and trace cells or tissues that lack a specific promoter during development and/or regeneration. Furthermore, the temporal induction of the Cre/*loxP* system using chemicals may have undesirable effects, including cytotoxicity and cellular perturbation[8,9]. Moreover, a critical limitation of conventional Cre/*loxP* recombination systems is their inability to selectively target specific sub-regions of tissues or cells with similar gene expression patterns.

In contrast to conventional lineage tracing approaches, light-inducible systems offer a significant advantage by enabling noninvasive photoactivation in deep tissues with high spatial and temporal resolution[10,11]. Consequently, several attempts have been made to

99 develop optically controlled Cre systems in zebrafish, as reported in previous studies[12–16].
100 Despite these remarkable advancements, current light-inducible systems tested and used in
101 zebrafish suffer from low efficiency, leakiness in the dark, the need of prolonged exposure to
102 UV light resulting in cytotoxicity, the requirement of advanced microscopy setups, the
103 addition of exogenous chromophores, or the use of harmful chemicals such as caged
104 biomolecules or tamoxifen[10,12–17].
105
106 On the other hand, a highly efficient genetically encoded photoactivatable Cre recombinase
107 (PA-Cre) has recently been reported in mammals[18,19]. This PA-Cre is based on the split Cre
108 complementation and the Magnet dimerization systems[18,20]. In this new system, Cre is split
109 into two fragments that individually lack Cre catalytic activity, and their complementation is
110 driven by two photoswitches named positive Magnet (pMag) and negative Magnet
111 (nMag)[21,22]. The Magnet system has also been shown to be efficient in inducing light-
112 controlled genetic recombination *in vitro* and *in vivo* when used with Dre and Flp
113 recombinases in mammals[23,24]. Furthermore, it has been recently reported that the Magnets-
114 based photoactivatable split Gal4 system enables rapid and robust spatiotemporal gene
115 expression control in *Drosophila* in vivo[25]. However, to date, there are no stable transgenic
116 zebrafish models for optogenetic-based Cre/*loxP* recombination. Therefore, we aimed to
117 develop a PA-Cre system to enable light-inducible lineage tracing *in vivo*, leveraging on the
118 unique strengths of the zebrafish model.
119
120 In this study, we evaluated the functionality of the PA-Cre system in zebrafish. We
121 demonstrate that the original PA-Cre system previously used in mammalian cells[18], induces
122 efficient Cre/*loxP* recombination in zebrafish embryos upon simple LED-mediated blue light

123 illumination. We further developed and assessed the performance of the PA-Cre system
124 under various zebrafish-specific promoters. We illustrate that our new system exhibits no
125 phototoxicity or leakiness in the dark and enables efficient *loxP*-mediated DNA recombination
126 *in vivo*. Finally, we generated a new transgenic zebrafish line in which the expression of the
127 PA-Cre system is under the control of the zebrafish ubiquitin (*ubb*) promoter [26]. By utilizing
128 this *Tg(ubb:PA-Cre)* line, we show that our system allows for efficient and robust Cre/*loxP*
129 recombination in diverse tissues and cell types through noninvasive blue light illumination.
130 Collectively, our newly developed versatile tool is anticipated to pave the way for light-
131 controlled tracing of cell fate and migration *in vivo*, opening up new opportunities for
132 zebrafish research.
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147 **Results**

148 **The PA-Cre system enables *in vivo loxP*-mediated DNA recombination in zebrafish embryos.**

149 To assess the efficacy of the PA-Cre system (Figure 1A) to induce *loxP*-mediated DNA
150 recombination in zebrafish, we injected the original PA-Cre construct[18] (Figure 1B) into
151 zebrafish eggs. The PA-Cre construct, in which expression is driven by the cytomegalovirus
152 (CMV) promoter[18] (Figure 1B), was injected into *Tg(-3.5ubb:loxP-lacZ-loxP*-eGFP; *cry:GFP)*
153 (referred to hereafter as *Tg(ubb:HULK)*[27] embryos at one-cell stage and then subjected to
154 global blue light illumination with from 6 hours post-fertilization (hpf) to 18 hpf (Figure 1C)
155 utilizing a simple low-cost LED light. It has been shown that The *Tg(ubb:HULK)* zebrafish
156 exhibits ubiquitous green fluorescence protein (GFP) expression only upon Cre-mediated
157 recombination[27]. In injected embryos, blue light illumination at 6 hpf resulted in Cre/*loxP*
158 recombination and generated a mosaic GFP expression in various tissues throughout the
159 embryo body (Figure 1D). All embryos that were injected and exposed to blue light
160 illumination showed mosaic GFP expression. Importantly, we observed no GFP expression in
161 embryos that were injected and kept in the dark(Figure 1D), indicating that the Cre-mediated
162 recombination is solely driven by blue light illumination and does not undergo stochastic
163 magnets dimerization. Fluorescence imaging showed that zebrafish embryos exposed to blue
164 light exhibited a 29-fold induction over embryos that were kept in the dark (Figure 1E). In
165 addition, we observed no photo-induced cytotoxicity upon blue light illumination, as we
166 found comparable rates of abnormalities between light-exposed embryos (13.3%) and non-
167 exposed embryos (15.2%). These results demonstrate that transient photoactivation of the
168 PA-Cre system in zebrafish embryos allows robust light-induced Cre/*loxP* recombination in
169 multiple tissues. Importantly, no background DNA recombination was detected in the
170 absence of illumination and no photo-induced cytotoxicity was observed.

171 **Generation of a stable functional zPA-Cre transgenic line in Zebrafish.**

172 Having established the functionality of the PA-Cre system is in zebrafish embryos, we tested
173 its suitability for generating a stable transgenic line. To this end, we decided to use the
174 zebrafish *ubb* promoter[26] in place of the CMV promoter to control the expression of the PA-
175 Cre system. The CMV promoter has been associated with potential silencing of promoter
176 activity *in vivo*, making it less suitable for this type of applications. In contrast, the *ubb*
177 promoter has been shown to have consistent activity throughout zebrafish development
178 stages and does not exhibit silencing issues[26,28]. Therefore, we created the *Tg(-3.5ubb:PA-Cre;*
179 *cmlc2-EGFP)* line (referred to hereafter as zPA-Cre) in which the expression of the PA-Cre
180 system is driven by the *ubb* promoter. Additionally, the line features *cmlc2-EGFP* as a
181 transgenesis marker in the heart (Figure 2A).

182 To test whether the newly developed line is capable of inducing DNA recombination upon
183 blue light illumination, we crossed it with the *Tg(ubb:HULK)* (Figure 2B). In double transgenic
184 zebrafish embryos, blue light illumination from 6 hpf to 18 hpf resulted in a robust Cre/*loxP*
185 recombination, leading to robust GFP expression in various tissues at 24 hpf (Figure 2C) and
186 48 hpf (Figure 2D-E). All embryos exposed to blue light illumination showed ubiquitous GFP
187 expression, while no GFP expression was observed in embryos kept in the dark (Figure 2C-E),
188 confirming the fidelity of the zPA-Cre line and its inducibility exclusively upon blue light
189 illumination. Furthermore, we did not observe any photo-induced cytotoxicity, as the
190 abnormality rate of blue light exposed embryos (10.47%) was not considerably different from
191 that of non-exposed embryos (10.28%). In addition, fluorescence imaging demonstrated a
192 248.8-fold induction of GFP expression in zebrafish embryos exposed to blue light compared
193 to those kept in the dark (Figure 2F). Altogether, these findings demonstrate the robust and

194 efficient induction of DNA recombination *in vivo* upon blue light illumination by the zPA-Cre
195 line.
196
197 **zPA-Cre is effective in diverse cell types.**
198 To assess the versatility of the zPA-Cre system, we investigated its capacity to induce DNA
199 recombination in various Cre-responsive transgenic zebrafish lines. We crossed the zPA-Cre
200 line with distinct switch lines, in which specific cell types express DsRed under steady-state
201 conditions and switch to GFP expression upon activation of Cre. These included the
202 macrophage-specific *Tg(mpeg1:LoxP-DsRedx-LoxP-GFP-NTR)*[29] (Figure 3A), or the
203 erythrocyte-specific *Tg(α/β$_{a2}$-globin:loxP-DsRedx-loxP-GFP)*[30] line (Figure 3D) or the
204 *lymphocyte-specific Tg(lck:loxP-DsRedx-loxP-GFP)*[30,31] line (Figure 3G). In double transgenic
205 zebrafish embryos, exposure to blue light illumination from 6 hpf to 18 hpf resulted in
206 Cre/*loxP*, leading to robust GFP expression at 48 hpf in macrophages (Figure 3B) and in
207 erythrocytes (Figure 3E), and at 120 hpf in thymocytes (Figure 3H). To evaluate the efficiency
208 of the zPA-Cre system, we quantified the proportion of switched GFP-positive macrophages,
209 erythrocytes and thymocytes in the *Tg(mpeg1:LoxP-DsRedx-LoxP-GFP-NTR)* line, the
210 *Tg(α/β$_{a2}$-globin:loxP-DsRedx-loxP-GFP)* line and the *Tg(lck:loxP-DsRedx-loxP-GFP)* line,
211 respectively. We found that ~95% of macrophages (Figure 3C), ~90% of erythrocytes (Figure
212 3F) and ~82% of thymocytes in the thymus (Figure 3I) exhibited GFP expression, indicating the
213 high efficiency of our labelling system.
214
215 In addition, we tested the functionality of the PA-Cre system under the *kdrl* promoter[32,33],
216 which exhibits expression during gastrulation, in the lateral plate mesoderm, and in
217 endothelial cells[33–35]. To examine the potential of the *kdrl*-PA-Cre system for erythrocyte

218 labeling, we injected a *kdrl*-PA-Cre construct into *Tg(α/β$_{a2}$-globin:loxP-DsRedx-loxP-GFP)*
219 embryos at one-cell stage, as erythrocytes are known to be derived from the lateral plate
220 mesoderm[36–39]. Following blue light illumination from 6 hpf to 18 hpf in injected embryos,
221 GFP expression was observed in erythrocytes at 24 hpf, indicating successful Cre/loxP
222 recombination. Conversely, non-exposed embryos did not exhibit GFP expression in
223 erythrocytes (supplemental Figure 1). These results indicate that the zPA-cre system enables
224 DNA recombination in different cell types, and that the PA-Cre system expression can be
225 driven by distinct promoters. Collectively, our data establish the zPA-Cre system as a versatile
226 tool for DNA recombination in zebrafish.
227

228 **zPA-Cre enables light-inducible Cre/*loxP* recombination at different developmental time**
229 **points and at a specific region of interest.**
230
231 We then evaluated the efficiency of our system in comparison to widely used conventional
232 non-photoactivatable Cre systems. To this end, we utilized the *Tg(ubb:creERT2)* line, in which
233 Cre is ubiquitously expressed but its activity is induced only by incubation with tamoxifen,
234 that allows Cre to enter to the nucleus. We found that *Tg(zPA-Cre; ubb:HULK)* zebrafish
235 embryos exposed to blue light overnight at 6 hpf exhibited a 23.2-fold induction compared to
236 *Tg(ubb:creERT2*[26]*; ubb:HULK)* embryos treated with 4-OH-tamoxifen (4-OHT) (Figure 4A, C),
237 indicating that our system exhibits superior induction efficiency compared to other
238 conventional inducible tools.
239 The use of light-inducible tools in zebrafish beyond the embryonic stages can be challenging
240 due to limited light penetration in larval tissues. Therefore, we assessed the efficiency of DNA
241 recombination using the zPA-Cre system at different developmental time points. To this end,
242 we exposed double transgenic *Tg(zPA-Cre; ubb:HULK)* fish to blue light overnight at 24 hpf,

243 48 hpf, and 72 hpf, and imaged them at 120 hpf (Figure 4B). We observed GFP expression in
244 all embryos or larvae exposed to blue light, although at lower levels compared to fish exposed
245 to blue light at 6 hpf. Of note, control siblings that were kept in the dark did not show GFP
246 expression (Figure 4B). Moreover, illuminating 6 hpf embryos with blue light for only 2 hours
247 resulted in robust GFP expression at 120 hpf. These findings demonstrate that while the
248 highest efficiency is achieved during early developmental stages, our system remains
249 effective at later developmental stages.
250
251 To test the capability of the zPA-Cre system to drive DNA recombination in specific tissue
252 regions, we used the *Tg(zPA-Cre; ubb:HULK)* embryos and performed photoactivation in
253 targeted regions of interest at 4 hpf (Figure 4D) or at 48 hpf (Figure 4E) making use of
254 conventional confocal microscopy. Photoactivation with blue light using a 488 nm laser at
255 100% laser power (2 mW/mm2) in a specific region (a 25 μm diameter circle) for 5 minutes at
256 4 hpf resulted in mosaic GFP expression in various tissues later at 19 hpf (Figure 4D). In
257 contrast, photoactivation of a 25 μm diameter circle within a single skeletal muscle at 48 hpf
258 using the same laser for 5 minutes resulted in GFP expression in the activated region with
259 little impact on surrounding tissues (Figure 4E).
260 Lastly, we utilized the *Tg(zPA-Cre)* line in combination with the *Tg(α/β_{a2}-globin:loxP-DsRedx-*
261 *loxP-GFP)* line to test if *loxp*-mediated DNA recombination could be induced in a small number
262 of few erythrocytes present in the posterior blood island (PBI), a transient hematopoietic
263 tissue in zebrafish. Photoactivation with blue light using a 488 nm laser at 100% laser power
264 (2 mW/mm2) in few erythrocytes in the PBI (with a 25 μm diameter circle) for 5 minutes at
265 22 hpf resulted in the presence of GFP^+ erythrocytes in the caudal hematopoietic tissue (CHT),
266 the fetal liver counterpart in zebrafish[9] (Figure 4F), as well as in the circulation (supplemental

video) at 48 hpf. In conclusion, these experiments demonstrate that the zPA-Cre system enables efficient and precise spatial and temporal control of *loxP*-mediated DNA recombination in zebrafish embryos with high resolution.

Discussion

Optogenetic tools play a crucial role in studying development and tissue regeneration in various organisms, offering precise spatial and temporal resolution. Numerous light-inducible tools for Cre/*loxP* recombination have been developed utilizing UV light, blue light, or red light. Consequently, several attempts have been made to develop optically controlled Cre systems in zebrafish, as reported in previous studies[12–16]. Despite significant advances, current light-inducible systems tested in zebrafish have been performed in transient, and suffer from low efficiency, leakiness, require long light exposure resulting in cytotoxicity, require exogenous chromophores or need harmful chemicals as caged biomolecules or tamoxifen [10,12–17].

Here, we demonstrated the successful development of a novel stable optogenetic zebrafish line in which the PA-Cre system is controlled by the zebrafish ubiquitin promoter[26]. By utilizing the *Tg(ubb:PA-Cre)* line, we provide evidence that our system allows for efficient and robust Cre/*loxP* recombination in various tissues and cell types through noninvasive blue light illumination .

As a general rule, it is crucial for a light-inducible Cre recombinase to maintain low levels of background recombination[10]. In this regard, we show that the *Tg(ubb:PA-Cre)* line exhibits no background recombination in the absence of light stimulation, while displaying a highly

291 inducible efficiency of DNA recombination upon exposure to blue light. Furthermore, we
292 showcase the versatility of our system by demonstrating its ability to induce recombination
293 at different developmental stages and achieve spatial control within specific regions upon
294 blue light illumination. These distinctive advantages offered by the *Tg(ubb:PA-Cre)* line pave
295 the way for *in vivo* zebrafish studies during development with high spatiotemporal resolution.
296 As shown in this study, the newly generated *Tg(ubb:PA-Cre)* line enables precise spatial and
297 temporal control of Cre/*loxP* recombination through blue light illumination. This versatile
298 DNA recombination system holds potential for applications such as fate mapping or the
299 induction of carcinogenesis in specific cell types upon illumination. Moreover, the utilization
300 of the PA-Cre system in zebrafish reveals a striking simplicity and efficiency, thus expanding
301 the repertoire of available genetic manipulation methods.
302
303 Our newly developed versatile tool is expected to pave the way for light-controlled DNA
304 recombination *in vivo* in cells or tissues that may not have a specific promoter, thereby
305 facilitating studies on lineage tracing, development, and disease. Furthermore, as we
306 generated a stable transgenic line in which the PA-Cre system is driven by the *ubb* promoter,
307 it holds a particular promise for investigating the utility of our tool in adult zebrafish. Exploring
308 the performance and efficiency of the PA-Cre system under different promoters in zebrafish
309 is also an enticing avenue to achieve more precise cell-specific DNA recombination. Lastly,
310 with the development of the PA-Dre[23] and PA-Flp[24] systems, it will be become feasible to build
311 additional optogenetic tools in zebrafish and perform intersectional genetics involving more
312 than one recombinase.

313 Taken together, our findings indicate that the *Tg(ubb:PA-Cre)* line is an efficient optogenetic
314 tool for gene manipulation with greatly expandable applications in developmental biology
315 research.
316

317 **Materials availability**

318 **Materials and Data availability**

319 All reagents and data sets generated during the current study are available from the
320 corresponding authors upon request.

321 **Materials and Methods**

322 **Zebrafish.** Embryonic, larval and adult zebrafish (*Danio rerio*) were maintained at 28°C on a
323 14-hour light/10-hour dark cycle. The collected embryos were raised in fish water containing
324 0.01% methylene blue to prevent fungal growth. All fish are housed in the fish facility of our
325 laboratory, which was built according to the local animal welfare standards. All animal
326 procedures were performed in accordance with French and European Union animal welfare
327 guidelines and were approved by the ethic committee of the Curie Institute (Paris, France).
328
329 **Transgenic lines.** The following lines of the AB stain were used*: Tg(ubb:HULK)*[27]*,*
330 *Tg(mpeg1:LoxP-DsRedx-LoxP-GFP-NTR)*[29]*,Tg(globin:loxP-DsRedx-loxP-GFP)*[30],
331 *Tg(ubb:CreERT2; cmlc2-GFP)*[26] and *Tg(lck:loxP-DsRedx-loxP-GFP)*[30]*.*
332
333 **Plasmids.** The original PA-Cre construct driven by the CMV promoter was purchased from
334 Addgene (Addgene no. 122960). The PA-Cre coding sequence was synthesized by Genescript
335 and listed in the supplementary note. The (*-3.5ubb:PA-Cre; cmlc2-EGFP)* constructs were
336 generated by inserting 3.5 kb ubi promoter fragment, together with the coding sequence of
337 PA-Cre, via Gibson assembly into pTol2 vector that carries cmlc2-EGFP as a transgenesis
338 marker. To generate the *kdrl:PA-Cre* constructs, the *kdrl* promoter and the coding sequence
339 of PA-Cre were cloned via Gibson assembly into the pTol2 vector.
340
341 **Zebrafish embryo microinjection.** The *CMV:PA-Cre* and *kdrl:PA-Cre* DNA constructs (25 ng/µl)
342 were injected into zebrafish embryos at the one cell stage using pressure-controlled
343 microinjector and a micromanipulator. After injection, the embryos were cultured in E3
344 embryo medium with methylene blue in the dark at 28.5°C.
345
346 **Generation of transgenic lines.** The *-3.5ubb:PA-Cre;cmlc2-EGFP* constructs (25 ng/µl) were
347 then co-injected with mRNA transposase (50 ng/µl) into 1-cell stage WT embryos[40] and the
348 resulting embryos were grown to adulthood for stable line screening.
349
350 **Photoactivation with global blue light.** Global light induction was performed using a MARS
351 AQUA-YWT-165W LED aquarium hood. 6 hpf embryos were illuminated with constant blue
352 light for 12 h. To ensure all cells were exposed, embryos were periodically swirled during

353 irradiation. In experiments presented in Figure 4, embryos and larvae at each indicated
354 developmental stage were illuminated with constant blue light overnight. Dark controls were
355 placed in a lightproof box within the same 28.5°C incubator as the light-treated samples.
356
357 **Regional activation.** The blue-light activation was performed on a Zeiss LSM780 confocal
358 microscope. The desired region of the embryo was focused under a 25X lens (Zeiss Plan-
359 Apochromat 25×, NA = 0.8 WD = 0.55), with the background fluorescence signal excited by a
360 405 nm laser. The desired region was selected under the bleach mode in ZEN software. A 2
361 mW 488 nm laser was used for activation the region for 5 minutes at 100% laser power. After
362 activation, the embryos were incubated in dark at 28.5°C and then examined at indicated
363 developmental stages.
364
365 **Fluorescence Microscopy.** Zebrafish embryos, larvae and adults were anesthetized with
366 0.01% tricaine (A5040, Sigma) and mounted in 2.5% methylcellulose in 35-mm imaging dishes
367 (MatTek) as described previously[41]. Fluorescent imaging was performed with either Zeiss Axio
368 Zoom V.16 upright microscope with an AxioCam HRm Zeiss camera and Zeiss Zen 3.3 software
369 or with Leica thunder imaging system with Leica LAS-X software. Fluorescence was detected
370 with mCherry, Texas Red (for dsRed labelled cells), and green fluorescent protein (GFP) filters.
371
372 **Image Analysis.** All images were analyzed using FIJI software [42].
373
374 **Quantification and statistical analysis.** Statistical analyses were performed by the GraphPad
375 Prism software (Prism 9). All experiments with only two groups and one dependent variable
376 were compared using an unpaired t-test with Welch's correction. Statistical data show mean
377 ± s.e.m. Each dot plot value represents an independent embryo, and every experiment was
378 conducted three times independently. The corrected total cell fluorescence (CTCF) was
379 calculated using the formula 'Integrated density whole – (area whole embryo x mean
380 fluorescence background)'. This formula is loosely based on a method described for
381 calculating cell-fluorescence [43]. To calculate the CTCF percentage for DsRed+ cells, the CTCF
382 value of DsRed was divided by the total summation of CTCF values for both DsRed and GFP in
383 each larvae. The same was done for calculating CTCF percentage for GFP+ cells. For
384 fluorescence intensity quantification, the same parameters (excitation/emission, gain for
385 detectors, lasers intensity) were applied for images and fluorescence intensity was measured
386 using FIJI.
387
388
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405 **Author contribution**

406 Conceptualization: R.E. and P.P.H.; Methodology and data collection: R.E., A.M., P.D. and G.G.;
407 Writing - Original Draft: R.E.; Writing - Review & Editing: R.E. and P.P.H.; Funding Acquisition:
408 P.P.H.
409

410 **Conflict of Interest**

411 The authors declare no competing interests.
412
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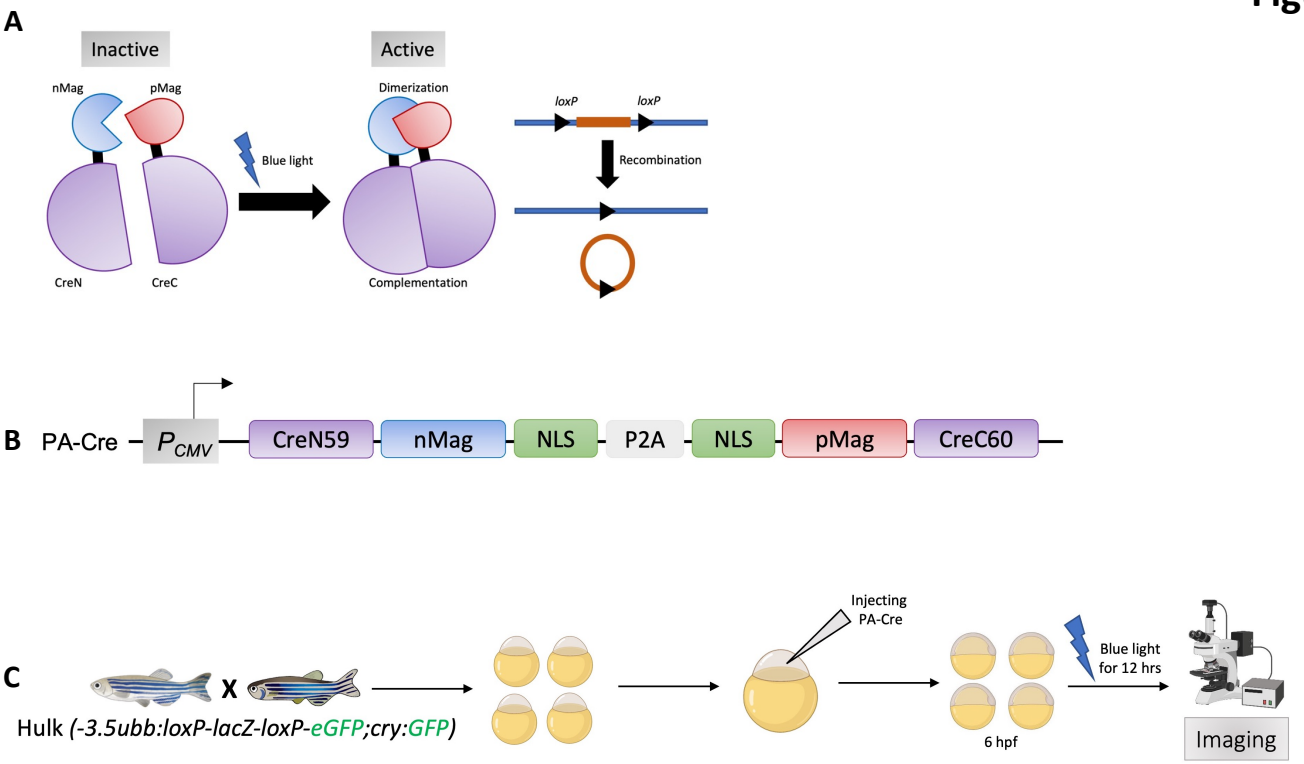
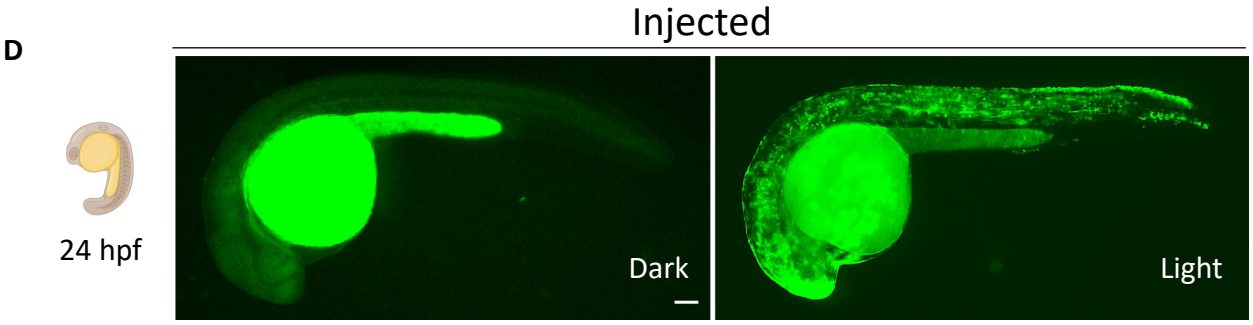
Figure 1


A
Inactive
Active
nMag
pMag
Blue light
Dimerization
CreN
CreC
Complementation
loxP
loxP
Recombination
B
PA-Cre
P_{CMV}
CreN59
nMag
NLS
P2A
NLS
pMag
CreC60
C
X
Hulk (-3.5ubb:loxP-lacZ-loxP-eGFP;cry:GFP)
Injecting
PA-Cre
6 hpf
Blue light
for 12 hrs
Imaging


D
Injected
24 hpf
Dark
Light

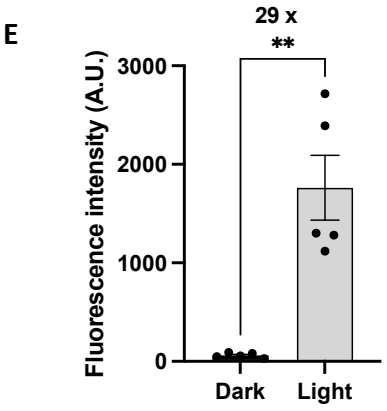

E
29 x
**
Fluorescence intensity (A.U.)
3000
2000
1000
0
Dark
Light

Figure 1: PA-Cre mediated genetic recombination in zebrafish embryos.

(A) Schematic representation of the optogenetic PA-Cre system. CreN and CreC are respectively fused to nMagnet and pMagnet tandems. These fusions are monomers in the dark state (inactive PA-Cre). Upon blue light photoactivation, dimerization of nMag-pMag leads to complementation of CreN-CreC and thus induction of genetic recombination and activation of gene expression (active PA-Cre). nMag, negative Magnet; pMag, positive Magnet; CreN and CreC: two pairs of split Cre fragments.
(B) Schematic representation of the PA-Cre genetic construct. The PA-Cre system is driven by the CMV promoter. P, promoter; nMag, negative Magnet; pMag, positive Magnet; NLS, nuclear localization signal; CreN59, split Cre fragment (residues 19–59); CreC60, split Cre fragment (residues 60–343); P2A, *2A self-cleaving peptides*.
(C) Schematic representation strategy used to induce genetic recombination in zebrafish embryos. *Tg (ubb:Hulk)* embryos were injected at one cell stage with PA-Cre construct, photoactivated with blue light at 6 hpf for 12 hours and then imaged at the desired developmental stage.
(D) Representative fluorescent images at 24 hpf of injected *Tg(ubb:Hulk)* embryos kept in dark (left) or exposed to blue light illumination at 6 hpf for 12 hours (right). GFP signal are shown in various tissues. Scale bar, 100 µm.
(E) Quantification of fluorescence intensity at 48 hpf of embryos injected with PA-Cre construct and kept in dark (n=5) or exposed to blue light illumination at 6 hpf for 12 hours (n=5). 29x represents the fold induction of the PA-cre system. Mean ± SEM of the fluorescence intensity is shown. Unpaired t-test with Welch's correction was used for this analysis. $^{**}p \leq 0.01$. A.U., Arbitrary Unit.

Figure 2

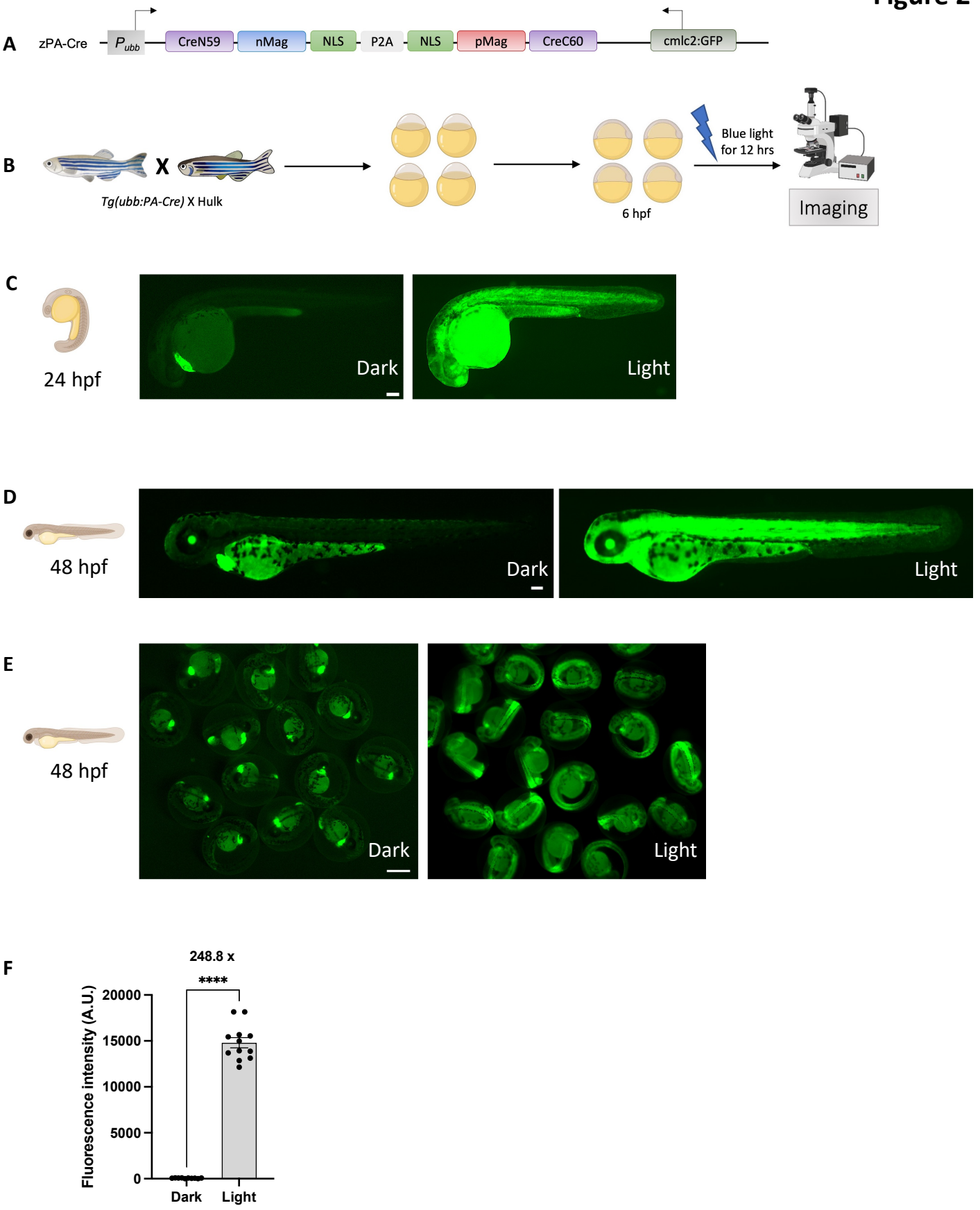

A
zPA-Cre
P_{ubb}
CreN59
nMag
NLS
P2A
NLS
pMag
CreC60
cmlc2:GFP
B
Tg(ubb:PA-Cre) X Hulk
6 hpf
Blue light for 12 hrs
Imaging
C
24 hpf
Dark
Light
D
48 hpf
Dark
Light
E
48 hpf
Dark
Light
F
248.8 x
****
Fluorescence intensity (A.U.)
20000
15000
10000
5000
0
Dark
Light

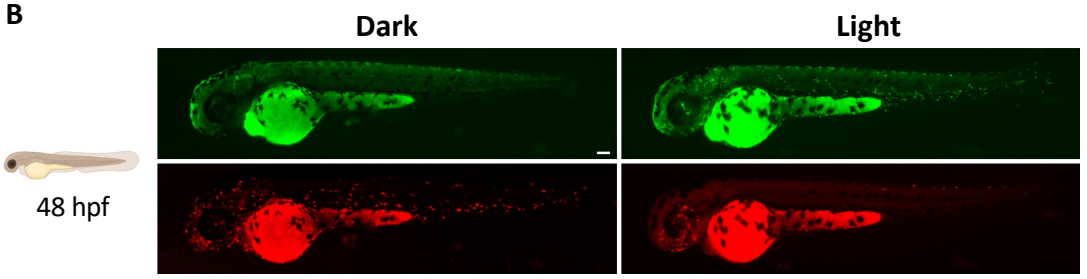
Figure 2: The sable transgenic line *Tg(ubb:PA-Cre)* enables light-induced genetic recombination in zebrafish embryos.

(A) Schematic representation of the zebrafish PA-Cre genetic construct (zPA-Cre). The zPA-Cre system is driven by the *ubb* promoter. The green heart is used as a transgenesis marker *(cmlc2:GFP)*. P, promoter; nMag, negative Magnet; pMag, positive Magnet; NLS, nuclear localization signal; CreN59, split Cre fragment (residues 19–59); CreC60, split Cre fragment (residues 60–343); P2A, 2A self-cleaving peptides; cmlc2, cardiac myosin light chain 2.
(B) Schematic representation strategy used to induce genetic recombination in zebrafish embryos. The stable transgenic line *Tg(ubb:PA-Cre)* was crossed to *Tg (ubb:Hulk)*. The double transgenic embryos *Tg (ubb:PA-Cre; ubb:Hulk)* were photoactivated with blue light at 6 hpf for 12 hours and then imaged at the desired developmental stage.
(C) Representative fluorescent images at 24 hpf of *Tg (ubb:PA-Cre; ubb:Hulk)* embryos kept in dark (left) or exposed to blue light illumination at 6 hpf for 12 hours (right). Scale bar, 100 µm.
(D) Representative fluorescent images at 48 hpf of *Tg (ubb:PA-Cre; ubb:Hulk)* embryos kept in dark (left) or exposed to blue light illumination (right) at 6 hpf for 12 hours. Scale bar, 100 µm.
(E) Representative fluorescent images at 48 hpf of a group of *Tg (ubb:PA-Cre; Hulk)* embryos kept in dark (left) or exposed to blue light illumination at 6 hpf for 12 hours (right). Scale bar, 100 µm.
(F) Quantification of fluorescence intensity at 48 hpf of *Tg (ubb:PA-Cre; ubb:Hulk)* embryos kept in dark (n=10) or exposed to blue light illumination at 6 hpf for 12 hours (n=12). 248.8x represents the fold induction of the zPA-Cre system. Mean ± SEM of the fluorescence intensity is shown. Unpaired t-test with Welch's correction was used for this analysis. $^{****}p \leq 0.0001$. A.U., Arbitrary Unit.

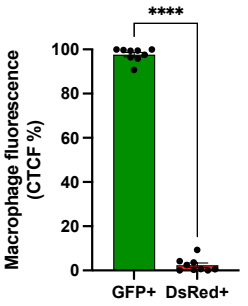
Figure 3

A **X** *Tg(ubb:PA-Cre)* **X** *mpeg1:loxP-**DsRed**-STOP-loxP-**GFP**-**NTR***

B

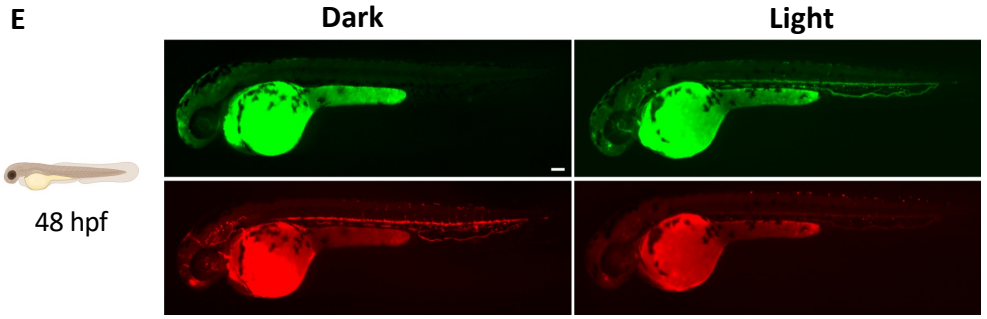


C



D **X** *Tg(ubb:PA-Cre)* **X** *globin:loxP-**DsRed**-STOP-loxP-**GFP***

E



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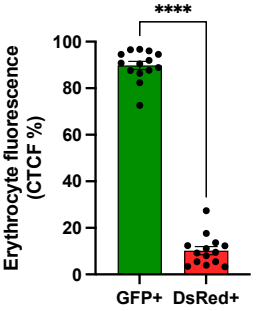
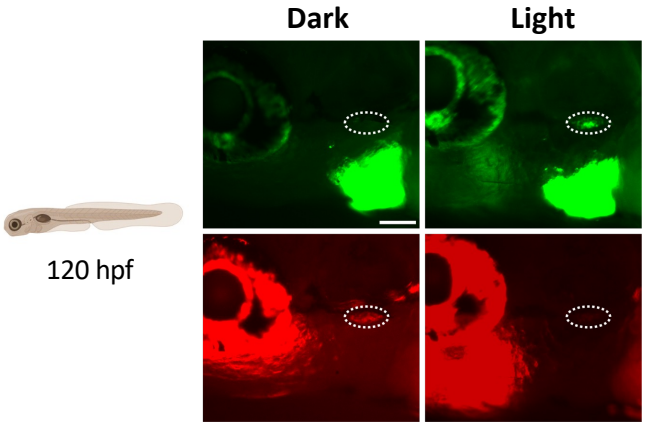


G **X** *Tg(ubb:PA-Cre)* **X** *lck:loxP-**DsRed**-STOP-loxP-**GFP***

H



I

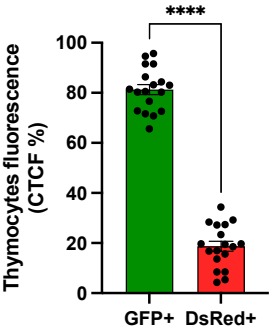


Figure 3: *Tg(ubb:PA-Cre)* enables light-induced genetic recombination with different Cre reporter zebrafish transgenic lines.

(A) The stable transgenic line *Tg(ubb:PA-Cre)* was crossed to *Tg(mpeg1:LoxP-DsRedx-LoxP-GFP-NTR)*. The double transgenic embryos *Tg(ubb:PA-Cre; mpeg1:Switch)* were photoactivated with blue light at 6 hpf for 12 hours and then imaged at the desired developmental stage.
(B) Representative fluorescent images at 48 hpf of *Tg (ubb:PA-Cre; mpeg1:Switch*) embryos kept in dark (left) or exposed to blue light illumination at 6 hpf for 12 hours (right). Scale bar, 100 µm.
(C) Quantification of macrophage fluorescence intensity was measured at 48 hpf in photoactivated *Tg (ubb:PA-Cre; mpeg1:Switch*) embryos (n=9). Mean ± SEM of the $DsRed^+$ and GFP^+ corrected total cell fluorescence (CTCF) percentage is shown. Unpaired t-test with Welch's correction was used for this analysis. $^{****}p \leq 0.0001$.
(D) The stable transgenic line *Tg(ubb:PA-Cre)* was crossed to *Tg (α/β_{a2}-globin:loxP-DsRedx-loxP-GFP)*. The double transgenic embryos *Tg(ubb:PA-Cre; globin:Switch)* were photoactivated with blue light at 6 hpf for 12 hours and then imaged at the desired developmental stage.
(E) Representative fluorescent images at 48 hpf of *Tg (ubb:PA-Cre; globin:Switch)* embryos kept in dark (left) or exposed to blue light illumination at 6 hpf for 12 hours (right). Scale bar, 100 µm.
(F) Quantification of erythrocyte fluorescence intensity was measured at 48 hpf in photoactivated *Tg (ubb:PA-Cre; globin:Switch*) embryos (n=14). Mean ± SEM of the $DsRed^+$ and GFP^+ corrected total cell fluorescence (CTCF) percentage is shown. Unpaired t-test with Welch's correction was used for this analysis. $^{****}p \leq 0.0001$.
(G) The stable transgenic line *Tg(ubb:PA-Cre)* was crossed to *Tg (lck:loxP-DsRedx-loxP-GFP)*. The double transgenic embryos *Tg (ubb:PA-Cre; lck:Switch)* were photoactivated with blue light at 6 hpf for 12 hours and then imaged at the desired developmental stage.
(H) Representative fluorescent images at 120 hpf of *Tg (ubb:PA-Cre; lck:Switch)* embryos kept in dark (left) or exposed to blue light illumination at 6 hpf for 12 hours (right). White dashes mark the thymus. Scale bar, 100 µm.
(I) Quantification of thymocyte fluorescence intensity was measured at 120 hpf in photoactivated *Tg (ubb:PA-Cre; lck:Switch*) embryos (n=18). Mean ± SEM of the $DsRed^+$ and GFP^+ corrected total cell fluorescence (CTCF) percentage is shown. Unpaired t-test with Welch's correction was used for this analysis. $^{****}p \leq 0.0001$.

Figure 4

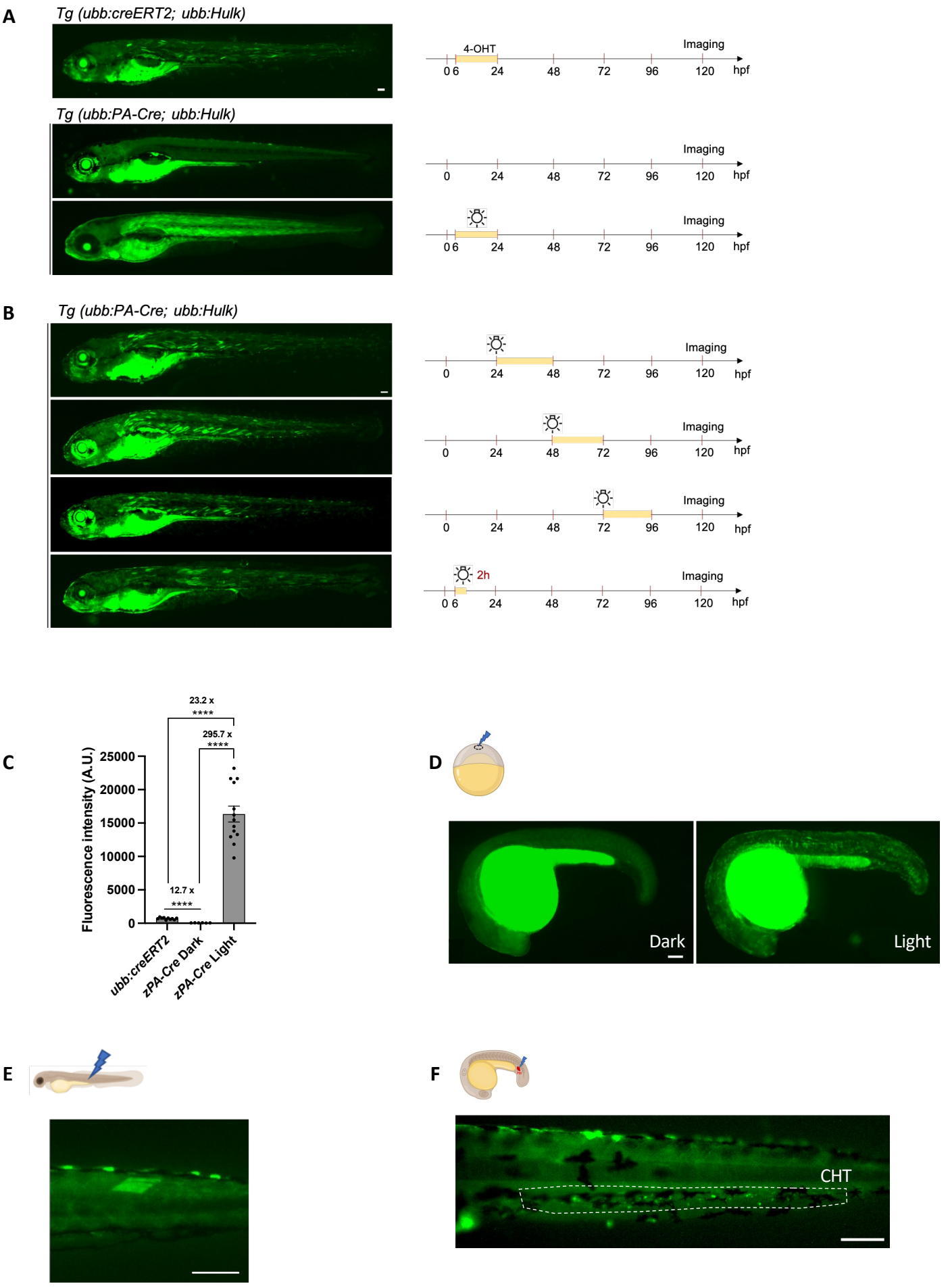
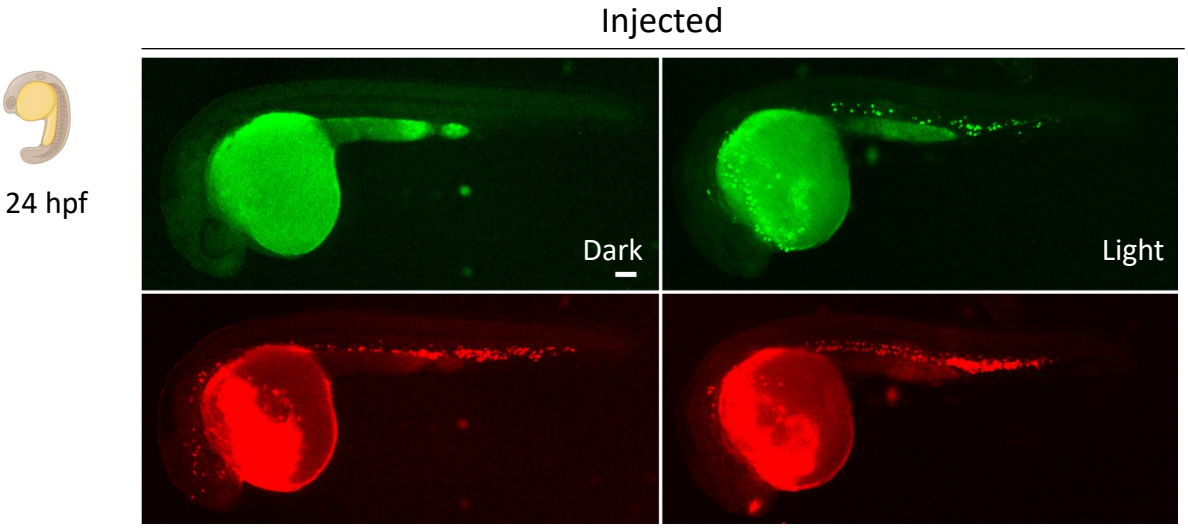

A
Tg (ubb:creERT2; ubb:Hulk)
4-OHT
Imaging
0 6 24 48 72 96 120 hpf
Tg (ubb:PA-Cre; ubb:Hulk)
Imaging
0 24 48 72 96 120 hpf
Imaging
0 6 24 48 72 96 120 hpf
B
Tg (ubb:PA-Cre; ubb:Hulk)
Imaging
0 24 48 72 96 120 hpf
Imaging
0 24 48 72 96 120 hpf
Imaging
0 24 48 72 96 120 hpf
2h
Imaging
0 6 24 48 72 96 120 hpf
C
23.2 x
****
295.7 x
****
12.7 x
****
Fluorescence intensity (A.U.)
25000
20000
15000
10000
5000
0
ubb:creERT2
zPA-Cre Dark
zPA-Cre Light
D
Dark
Light
E
F
CHT

Figure 4: *Tg(ubb:PA-Cre)* enables light-induced spatial and temporal genetic recombination.

(A) Representative fluorescent images at 120 hpf of 4-OHT-induced *Tg (ubb:creERT2; ubb:Hulk)* larvae or *Tg (ubb:PA-Cre; ubb:Hulk)* larvae kept in dark or exposed to blue light illumination at 6 hpf overnight (left panel). Scale bar, 100 µm. Schematic representation strategy used to induce genetic recombination in zebrafish *Tg (ubb:creERT2; ubb:Hulk)* or *Tg (ubb:PA-Cre; ubb:Hulk)* larvae (right panel).
(B) Representative fluorescent images at 120 hpf of *Tg (ubb:PA-Cre; ubb:Hulk)* larvae exposed to blue light illumination at 24 hpf over-night, at 48 hpf over-night, at 72 hpf over-night and at 6 hpf for 2 hours (left panel). Scale bar, 100 µm. Schematic representation strategy used to induce genetic recombination in zebrafish *Tg (ubb:PA-Cre; ubb:Hulk)* larvae at different developmental stages (right panel).
(C) Quantification of fluorescence intensity at 120 hpf of 4-OHT-induced *Tg (ubb:creERT2; ubb:Hulk)* (n=10), *Tg (ubb:PA-Cre; ubb:Hulk)* larvae kept in dark (n=6) or exposed to blue light illumination at 6 hpf over-night (n=13). 23.2x and 295.7 x represent the fold induction comparison between the different groups. Mean ± SEM of the fluorescence intensity is shown. Unpaired t-test with Welch's correction was used for this analysis. $^{****}p \leq 0.0001$. A.U., Arbitrary Unit.
(D) Schematic representation strategy used to induce local photoactivation with blue-light laser at 4 hpf. A 25um diameter circle were targeted by a 488 nm laser for 5 mins. Representative fluorescent images of *Tg(ubb:PA-Cre; ubb:Hulk)* embryo kept in dark (left, n=2) or exposed to blue light laser (right, n=3) at 19 hpf. Scale bar, 100 µm.
(E) Schematic representation strategy used to induce local photoactivation within a single skeletal muscle using blue-light laser at 48 hpf. A 25um diameter circle within a single skeletal muscle were targeted by a 488 nm laser for 5 mins (n=6). Representative fluorescent images of *Tg(ubb:PA-Cre; ubb:Hulk)* showing GFP^+ skeletal muscle at 72 hpf. Scale bar, 100 µm.
(F) Schematic representation strategy used to induce local photoactivation with blue-light laser at 22 hpf. Few cells in the PBI (a 25um diameter circle) were targeted by a 488 nm laser for 5 mins (n=6). Representative fluorescent image of *Tg(ubb:PA-Cre; globin:Switch)* embryo showing GFP^+ erythrocytes in the CHT at 48 hpf. Scale bar, 100 µm.

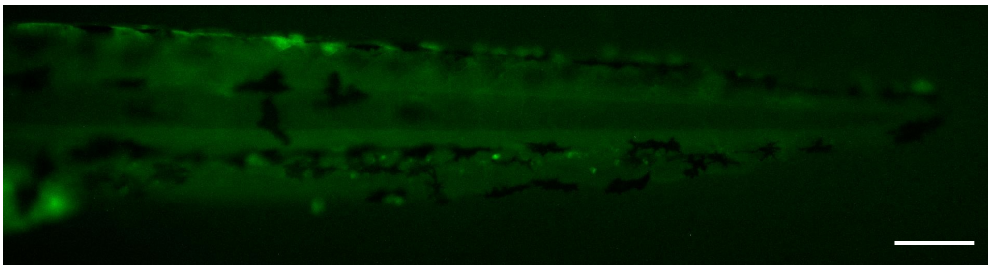
Supp Figure

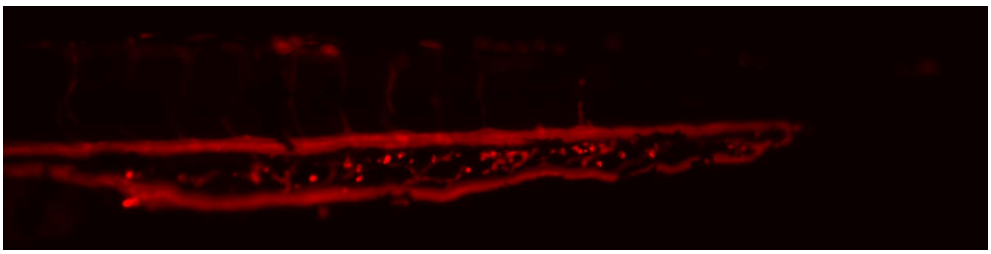


Supp. Figure

Representative fluorescent images at 24 hpf of *Tg(globin:Switch)* embryos injected with the *kdrl*-PA-Cre construct at the one-cell stage and kept in dark (left) or exposed to blue light illumination at 6 hpf for 12 hours (right). Scale bar, 100 µm.

48 hpf





Supp. Videos

Real-time imaging in *Tg(ubb:PA-Cre; globin:Switch)* at 48hpf. A 22 hpf embryo was used to induce local photoactivation by a 488 nm laser for 5 mins within a 25μm diameter circle in the posterior blood island (PBI). Lateral view; anterior to the left and dorsal to the top. Scale bar, 100 μm.

Supplementary Note. Nucleic acid sequences of PA-Cre used in the this study.

accacc**atg**gccacctctgatgaagtcaggaagaacctgatggacatgttcagggacaggcaggccttctctgaacacacctggaagatgctcctgtctgtgtgcagatc
ctgggctgcctggtgcaagctgaacggtacccatactctttatgcccccggtggatatgacattatgggatatctggaccagatcggcaaccggccaaacccgcaggtgg
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ggcgagtatcgatactctatgggattccagtgcgaaacagaaggcggaagcggtggcgtgcccaagaagaagaggaaagtcggatccggcagcggcgccaccaactt
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agacaccccgatagtttacgcgagtgaagccttcctctacatgacaggttacagcaacgcagaggtgctgggccggaattgccggtttctgcaaagccctgacggcatgg
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gagatacctggcctggtctggccactctgccagagtgggtgctgccagggacatggccagggctggtgtgtccatccctgaaatcatgcaggctggtggctggaccaatg
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