ImPaqT - A Golden Gate-based Toolkit for Zebrafish Transgenesis

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Abstract

Transgenic animals continue to play an essential role in many aspects of zebrafish research, including the development of disease models. The most widely used system for zebrafish transgenesis is the Tol2 transposon system. Here, we have developed ImPaqT (**Im**munological toolkit for **Paq**CI-based Golden Gate Assembly of Tol2 **T**ransgenes), a new Tol2-based transgenesis system that utilizes Golden Gate assembly to facilitate the production of transgenic zebrafish lines. This system allows for rapid assembly of multiple fragments into a single transgene, facile swapping of individual sequences to generate new transgenes and an easy cloning workflow to incorporate new genetic elements into the existing kit. Within this toolkit framework, we have generated a number of reagents to enable gene expression within immune and non-immune cell types, an array of best-in-class fluorescent proteins to visualize cell populations and transgenes as well as tools to simplify genetic manipulation, purification and ablation of targeted cells. Unlike recombination-based systems, the Golden Gate approach is also expandable, allowing the incorporation of complex designs such as multi-fragment promoters within the established modular framework of ImPaqT. Here, we demonstrate the function of this new system by generating a number of novel transgenic immune reporter lines. While our toolkit is focused on the immune system as an emerging area of study within zebrafish research, the ImPaqT approach can be broadly adapted to the construction of almost any zebrafish transgene, offering new tools for the generation of transgenes within the zebrafish community.

33 **Introduction**

34 The zebrafish (*Danio rerio*) has emerged as a powerful model organism and is increasingly
35 used in various research fields and disciplines. Zebrafish share 70% of their genome and more
36 than 80% of disease genes with humans (Howe et al. 2013). This has led to the development
37 of many distinct zebrafish disease models (Santoriello and Zon 2012) including models of
38 hematopoietic and neurodegenerative diseases, cardiovascular diseases (Bowley et al. 2022)
39 such as atherosclerosis (Tang et al. 2021), cancer (Liu and Leach 2011; Russo et al. 2022),
40 and tuberculosis (Bouz and Al Hasawi 2018; Varela and Meijer 2022), among others.
41 Transgenic animals continue to play a key role for many experimental strategies, including
42 study of gene function, genetic screens, and development of disease models. Transgenic
43 zebrafish lines have been widely generated by applying the Tol2 transposon system with a
44 transgenesis rate of 50% (Kawakami et al. 2004). In this system, a transposon-donor plasmid
45 containing a DNA sequence of interest flanked by *Tol2* sites and synthetic mRNA encoding
46 the Tol2 transposase are introduced into the single cell of fertilized eggs by microinjection.
47 Upon translation, the transposase protein integrates the *Tol2* flanked gene fragment randomly
48 into the genome through a cut-and-paste mechanism without causing rearrangement or
49 modifications at the target site (Kawakami and Shima 1999; Kawakami, Shima, and Kawakami
50 2000; Kawakami et al. 2004; Kawakami 2007).

51 A number of approaches have previously been used to generate Tol2 transgenesis constructs.
52 Traditionally, multi-step restriction enzyme and ligase based subcloning was used. However,
53 this has given way to many advanced cloning approaches, including Gateway cloning (Hartley,
54 Temple, and Brasch 2000; Kwan et al. 2007; Katzen 2007), Gibson Assembly (Gibson et al.
55 2009), In-Fusion cloning (Zhu et al. 2007; Sleight et al. 2010) and Golden Gate cloning, which
56 have been used to speed up and simplify the procedure of transgene assembly. Each of these
57 approaches have different advantages and disadvantages. Gateway cloning allows for the
58 establishment and reuse of a library of fragments (e.g. promoters, genes and fluorescent
59 proteins) but requires the use of lambda phage-derived *att* sequences to facilitate insertion of

60 the desired sequences. These *att* sequences result in the addition of ~20-25 bp of additional
61 sequence in between each element and expansion of the number of fragments to be
62 assembled can require recloning of the sequence with new *att* ends (Katzen 2007; Engler,
63 Kandzia, and Marillonnet 2008; Sone et al. 2008). By contrast, Gibson and In-Fusion cloning
64 allow for joining of short (15-20 bp) regions of homology, enabling the seamless cloning of
65 many transgenes. However, the requirement for homology for seamless cloning generally
66 means that individual components have to be adapted to upstream and downstream elements
67 to generate new transgenes and have an upper limit of approximately 5-6 fragments with
68 currently available kits. Golden Gate cloning utilizes type IIS restriction enzymes to cleave and
69 join multiple fragments in a one pot reaction (Padgett and Sorge 1996; Engler, Kandzia, and
70 Marillonnet 2008). It balances the tradeoffs of the previous cloning systems, requiring inclusion
71 of only a short 4 base pair sequence, while enabling researchers to generate a library of
72 vectors that can be combinatorially assembled to generate distinct transgenes. Large numbers
73 of DNA fragments can also be assembled by Golden Gate with reports of assembly of up to
74 52 fragments in a single reaction (Pryor et al. 2022). Despite the advantages of Golden Gate
75 cloning, this approach has not been widely applied in zebrafish transgene assembly. Only a
76 few examples of this approach have been described for Tol2 transgenesis including the
77 recently described GoldenFish toolkit which uses Golden Gate cloning to gain more flexibility
78 in transgene design (Jiang et al. 2023) and the Golden Gateway tool (Kirchmaier, Lust, and
79 Wittbrodt 2013) which combines the well-established cloning tools Gateway and Golden Gate
80 cloning to simplify creation of new components for Gateway assembly of transgenes.

81 Here, we have sought to apply Golden Gate cloning approaches to create a toolkit for zebrafish
82 transgenesis. We have developed ImPaqT, a new **Im**munological toolkit for **Paq**CI-based
83 Golden Gate Assembly of Tol2 **T**ransgenes, to facilitate the production of transgenic animals.
84 Studies of immune function have been of growing interest in the zebrafish community due to
85 the close genetic similarity of the immune systems of humans and zebrafish (Howe et al. 2013),
86 but there are few compiled resources for these cell types. ImPaqT provides a collection of

87 commonly used immunological promoters targeting distinct lineages of vertebrate immunity,
88 genetic tools and fluorophores supporting flexible generation of transgenes for immunological
89 research. This toolkit utilizes Golden Gate cloning due to the advantages of this technique and
90 incorporates a newly described type IIS restriction enzyme, PaqCI, with a 7 base recognition
91 sequence to reduce the frequency of off target cutting (from 1 in 4,096 bases (4^6) for a 6-base
92 cutter to 1 in 16,384 bases (4^7), assuming random cutting). We designed the kit to have
93 consistent overhang sequences for each position in the backbone (e.g. 5', middle, and 3'
94 element) in order to make the toolkit modular and simplify the construction of additional
95 transgenes. As proof-of-concept, we show that this toolkit can facilitate the production of
96 expression constructs and we demonstrate the generation of transgenic animals using the Tol2
97 transgenesis with these constructs. ImPaqT is modular, enabling easy swapping of gene
98 sequences, easily expandable, allowing the introduction of multiple new genetic elements if
99 needed, and relies only on the use of short (4 bp) overlaps that can also be designed to be
100 scarless if desired. To establish an immunological toolkit that will be broadly useful to zebrafish
101 researchers, we have generated a variety of immunological and non-immunological promoters,
102 including *mfap4* (macrophages)*, irg1* (macrophages, induced by infection) *, lyz* (neutrophils),
103 *lck* (T cells), *ubb* (ubiquitious) *and hsp70* (heat shock inducible), tools for genetic
104 recombination (*icre*), cell killing (*kid, nitroreductase)* and cell isolation tools (*LNGFR*), and an
105 array of fluorescence proteins including *mTurquoise2*, *tdStayGold*, *mCitrine*, *mNeonGreen*,
106 *dLanYFP* and td*Tomato* that can be used to rapidly develop new transgenes and new
107 transgenic lines.

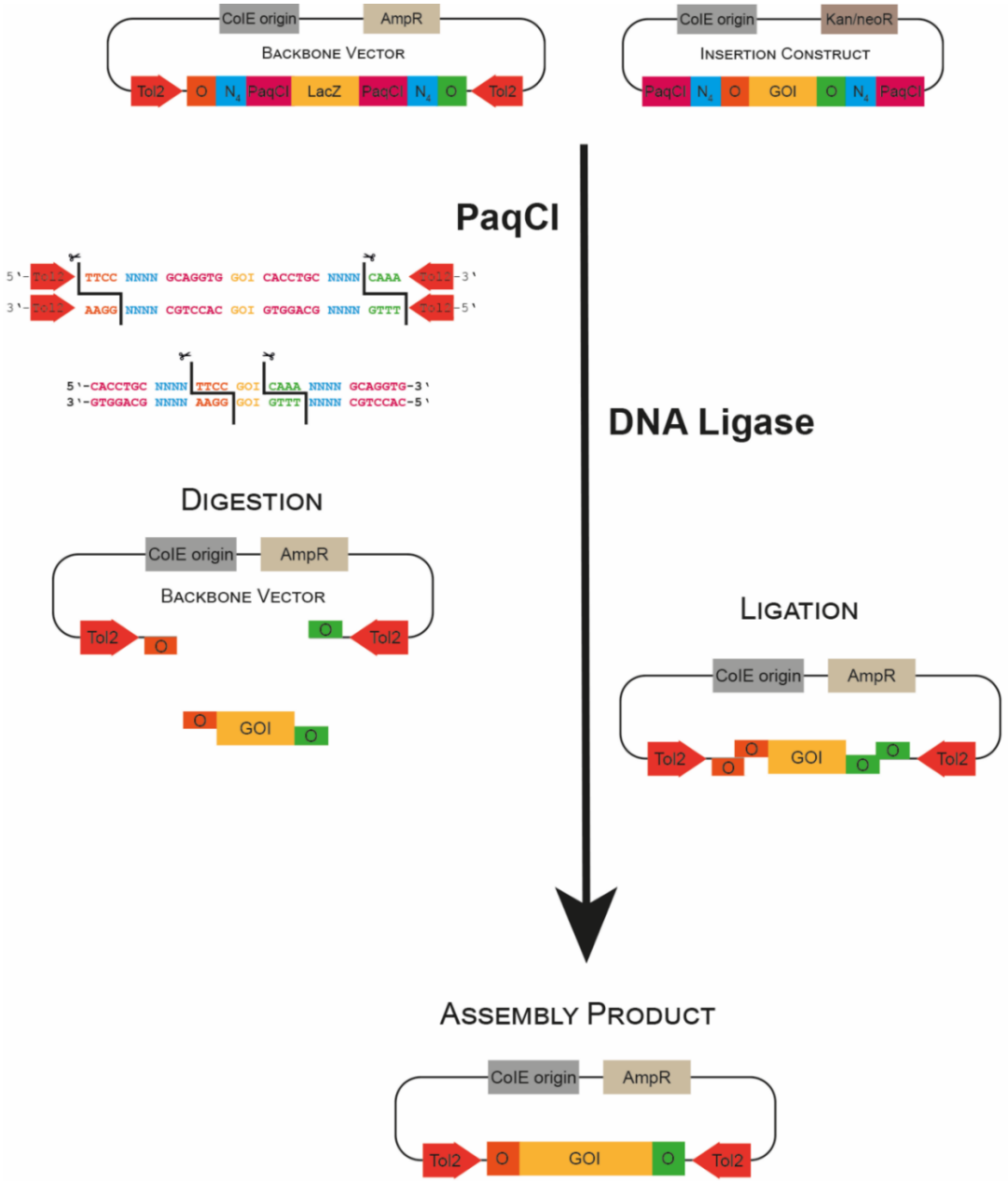
108 **Results**

109 PaqCI-based Golden Gate cloning enables the construction of Tol2 expression vectors

110 We sought to create a toolkit for immune transgene construction for the zebrafish community.
111 Ultimately, we decided to design this toolkit to use Golden Gate cloning, given the short regions
112 of homology that this approach required, the ability to structure this toolkit in a modular fashion
113 and the expandability of the Golden Gate cloning approach, enabling incorporation of
114 additional genetic elements as needed. Golden Gate assembly uses type IIS restriction
115 enzymes to generate a set of unique overhangs that are used to join fragments in a desired
116 order. To create a system for modular cloning of transgenes, we chose consistent 4-base
117 overhang sequences for each of the three elements such that DNA fragments amplified with
118 these overhangs can be cloned in the same position in the final construct, allowing these
119 elements to be used combinatorially. As zebrafish transgenes generally consist of a cell-type
120 specific promoter, a gene of interest, and a fluorescent marker, we structured our toolkit to
121 have three elements that would ultimately be joined within a backbone vector possessing Tol2
122 repeats for transgenesis. These elements were named based on their location within the final
123 construct - 5' element (5E) for the first position, a middle element (ME) and a 3' element (3E)
124 for the final position within the backbone.

125 To generate the overhangs required to join individual components for Golden Gate assembly,
126 we chose the 7-base cutter PaqCI to limit off-target cutting of inserts used in assembly. Genes
127 and regions of interest were generated by PCR amplification using primers containing the 7-
128 base PaqCI recognition sequence along with a spacer consisting of any four nucleotides that
129 will be excised from the final construct, and a 4-base overhang sequence specific to the
130 location of the sequence of interest in the final construct (Fig. S1). These PCR products were
131 cloned into vectors and sequence verified to generate a stable library of constructs for
132 transgene assembly. Cleavage with PaqCI leads to the creation of specific 4-base overhangs
133 for each position at their 5' and 3' end enabling proper assembly and single-step ligation into
134 a backbone vector for transgene generation. These backbone vectors possess inverted *Tol2*

135 repeats for transgenesis surrounding a PaqCI-flanked LacZ gene for blue-white screening to
136 identify backbone constructs containing transgene components. A simplified schematic for the
137 assembly using one insert is illustrated in Fig. 1. As we demonstrated below, we have found
138 that this approach allows for efficient, combinatorial transgene generation.



139

140 **Figure 1: Basic principles of Golden Gate cloning for Tol2 Transgenesis**
141 Schematic of a Golden Gate cloning reaction using one insert. The insertion construct and backbone
142 vector are assembled in a single-tube reaction using PaqCI and ligase. PaqCI is a type IIS restriction
143 enzyme. Its recognition site and cleavage are indicated. The font color corresponds to the color of the
144 respective feature indicated in the cartoon. The backbone vector consists of Tol2 sites, PaqCI
145 recognition sites (PaqCI) along with 4-base spacer sequences (N_4) and two overhangs (O). The insertion
146 construct includes the gene of interest (GOI) flanked by PaqCI, N_4 and the same two overhangs.
147 Assembly involves digestion of backbone and insertion constructs by PaqCI producing matching
148 overhangs and ligation through the DNA ligase forming the final assembly product.

149 ImPaqT facilitates the flexibility of transgenes design and the generation of transgenic
150 zebrafish

151 Having established this Golden Gate cloning-based technique for the assembly of Tol2
152 transposon vectors, we sought to create an array of constructs that would be beneficial to the
153 immunology community to generate transgenic animals. To these ends, we have generated
154 constructs for expression in commonly studied zebrafish immune populations, including T cells
155 (*lck*) (Langenau et al. 2004), neutrophils (*lyz)* (Hall et al. 2007) and macrophages (*mfap4* and
156 the infection-inducible *irg1*) (Walton et al. 2015; Hall et al. 2013). To enable lineage tracing
157 approaches for labeling of hematopoietic derived cells, we also included the hematopoietic
158 stem cell (HSC)-specific promoter *runx1 +23* (Tamplin et al. 2015) as well as tools for broader
159 expression including the ubiquitious promoter *ubiquitin B* (*ubb*) (Mosimann et al. 2011), and
160 the temperature inducible heat shock protein 70-like promoter (*hsp70*) (Shoji, Yee, and
161 Kuwada 1998; Halloran et al. 2000; Kwan et al. 2007). We have also integrated a number of
162 tools to allow for manipulation of these immune populations, including tools for genetic
163 manipulation (*icre* (Shimshek et al. 2002)), for rapid purification of cell populations (truncated
164 human *LNGFR* (Wattrus and Zon 2020)) and cell-specific ablation (*nitroreductase* (Curado et
165 al. 2007; Curado, Stainier, and Anderson 2008) and *kid* (Labbaf et al. 2022)). To visualize
166 labeled cell populations and genes, we provide a diverse array of best-in-class fluorescent
167 proteins in a range of colors to facilitate simultaneous imaging of distinct cell types. These
168 include *tdStayGold* (Hirano et al. 2022), *mTurquoise2* (Goedhart et al. 2012), *tdTomato*
169 (Shaner et al. 2004), m*Citrine* (Zacharias et al. 2002), *mNeonGreen* (Shaner et al. 2013) and
170 *dLanYFP* (Shaner et al. 2013). A summary of all available constructs is shown in Table 1.
171 ImPaqT is also designed to be easily expandable through simple cloning approaches.

172 **Table 1: Library of insertion constructs.**
173 5' element (5E), middle element (ME), 3' element (3E).

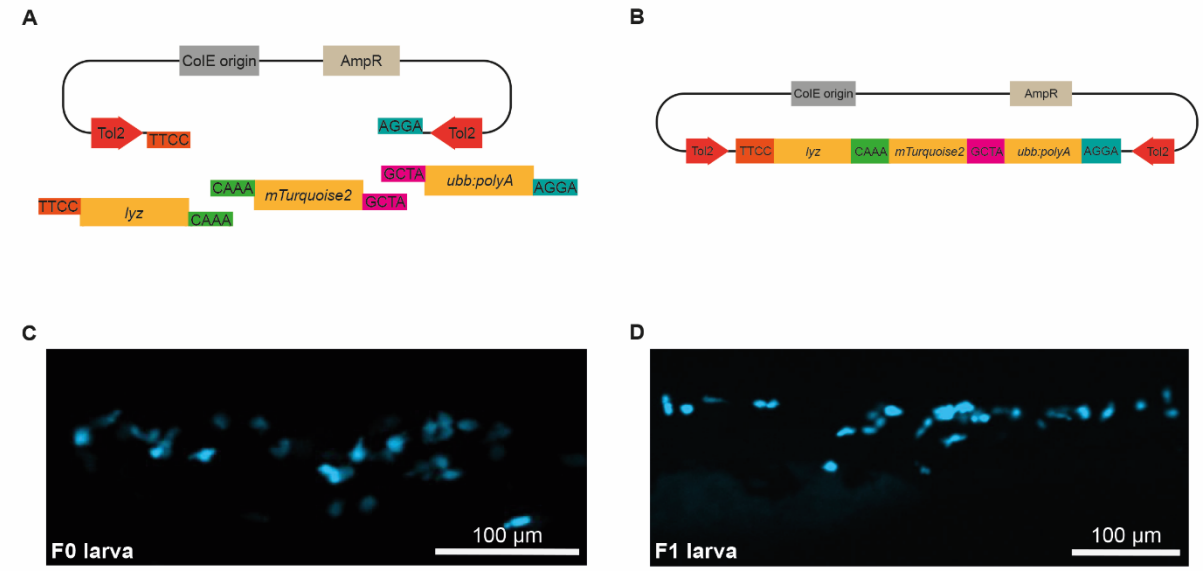
Insertion Elements	Position	Function	Reference
irg1	5E	Infection-inducible macrophage-specific promoter	(Hall et al. 2013)
mfap4	5E	Macrophage-specific promoter	(Walton et al. 2015)
lyz	5E	Neutrophil-specific promoter	(Hall et al. 2007)
ubiquitin B (*ubb*)	5E	Promoter for ubiquitious expression	(Mosimann et al. 2011)
ubb:loxP GFP loxP	5E	Promoter for ubiquitious expression of floxed GFP for use in combination with Cre	(Mosimann et al. 2011)
hsp70	5E	heat shock protein 70-like promoter for inducible gene expression	(Shoji, Yee, and Kuwada 1998; Halloran et al. 2000; Kwan et al. 2007)
lck	5E	T cell-specific promoter	(Langenau et al. 2004)
runx1 + 23	5E	Promoter for expression in hematopoietic stem cells	(Tamplin et al. 2015)
icre	ME	Cre recombinase for inducible recombination of DNA between loxP sites	(Shimshek et al. 2002)
(p2A) dLanYFP	3E	Yellow fluorescent protein	(Shaner et al. 2013)
mNeonGreen	ME, 3E	Green/ yellow fluorescent protein	(Shaner et al. 2013)

LNGFR	ME	Low affinity nerve growth factor receptor for rapid cell enrichment	(Wattrus and Zon 2020)
nitroreductase	ME	Inducible ablation of specific cell lineages	(Curado et al. 2007; Curado, Stainier, and Anderson 2008)
kid	ME	Ablation of specific cell lineages	(Labbaf et al. 2022; de la Cueva-Mendez et al. 2003)
(p2A) tdTomato	ME, 3E	Red fluorescent protein	(Shaner et al. 2004)
tdTomato CAAX	ME	Red fluorescent protein with membrane localization protein	(Walton et al. 2015)
(p2A) mTurquoise2	ME, 3E	Cyan fluorescent protein	(Goedhart et al. 2012)
mCitrine	ME	Yellow fluorescent protein	(Zacharias et al. 2002)
tdStayGold	ME	Green fluorescent protein	(Hirano et al. 2022)
ubb:polyA	3E	Ubiquitious expression of a SV40 poly A	(Walton et al. 2015)

174

175 Generation of new transgenic zebrafish lines using ImPaqT

176 To test our PaqCI-based Golden Gate assembly toolkit as well as generate new immunological
177 zebrafish lines for the community, we used ImPaqT to assemble a number of new transgenic
178 lines. Using the neutrophil-specific promoter *lyz*, we generated a T*g(lyz:mTurquoise2)*^{ber2}
179 construct through Golden Gate assembly of 5E *lyz*, ME *mTurquoise2* and 3E *ubb:polyA* to
180 create the *lyz:*m*Turquoise2* transgene construct (Fig. 2A-B). The construct was coinjected with
181 *Tol2* transposase mRNA into single cell of fertilized eggs and at 2 dpi we observed strong blue
182 fluorescence signal within likely neutrophil populations in the CHT (Fig. 2C). Positive animals
183 were raised to adulthood and screening of F1 progeny of these animals revealed robust
184 expression of mTurquoise2 within putative neutrophil populations throughout the animal (Fig.
185 2D). Crossing to the already established neutrophil reporter *Tg(lyz:EGFP)*^{nz117} (Hall et al. 2007)
186 zebrafish confirmed specific expression of our transgene within neutrophils (Fig. S2).

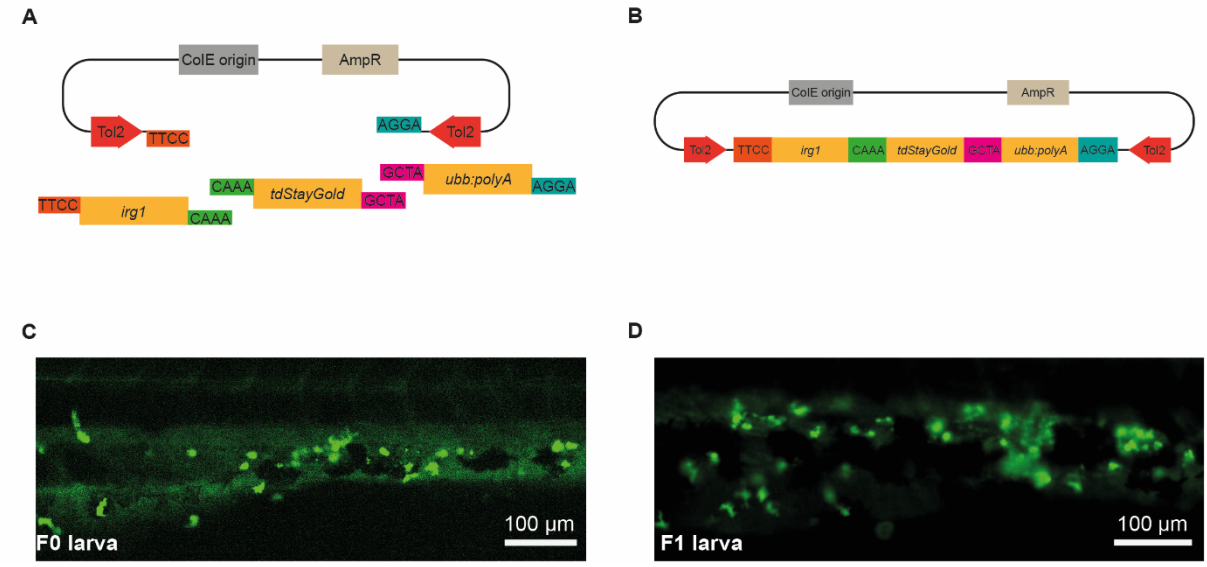


187

188 **Figure 2: Generation of *Tg(lyz:mTurquoise2)* zebrafish**
189 A: Schematic of the Golden Gate assembly of *Tg(lyz:mTurquoise2)*. The backbone vector contains
190 overhang sequences O1 (TTCC) and O4 (AGGA). *lyz* is a neutrophil-specific promoter containing
191 overhang sequences O1 and O2 (CAAA). *mTurquoise2* encodes a bright cyan fluorescent protein with
192 overhangs O2 and O3 (GCTA). *ubb:polyA* is assembled at the final position within the backbone with
193 O3 and O4.B: Schematic of the final assembly product of *Tg(lyz:mTurquoise2)*. C-D: Images of a
194 *Tg(lyz:mTurquoise2)* F0 (C) and F1 (D) larva showing neutrophil response in cyan at 2 days post
195 fertilization.

196

197 Similarly, we used the ImPaqT toolkit to generate a new macrophage-specific transgene using
198 the infection-inducible macrophage-specific promoter *irg1* and the recently described bright
199 green fluorescent protein *tdStayGold* (Hirano et al., 2022). To generate the
200 *Tg(irg1:tdStayGold)*^{ber3} construct, the 5E *irg1,* ME *tdStayGold* and 3E *ubb:polyA* elements were
201 combined into the backbone vector (Fig. 3 A-B). We injected zebrafish with this construct and
202 as *irg1* is an inflammation responsive promoter that is only weakly expressed in the absence
203 of immune stimulation, we injected larvae with LPS before screening. After LPS injection, we
204 were able to identify a number of F0 founders with strong green fluorescence within
205 subpopulations of their macrophages (Fig. 3C). These positive F0 larvae were raised to
206 adulthood and screening of F1 progeny revealed expression of tdStayGold throughout
207 macrophage populations upon stimulation with LPS (Fig. 3 D).

208 

209 **Figure 3: Generation of *Tg(irg1:tdStayGold)* zebrafish**
210 A: Schematic of the Golden Gate assembly of *Tg(irg1:tdStayGold)*. The backbone vector contains
211 overhang sequences O1 (TTCC) and O4 (AGGA). *irg1* is a macrophage-specific promoter containing
212 overhang sequences O1 and O2 (CAAA). *tdStayGold* encodes a bright green fluorescent protein with
213 overhangs O2 and O3 (GCTA). *ubb:polyA* is assembled at the final position within the backbone with
214 O3 and O4. B: Illustration of the final assembly product of *Tg(irg1:tdStayGold)*. C-D: Images of a
215 *Tg(irg1:tdStayGold)* F0 (C) and F1 (D) larva showing macrophage-specific tdStayGold expression.

216

217 We also aimed to test the utility of the tools that we have designed within our kit. To these
218 ends, we created an infection-inducible, macrophage specific *icre* transgene construct,
219 *Tg(irg1:icre-p2A mTurquoise2)*. This construct was generated by combining the 5E *irg1,*

220 ME *icre* and 3E *p2A mTurquoise2* (Fig. 4A and 4B). Animals injected with this transgene
221 showed macrophage-specific mTurquoise2 signal after injection with LPS (Fig. 4C). To further
222 confirm that this construct was able to drive rearrangement of floxed reporters, we injected
223 *Tg(-3.5ubb:LOXP-EGFP-LOXP-mCherry)*^{cz1701} (Mosimann et al. 2011) zebrafish with this
224 construct. These animals were injected with LPS and imaged for 48 h to visualize
225 rearrangement of the floxed construct. LPS treatment drove the induction of *icre* and the co-
226 translationally cleaved *p2a mTurquoise2* transgene within macrophages starting ca. 4 h post
227 LPS injection. While the induction of the *irg1:icre-p2a mTurquoise2* transgene was transitory,
228 this short-term induction of Cre could induce permanent rearrangement of the *loxP-EGFP-*
229 *loxP-mCherry* construct as seen by induction of red fluorescent mCherry within macrophage
230 populations (Fig. 4D, S3, Movie S1). Control animals without the *icre*-construct neither showed
231 mTurquoise2 nor mCherry signal in macrophages (Fig. S3, Movie S 2). This experiment
232 showed not only the functionality of the individual insertion constructs but also the range of
233 assembly constructs that can be generated with them.

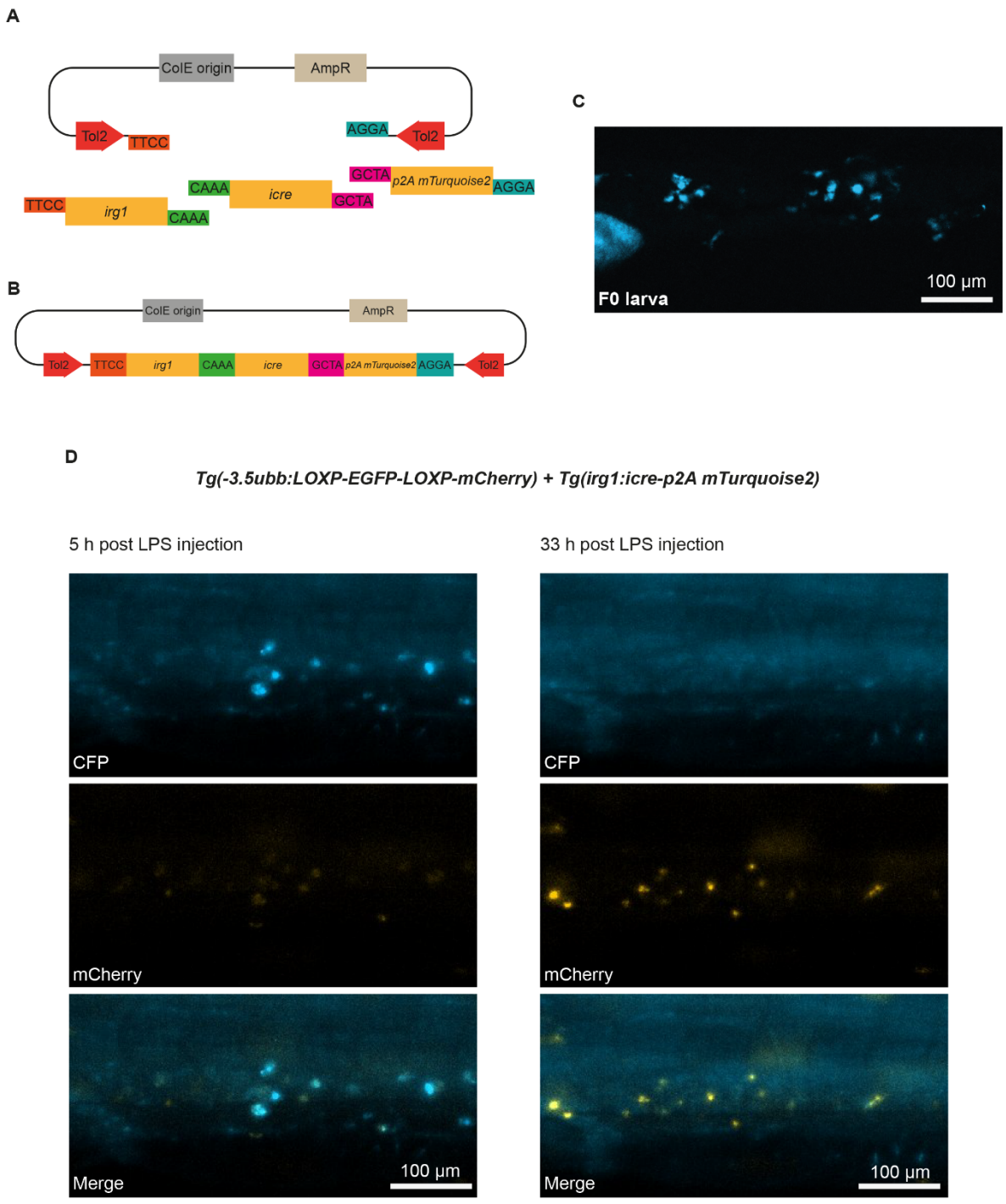


Figure 4: Generation of *Tg(irg1:icre-p2A mTurquoise2)* zebrafish for lineage tracing of individual macrophages

A: Schematic of the Golden Gate assembly of *Tg(irg1:icre-p2A mTurquoise2)*. The backbone vector contains overhang sequences O1 (TTCC) and O4 (AGGA). The macrophage promoter *irg1* contains overhang sequences O1 and O2 (CAAA). *icre* contains overhangs O2 and O3 (GCTA) for the middle position. *p2A mTurquoise2* is assembled at the final position within the backbone with O3 and O4. B: Illustration of the final assembly product of *Tg(irg1:icre-p2A mTurquoise2)*. C: Image of a *Tg(irg1:icre-p2A mTurquoise2)* F0 larva showing macrophage-specific mTurquoise2 signal after stimulation with LPS. D: Images of a *Tg(-3.5ubb:LOXP-EGFP-LOXP-mCherry)* larva that was introduced to the *Tg(irg1:icre-p2A mTurquoise2)* construct at single cell stage and was treated with PTU and LPS. The images show a macrophage-specific mTurquoise2 signal (e.g. 5 h post LPS injection) and a macrophage-specific mCherry signal (e.g. 33 h post LPS injection).

247 ImPaqT is expandable through the introduction of new overhang sequences

248 A limitation of many existing systems for transgenesis is that these systems have a fixed
249 number of elements that can be combined to generate transgenes. Golden Gate methods
250 however, are expandable through the addition of new overhangs and have been used to join
251 10-24 or even more than 50 fragments of DNA in a single reaction (Kucera 2021; Pryor et al.
252 2022). The ability to expand this kit to include larger numbers of constructs would simplify the
253 generation of complex transgenes including multifragment promoters, loxP sites, localization
254 tags (e.g NLS or prenylation signal) or multigene constructs for example. To provide proof of
255 concept for the expandability of ImPaqT, vectors were designed to enable the construction of
256 a *ubb* promoter-driven construct from three similar sized *ubb* promoter fragments, ubb_1, ubb_2
257 and ubb_3. These fragments were constructed such that the first fragment (ubb_1) and the last
258 fragment (ubb_3) use the same O1 and O2 overhangs used by 5E constructs so that the array
259 of promoter fragments can be cloned into the existing position of 5E elements and used
260 alongside already existing ME and 3E constructs (Fig. 5A-B). New internal overhangs were
261 designed for the ubb_1, ubb_2 and ubb_3 fragments to combine these distinct fragments (Fig. 5A-
262 D). Individual *ubb* fragments were used for Golden Gate assembly in combination with
263 ME *mTurquoise2* and 3E *ubb:polyA* (Fig. 5C) which led to the expected transgenesis construct
264 *Tg(ubb:mTurquoise2)*^{ber4} (Fig. 5D) that was injected into embryos at single cell stage.
265 Screening of injected animals confirmed that transgenic embryos established with this
266 construct had the expected expression pattern (Fig. 5E). We also found robust expression in
267 the F1 progeny (Fig. 5F) after raising positive F0 animals, confirming that injected animals are
268 viable and able to pass on the transgene to the next generation. Together, these findings
269 demonstrated proof-of-concept for the expansion of this transgene assembly system enabling
270 users to add fragments as needed through the addition of novel unique 4-base overhangs
271 while maintaining compatibility with existing ImPaqT constructs.

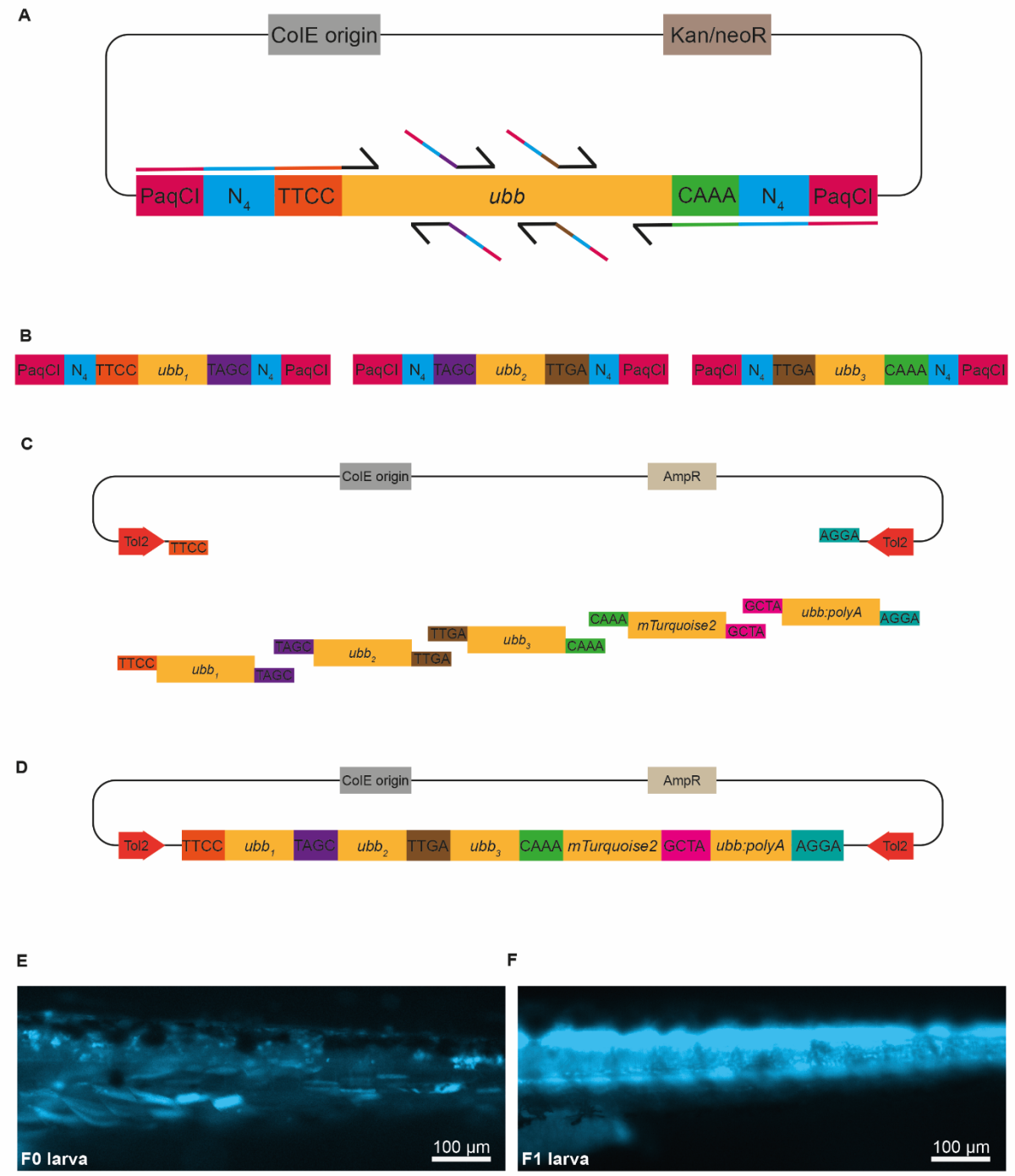


Figure 5: ImPaqT allows for expandability of individual positions while retaining compatibility with already established transposon components.
A: Schematic of the 5E *ubiquitin B* (*ubb*) promoter construct containing PaqCI recognition sites (PaqCI) along with 4-base spacer sequences (N_4) and two overhangs (O1: TTCC, O2: CAAA) for positioning at the 5' end of the assembly construct. Primers were designed to break up *ubb* into three fragments with new overhang sequences. B: Schematic of PCR amplified *ubb* fragments $ubb_{1\text{-}3}$ with PaqCI, N_4 and O1 and O1A (TAGC), O1A and O1B (TTGA), and O1B and O2, respectively. C: Schematic of the Golden Gate assembly of 5E $ubb_{1\text{-}3}$, ME *mTurquoise2* and 3E *ubb:polyA* into the backbone vector. D: Illustration of the final assembly product of *Tg(*$ubb_{1\text{-}3}$*:mTurquoise2)*. E-F: Images of a *Tg(ubb:mTurquoise2)* F0 (E) and F1 (F) larva showing ubiquitous mTurquoise2 signal.

Discussion

Transgenic animals remain essential for many research questions. They enable the visualization of cells and tissues, molecular pathways, immune responses and the perturbation of these pathways to identify the molecular mechanisms driving pathology. Use of the zebrafish as a model organism has been steadily increasing in many research disciplines due to the unique advantages of this model. This is true in immunology and host-pathogen interactions, where the optical transparency afforded by the zebrafish model have led to many research discoveries, but where, as a new immunology model, there are limited transgenic tools. Here, we demonstrate a new cloning toolkit for the generation of immunological transgenes that is based on Golden Gate cloning. The Golden Gate approach results in shorter overlap regions (4 bp) than recombination-based techniques like Gateway cloning, while compared to seamless, homology based cloning methods such as Gibson cloning, it allows for straightforward modular cloning approaches. The Golden Gate approach also takes advantage of off-the-shelf restriction enzymes and ligase, compared to other cloning approaches which use proprietary enzymes for cloning.

We have termed this toolkit ImPaqT - **Im**munological toolkit for **Paq**CI-based Golden Gate Assembly of Tol2 **T**ransgenes. It includes a range of immunologically focused promoters along with genetic tools and fluorescent proteins that can be assembled using PaqCI-based Golden Gate cloning. These tools will enable the flexible assembly of transgenes to drive expression and to genetically manipulate, purify and ablate immune cell populations of interest. While zebrafish possess the same immune lineages of other vertebrates, as a relatively new model for immunology studies, the zebrafish has a more limited array of cell type-specific promoters than mouse models. For instance, despite the identification of potential NK- and ILC-specific (Hernandez et al. 2018) as well as mast cell-specific (Dobson et al. 2008) genes, promoters specific for these cell populations have not been described. As new immune-specific promoters are identified, these can be readily added to this toolkit. While we have targeted the immune system as a particular area of need, this Golden Gate approach can also be adapted to any other tissue or cell lineages within the zebrafish.

312 While Golden Gate cloning enables the use of shorter homology stretches and the
313 combinatorial use of constructs for the assembly of diverse transgenes, as a restriction
314 enzyme-based system, a limitation of Golden Gate systems, compared to other assembly
315 approaches, has been the risk of off-target cutting within the cloned sequences. This off-target
316 cutting requires mutation of insert sequences to eliminate these sites (termed domestication),
317 (Marillonnet and Grutzner 2020; Bird, Marles-Wright, and Giachino 2022; Grützner and
318 Marillonnet 2020), which can potentially alter function of domesticated promoters and genes.
319 While domestication can result in changes in the sequence that may alter behavior of the
320 genetic element, these in principle can be managed for genes of interest using silent mutations
321 in critical bases. While promoters cannot be managed through the same approach, promoter
322 fragments used for zebrafish are frequently large (commonly 2-6 kb) and it is unlikely that the
323 mutation of a single base pair will inadvertently ablate the promoter activity. Consistent with
324 this, we have not seen any changes in promoter activity or localization in the two promoters
325 that we domesticated for the ImPaqT toolkit, although further cloning of additional promoters
326 will more completely test this idea. Our choice of PaqCI itself seeks to address the limitations
327 of domestication, as PaqCI uses a 7 base recognition site. In contrast to the 6 base-cutters
328 that have been described in previous zebrafish Golden Gate approaches (Kirchmaier, Lust,
329 and Wittbrodt 2013; Jiang et al. 2023), the rare cutting of PaqCI (if random, 1 in 16,384 bases)
330 makes this enzyme better suited for the large DNA fragments that are frequently used for
331 zebrafish promoters.

332 A limitation of existing systems has been the use of a fixed number of constructs for assembly
333 of the transgene. One benefit of the Golden Gate directed system is that it can be expanded
334 for cloning of complex transgenes, with some Golden Gate assemblies encompassing 20 or
335 even more than 50 fragments (Kucera 2021; Pryor et al. 2022). While we have designed the
336 system to incorporate 4 distinct DNA segments (3 inserts and a backbone vector) the flexibility
337 of Golden Gate cloning means that our system can be readily expanded and contracted while
338 maintaining compatibility with the other components of the kit. As proof-of-concept, we were
339 able to break a single 5E encoding the *ubb* promoter into 3 distinct plasmids that we could

340 subsequently assemble together with existing ME, 3E and backbone elements to generate a
341 functional *ubb* driven transgene. The flexibility of this system will simplify the construction of
342 large and complex transgenes. Potential applications would include breaking up larger
343 promoter fragments into smaller and easier to clone elements, insertion of recombination sites
344 for enzymes like Cre or Flp for rearrangement, assembly of multipromoter constructs encoding
345 genes such as Cas9 and CRISPR gRNAs for cell type-specific knockout, or the inclusion of
346 epitope or localization tags.

347 Lastly, while we have applied this Golden Gate method for the construction of Tol2 transgenes,
348 the advantages of the PaqCI-Golden Gate method will likely be useful for the editing of
349 zebrafish by other approaches as well. For instance, CRISPR insertion has been increasingly
350 used to generate knock-in zebrafish lines, via either homologous recombination, non-
351 homologous methods or by using short homology stretches. Knock-in approaches require the
352 construction of donor vectors which incorporate varied sequences such as homology regions,
353 fluorescent proteins and stretches of genomic DNA (Albadri, Del Bene, and Revenu 2017).
354 Similarly, many groups also use I-SceI insertion approaches to generate transgenic zebrafish
355 (Hoshijima, Jurynec, and Grunwald 2016). The PaqCI Golden Gate method could be adapted
356 to simplify the construction of transgenes and editing constructs for these other approaches as
357 well.

358 **Materials and Methods**

359 **Ethics statement**

360 Zebrafish husbandry and experiments were approved by the “Landesamt für Gesundheit und
361 Soziales” (LaGeSo) Berlin (Germany).

362 **Zebrafish husbandry and handling**

363 Zebrafish (*Danio rerio*) embryos were raised in petri dishes containing E3 medium at 28°C for
364 up to five days. Animals older than 5 days were maintained in a circulating system with
365 constant water conditions of 28°C, pH 7.0 - 7.3, and a conductivity of 600-700 µS on a 12-hour
366 light/ 12-hour dark cycle. They were fed twice a day with artemia and once a day with dry food.

367 **Generation of transgenic zebrafish lines**

368 Zebrafish strains

369 All zebrafish strains, including *Tg(lyz:mTurquoise2)*^{ber2}*, Tg(irg1:tdStayGold)*^{ber3},*Tg(irg1:icre-*
370 *p2A mTurquoise2)* and *Tg(ubb:mTurquoise2)*^{ber4}*,* were in the *AB wildtype background and
371 generated in this study by applying the Tol2 transgenesis system as previously described
372 (Kawakami et al., 2004*). Tg(-3.5ubb:LOXP-EGFP-LOXP-mCherry)*^{cz1701} zebrafish were
373 obtained from the European Zebrafish Resource Center (EZRC). *Tg(-3.5ubb:LOXP-EGFP-*
374 *LOXP-mCherry)*^{cz1701} (Mosimann et al. 2011) and *Tg(lyz:EGFP)*^{nz117} (Hall et al. 2007) have
375 been previously described.

376 Preparation of the backbone vector and inserts

377 The backbone vector containing the LacZ gene flanked by PaqCI sites and *Tol2* arms in a
378 vector backbone with a pMB1 origin and an AmpR gene for bacterial selection (pTWIST Amp
379 High Copy) was purchased from Twist Bioscience. The complete sequence is shown in Figure
380 S4. Primer sequences are shown in Table S1. Gene fragments for *runx1+23* (with the
381 endogenous PaqCI site mutated), *tdStayGold* and truncated human *LNGFR* (zebrafish codon
382 optimized and with endogenous PaqCI sites mutated) were purchased from Twist Bioscience

383 and TOPO cloned into pCRII TOPO (Thermofisher Zero Blunt TOPO). The complete
384 sequences are shown in Figure S5.

385 PaqCI recognition sites with 4-base spacer sequences and 4-base overhangs were added to
386 DNA sequences via PCR amplification. Primer were designed to add PaqCI sites such as
387 cleavage resulted in a gene fragment with a 4-base overhang that is compatible to the
388 overhang of another gene fragment that will be adjacent to it in the final assembly. Appropriate
389 4-base overhang sequences were confirmed using the online tool NEBridge Ligase Fidelity
390 Viewer™. PCR amplification was performed using Q5 polymerase and analyzed by gel
391 electrophoresis. The PCR product was cleaned up or, in case of multiple bands, the band of
392 correct size was excised under 70% UV light using a clean scalpel and DNA was extracted
393 using Nucleo Spin Gel and PCR Clean-up Kit (Macherey-Nagel). DNA sequences were
394 confirmed by sequencing. Gel purified gene fragments were subcloned into a Zero Blunt TOPO
395 vector (Invitrogen) and transformed into competent NEB 5α *E. coli* cells (C2987H). Cells were
396 cultivated in LB medium containing 50 µg/ml Kanamycin and plasmids were isolated using the
397 Nucleo Spin Plasmid Quick Pure protocol (Macherey-Nagel).

398 Both the *ubb* and *lck* promoters had endogenous PaqCI cleavage sites in the promoter
399 sequence. To domesticate these promoters, we mutated these PaqCI sites with the indicated
400 PCR primers (Table S1) and assembled them with In-Fusion cloning (Takara Bio).

401 <u>Golden Gate based assembly of expression constructs</u>

402 Golden Gate assembly was performed according to the NEB protocol. In detail, 75 ng of the
403 backbone vector and each insertion construct was mixed with 10 U PaqCI, 0.5 µl PaqCI
404 activator (20 µM) and 400 U T4 DNA ligase in T4 DNA ligase buffer to a final volume of 20 µl.
405 Final assembly product was obtained after 60 Cycles of incubation at 37°C for 1 min and
406 incubation at 16°C for 1 min, followed by 5 min incubation at 37°C and 5 min incubation at
407 60°C using a thermocycler. The final product was transformed into competent NEB 5α *E. coli*
408 cells (C2987H) and cells were cultivated in LB medium containing 20 µg/ml Ticarcillin.
409 Plasmids were isolated using the Nucleo Spin Plasmid Quick Pure protocol (Macherey-Nagel)

410 and PCR purified using the Nucleo Spin Gel and PCR Clean-up Kit (Macherey-Nagel) before
411 microinjection.

412 Microinjections

413 The injection mixture contained 25 ng/µL polyadenylated mRNA encoding the Tol2
414 transposase, 50 ng/µL transposon donor plasmid and 10% phenol red in a final volume of 10 µl
415 1 X Tango Buffer (Thermo Fisher). The mMessage mMachine kit (Life Technologies) was used
416 to transcribe the mRNA from T3TS-Tol2 (Balciunas et al. 2006). A volume of ~1 nL was injected
417 into the single cell of fertilized eggs by using a microinjector.

418 Imaging

419 Injected larvae were screened for insertion of the transgene 2 days post injection (dpi) using a
420 Nikon ECLIPSE Ti2 microscope (Nikon) with a Dragonfly 200 confocal system (Oxford
421 Instruments). Animals that were positive for the transgene were raised to adulthood and
422 outcrossed to the *AB wildtype strain for selection of transgenic founder zebrafish. Before
423 screening *Tg(irg1:icre-p2A mTurquoise2)* and *Tg(irg1:tdStayGold)* embryos, the activity of the
424 *irg1* promoter was induced by injection of LPS. For that purpose, animals were anesthetized
425 with tricaine (MS-222) at a final concentration of 0.016% and injected into the caudal vein with
426 LPS (Sigma-Aldrich, F3665) (final: 0.8 mg/ml). Screening was performed at least 4 h after
427 injection. To reduce pigmentation, *Tg(-3.5ubb:LOXP-EGFP-LOXP-mCherry)* embryos were
428 treated with 45 µg/ml 1-phenyl-2-thiourea (PTU) starting ca. 20 h post fertilization. Image
429 analysis was performed using the Fiji ImageJ software (National Institute of Health, USA).

430 Line and reagent availability

431 All new lines and plasmids are available from the corresponding author upon contact and will
432 also be deposited to AddGene.

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Competing interests

No competing interests declared.

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Data availability

All relevant data can be found within the article and its supplementary information.

References

Albadri, S., F. Del Bene, and C. Revenu. 2017. 'Genome editing using CRISPR/Cas9-based knock-in approaches in zebrafish', *Methods*, 121-122: 77-85.

Balciunas, D., K. J. Wangensteen, A. Wilber, J. Bell, A. Geurts, S. Sivasubbu, X. Wang, P. B. Hackett, D. A. Largaespada, R. S. McIvor, and S. C. Ekker. 2006. 'Harnessing a high cargo-capacity transposon for genetic applications in vertebrates', *PLoS Genet*, 2: e169.

Bird, J. E., J. Marles-Wright, and A. Giachino. 2022. 'A User's Guide to Golden Gate Cloning Methods and Standards', *ACS Synth Biol*, 11: 3551-63.

Bouz, G., and N. Al Hasawi. 2018. 'The zebrafish model of tuberculosis - no lungs needed', *Crit Rev Microbiol*, 44: 779-92.

Bowley, G., E. Kugler, R. Wilkinson, A. Lawrie, F. van Eeden, T. J. A. Chico, P. C. Evans, E. S. Noel, and J. Serbanovic-Canic. 2022. 'Zebrafish as a tractable model of human cardiovascular disease', *Br J Pharmacol*, 179: 900-17.

Curado, S., R. M. Anderson, B. Jungblut, J. Mumm, E. Schroeter, and D. Y. R. Stainier. 2007. 'Conditional Targeted Cell Ablation inZebrafish: A New Tool for RegenerationStudies', *Developmental Dynamics*.

Curado, S., D. Y. Stainier, and R. M. Anderson. 2008. 'Nitroreductase-mediated cell/tissue ablation in zebrafish: a spatially and temporally controlled ablation method with applications in developmental and regeneration studies', *Nat Protoc*, 3: 948-54.

de la Cueva-Mendez, G., A. D. Mills, L. Clay-Farrace, R. Diaz-Orejas, and R. A. Laskey. 2003. 'Regulatable killing of eukaryotic cells by the prokaryotic proteins Kid and Kis', *EMBO*.

Dobson, J. T., J. Seibert, E. M. Teh, S. Da'as, R. B. Fraser, B. H. Paw, T. J. Lin, and J. N. Berman. 2008. 'Carboxypeptidase A5 identifies a novel mast cell lineage in the zebrafish providing new insight into mast cell fate determination', *Blood*, 112: 2969-72.

Engler, C., R. Kandzia, and S. Marillonnet. 2008. 'A one pot, one step, precision cloning method with high throughput capability', *PLoS One*, 3: e3647.

470 Gibson, D. G., L. Young, R. Y. Chuang, J. C. Venter, C. A. Hutchison, 3rd, and H. O. Smith. 2009.
471 'Enzymatic assembly of DNA molecules up to several hundred kilobases', *Nat Methods*, 6: 343-
472 5.
473 Goedhart, J., D. von Stetten, M. Noirclerc-Savoye, M. Lelimousin, L. Joosen, M. A. Hink, L. van Weeren,
474 T. W. Gadella, Jr., and A. Royant. 2012. 'Structure-guided evolution of cyan fluorescent
475 proteins towards a quantum yield of 93%', *Nat Commun*, 3: 751.
476 Grützner, R., and S. Marillonnet. 2020. 'Generation of MoClo Standard Parts Using Golden Gate
477 Cloning.' in Springer (ed.), *DNA Cloning and Assembly Methods and Protocols*.
478 Hall, C., M. V. Flores, T. Storm, K. Crosier, and P. Crosier. 2007. 'The zebrafish lysozyme C promoter
479 drives myeloid-specific expression in transgenic fish', *BMC Dev Biol*, 7: 42.
480 Hall, C. J., R. H. Boyle, J. W. Astin, M. V. Flores, S. H. Oehlers, L. E. Sanderson, F. Ellett, G. J. Lieschke, K.
481 E. Crosier, and P. S. Crosier. 2013. 'Immunoresponsive gene 1 augments bactericidal activity of
482 macrophage-lineage cells by regulating beta-oxidation-dependent mitochondrial ROS
483 production', *Cell Metab*, 18: 265-78.
484 Halloran, M. C., M. Sato-Maeda, Jr. Warren, J. T., F. Su, Z. Lele, P. H. Krone, J. Y. Kuwada, and W. Shoji.
485 2000. 'Laser-induced gene expression in specific cells of transgenic zebrafish', *Development*.
486 Hartley, J. L., G. F. Temple, and M. A. Brasch. 2000. 'DNA cloning using in vitro site-specific
487 recombination', *Genome Res*, 10: 1788-95.
488 Hernandez, P. P., P. M. Strzelecka, E. I. Athanasiadis, D. Hall, A. F. Robalo, C. M. Collins, P. Boudinot, J.
489 P. Levraud, and A. Cvejic. 2018. 'Single-cell transcriptional analysis reveals ILC-like cells in
490 zebrafish', *Sci Immunol*, 3.
491 Hirano, M., R. Ando, S. Shimozono, M. Sugiyama, N. Takeda, H. Kurokawa, R. Deguchi, K. Endo, K. Haga,
492 R. Takai-Todaka, S. Inaura, Y. Matsumura, H. Hama, Y. Okada, T. Fujiwara, T. Morimoto, K.
493 Katayama, and A. Miyawaki. 2022. 'A highly photostable and bright green fluorescent protein',
494 *Nat Biotechnol*, 40: 1132-42.
495 Hoshijima, K., M. J. Jurynec, and D. J. Grunwald. 2016. 'Precise Editing of the Zebrafish Genome Made
496 Simple and Efficient', *Dev Cell*, 36: 654-67.
497 Howe, K., M. D. Clark, C. F. Torroja, J. Torrance, C. Berthelot, M. Muffato, J. E. Collins, S. Humphray, K.
498 McLaren, L. Matthews, S. McLaren, I. Sealy, M. Caccamo, C. Churcher, C. Scott, J. C. Barrett, R.
499 Koch, G. J. Rauch, S. White, W. Chow, B. Kilian, L. T. Quintais, J. A. Guerra-Assuncao, Y. Zhou,
500 Y. Gu, J. Yen, J. H. Vogel, T. Eyre, S. Redmond, R. Banerjee, J. Chi, B. Fu, E. Langley, S. F. Maguire,
501 G. K. Laird, D. Lloyd, E. Kenyon, S. Donaldson, H. Sehra, J. Almeida-King, J. Loveland, S.
502 Trevanion, M. Jones, M. Quail, D. Willey, A. Hunt, J. Burton, S. Sims, K. McLay, B. Plumb, J.
503 Davis, C. Clee, K. Oliver, R. Clark, C. Riddle, D. Elliot, G. Threadgold, G. Harden, D. Ware, S.
504 Begum, B. Mortimore, G. Kerry, P. Heath, B. Phillimore, A. Tracey, N. Corby, M. Dunn, C.
505 Johnson, J. Wood, S. Clark, S. Pelan, G. Griffiths, M. Smith, R. Glithero, P. Howden, N. Barker,
506 C. Lloyd, C. Stevens, J. Harley, K. Holt, G. Panagiotidis, J. Lovell, H. Beasley, C. Henderson, D.
507 Gordon, K. Auger, D. Wright, J. Collins, C. Raisen, L. Dyer, K. Leung, L. Robertson, K. Ambridge,
508 D. Leongamornlert, S. McGuire, R. Gilderthorp, C. Griffiths, D. Manthravadi, S. Nichol, G.
509 Barker, S. Whitehead, M. Kay, J. Brown, C. Murnane, E. Gray, M. Humphries, N. Sycamore, D.
510 Barker, D. Saunders, J. Wallis, A. Babbage, S. Hammond, M. Mashreghi-Mohammadi, L. Barr,
511 S. Martin, P. Wray, A. Ellington, N. Matthews, M. Ellwood, R. Woodmansey, G. Clark, J. Cooper,
512 A. Tromans, D. Grafham, C. Skuce, R. Pandian, R. Andrews, E. Harrison, A. Kimberley, J. Garnett,
513 N. Fosker, R. Hall, P. Garner, D. Kelly, C. Bird, S. Palmer, I. Gehring, A. Berger, C. M. Dooley, Z.
514 Ersan-Urun, C. Eser, H. Geiger, M. Geisler, L. Karotki, A. Kirn, J. Konantz, M. Konantz, M.
515 Oberlander, S. Rudolph-Geiger, M. Teucke, C. Lanz, G. Raddatz, K. Osoegawa, B. Zhu, A. Rapp,
516 S. Widaa, C. Langford, F. Yang, S. C. Schuster, N. P. Carter, J. Harrow, Z. Ning, J. Herrero, S. M.
517 Searle, A. Enright, R. Geisler, R. H. Plasterk, C. Lee, M. Westerfield, P. J. de Jong, L. I. Zon, J. H.
518 Postlethwait, C. Nusslein-Volhard, T. J. Hubbard, H. Roest Crollius, J. Rogers, and D. L. Stemple.
519 2013. 'The zebrafish reference genome sequence and its relationship to the human genome',
520 *Nature*, 496: 498-503.

521 Iwanami, N. 2014. 'Zebrafish as a model for understanding the evolution of the vertebrate immune
522 system and human primary immunodeficiency', *Exp Hematol*, 42: 697-706.
523 Jiang, Z., L. Huang, J. Zhao, Y. Li, J. Ma, R. Ni, Q. Yang, L. Luo, Y. Yang, and J. Chen. 2023. 'GoldenFish: a
524 rapid and efficient system to customize constructs for zebrafish transgenesis', *J Mol Cell Biol*,
525 14.
526 Katzen, F. 2007. 'Gateway((R)) recombinational cloning: a biological operating system', *Expert Opin*
527 *Drug Discov*, 2: 571-89.
528 Kawakami, K. 2007. 'Tol2: a versatile gene transfer vector in vertebrates', *Genome Biol*, 8 Suppl 1: S7.
529 Kawakami, K. , and A. Shima. 1999. 'Identification of the Tol2 transposase of the medaka fish Oryzias
530 latipes that catalyzes excision of a nonautonomous Tol2 element in zebrafish Danio rerio',
531 *Elsevier*.
532 Kawakami, K., A. Shima, and N. Kawakami. 2000. 'Identification of a functional transposase of the Tol2
533 element, an Ac-like element from the Japanese medaka fish, and its transposition in the
534 zebrafish germ lineage', *Proc NatlAcad Sci USA*.
535 Kawakami, K., H. Takeda, N. Kawakami, M. Kobayashi, N. Matsuda, and M. Mishina. 2004. 'A
536 transposon-mediated gene trap approach identifies developmentally regulated genes in
537 zebrafish', *Dev Cell*, 7: 133-44.
538 Kirchmaier, S., K. Lust, and J. Wittbrodt. 2013. 'Golden GATEway cloning--a combinatorial approach to
539 generate fusion and recombination constructs', *PLoS One*, 8: e76117.
540 Kucera, R. 2021. "Using PaqCI™ for Golden Gate Assembly – What Makes it a Special Addition to NEB's
541 DNA Assembly Portfolio?" In.
542 Kwan, K. M., E. Fujimoto, C. Grabher, B. D. Mangum, M. E. Hardy, D. S. Campbell, J. M. Parant, H. J.
543 Yost, J. P. Kanki, and C. B. Chien. 2007. 'The Tol2kit: a multisite gateway-based construction kit
544 for Tol2 transposon transgenesis constructs', *Dev Dyn*, 236: 3088-99.
545 Labbaf, Z., K. Petratou, L. Ermlich, W. Backer, K. Tarbashevich, M. Reichman-Fried, S. Luschnig, S.
546 Schulte-Merker, and E. Raz. 2022. 'A robust and tunable system for targeted cell ablation in
547 developing embryos', *Dev Cell*, 57: 2026-40 e5.
548 Langenau, D. M., A. A. Ferrando, D. Traver, J. L. Kutok, J.-P. D. Hezel, J. P. Kanki, L. I. Zon, A. T. Look,
549 and N. S: Trede. 2004. 'In vivo tracking of T cell development, ablation, and engraftment in
550 transgenic zebrafish', *PNAS*.

551 Liu, S., and S. D. Leach. 2011. 'Zebrafish models for cancer', *Annu Rev Pathol*, 6: 71-93.
552 Marillonnet, S., and R. Grutzner. 2020. 'Synthetic DNA Assembly Using Golden Gate Cloning and the
553 Hierarchical Modular Cloning Pipeline', *Curr Protoc Mol Biol*, 130: e115.
554 Meeker, N. D., and N. S. Trede. 2008. 'Immunology and zebrafish: spawning new models of human
555 disease', *Dev Comp Immunol*, 32: 745-57.
556 Mosimann, C., C. K. Kaufman, P. Li, E. K. Pugach, O. J. Tamplin, and L. I. Zon. 2011. 'Ubiquitous transgene
557 expression and Cre-based recombination driven by the ubiquitin promoter in zebrafish',
558 *Development*, 138: 169-77.
559 Padgett, K. A., and J. A. Sorge. 1996. 'Creating seamless junctions independent of restriction sites in
560 PCR cloning', *Gene*.
561 Pryor, J. M., V. Potapov, K. Bilotti, N. Pokhrel, and G. J. S. Lohman. 2022. 'Rapid 40 kb Genome
562 Construction from 52 Parts through Data-optimized Assembly Design', *ACS Synth Biol*, 11:
563 2036-42.
564 Russo, I., E. Sartor, L. Fagotto, A. Colombo, N. Tiso, and M. Alaibac. 2022. 'The Zebrafish model in
565 dermatology: an update for clinicians', *Discov Oncol*, 13: 48.
566 Santoriello, C., and L. I. Zon. 2012. 'Hooked! Modeling human disease in zebrafish', *J Clin Invest*, 122:
567 2337-43.
568 Shaner, N. C., R. E. Campbell, P. A. Steinbach, B. N. Giepmans, A. E. Palmer, and R. Y. Tsien. 2004.
569 'Improved monomeric red, orange and yellow fluorescent proteins derived from Discosoma
570 sp. red fluorescent protein', *Nat Biotechnol*, 22: 1567-72.

571 Shaner, N. C., G. G. Lambert, A. Chammas, Y. Ni, P. J. Cranfill, M. A. Baird, B. R. Sell, J. R. Allen, R. N.
572 Day, M. Israelsson, M. W. Davidson, and J. Wang. 2013. 'A bright monomeric green fluorescent
573 protein derived from Branchiostoma lanceolatum', *Nat Methods*, 10: 407-9.
574 Shimshek, D. R., J. Kim, M. R. Hubner, D. J. Spergel, F. Buchholz, E. Casanova, A. F. Stewart, P. H.
575 Seeburg, and R. Sprengel. 2002. 'Codon-improved Cre recombinase (iCre) expression in the
576 mouse', *Genesis*, 32: 19-26.
577 Shoji, W., C. S. Yee, and J. Y. Kuwada. 1998. 'Zebrafish Semaphorin Z1a collapses specific growth cones
578 and alters their pathway in vivo', *Development*.

579 Sleight, Sean C., Bryan A. Bartley, Jane A. Lieviant, and Herbert M. Sauro. 2010. 'In-Fusion BioBrick
580 assembly and re-engineering', *Nucleic Acids Research*, 38: 2624-36.
581 Sone, T., K. Yahata, Y. Sasaki, J. Hotta, H. Kishine, J. D. Chesnut, and F. Imamoto. 2008. 'Multi-gene
582 gateway clone design for expression of multiple heterologous genes in living cells: modular
583 construction of multiple cDNA expression elements using recombinant cloning', *J Biotechnol*,
584 136: 113-21.
585 Tamplin, O. J., E. M. Durand, L. A. Carr, S. J. Childs, E. J. Hagedorn, P. Li, A. D. Yzaguirre, N. A. Speck, and
586 L. I. Zon. 2015. 'Hematopoietic stem cell arrival triggers dynamic remodeling of the perivascular
587 niche', *Cell*, 160: 241-52.
588 Tang, D., F. Geng, C. Yu, and R. Zhang. 2021. 'Recent Application of Zebrafish Models in Atherosclerosis
589 Research', *Front Cell Dev Biol*, 9: 643697.
590 Varela, M., and A. H. Meijer. 2022. 'A fresh look at mycobacterial pathogenicity with the zebrafish host
591 model', *Mol Microbiol*, 117: 661-69.
592 Walton, E. M., M. R. Cronan, R. W. Beerman, and D. M. Tobin. 2015. 'The Macrophage-Specific
593 Promoter mfap4 Allows Live, Long-Term Analysis of Macrophage Behavior during
594 Mycobacterial Infection in Zebrafish', *PLoS One*, 10: e0138949.
595 Wattrus, S. J., and L. I. Zon. 2020. 'A Transgenic System for Rapid Magnetic Enrichment of Rare
596 Embryonic Cells', *Zebrafish*, 17: 354-57.
597 Zacharias, D. A., J. D. Violin, A. C. Newton, and R.Y . Tsien. 2002. 'Partitioning of Lipid-Modified
598 Monomeric GFPs into Membrane Microdomains of Live Cells', *Science*.
599 Zhu, B., G. Cai, E. O. Hall, and G. J. Freeman. 2007. 'In-fusion assembly: seamless engineering of
600 multidomain fusion proteins, modular vectors, and mutations', *Biotechniques*, 43: 354-9.