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Rapid BAC selection for *tol2*-mediated transgenesis in zebrafish

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SUMMARY

The generation of zebrafish transgenic lines that express specific fluorophores in a cell- or tissue-specific manner is an important technique that takes full advantage of the optical clarity of the embryo. Identifying promoter fragments that faithfully recapitulate endogenous expression patterns and levels is often difficult and using large genomic DNA fragments, such as bacterial artificial chromosomes (BACs), makes the process of transgenesis less reliable. Here we provide a detailed protocol that allows for BAC selection and subsequent rapid modification through recombineering in *Escherichia coli*, resulting in BACs that can be injected into zebrafish embryos and, aided by *tol2*-mediated transgenesis, reliably yield stable transgenic lines. A number of BACs can be prepared in parallel, and injection of the BACs containing CFP/YFP/RFP or Gal4 cassettes allows for immediate testing of whether a particular BAC will yield the desired result. Furthermore, since injected embryos often show widespread expression, recombineered BACs provide an alternative to two-color in situ hybridizations: BACs injected into embryos of a different transgenic reporter line thus enable in vivo colocalization studies. Using this protocol, we have generated 66 stable lines for 23 different genes, with an average transgenesis rate above 10%. Importantly, we provide evidence that BAC size shows no apparent correlation to the transgenesis rate achieved and that there are no severe position effects.

KEY WORDS: Imaging, Transgenesis, Zebrafish

INTRODUCTION

The transparency of the zebrafish embryo is one reason why this organism is among the mostly widely used experimental systems at present. Because of the small size of its embryos it is possible to visualize and analyze organs in their entirety, and also to follow single cell movements over time within an organ or the whole embryo. The paucity of antibodies in the zebrafish field, but more importantly the desire to perform in vivo imaging, have led to an ever increasing need to generate transgenic lines that allow an appreciation of the organ-specific, cell type-specific or gene-specific expression of a fluorescent reporter (Koster and Fraser, 2001). In many cases this has been accomplished by generating transgenic lines that express GFP or RFP under the control of a specific promoter (or promoter fragment), but more recently the use of the Gal4/UAS binary system has become increasingly popular (Asakawa et al., 2008; Distel et al., 2009) as it allows, in combination with different UAS lines, an increased flexibility to control the expression of various reporter cassettes from the same promoter.

A persistent problem in generating transgenic lines has been the identification of functional promoter fragments, i.e. the identification of those elements in the 5′ region of a gene of interest that, when placed in front of a GFP cassette, will fully recapitulate the expression of the endogenous gene. Often, only certain aspects of the endogenous expression pattern are represented (Zhang and Rodaway, 2007), and frequently it is not possible at all to obtain the transgene of choice because of technical limitations in cloning sufficiently large promoter fragments that fully encompass all the necessary regulatory elements. One way around this particular problem is the use of bacterial artificial chromosomes (BACs) (Yang et al., 2006; Suster et al., 2009a), which can contain up to 350 kb of genomic DNA, although there are technical challenges in dealing with these rather large fragments and it is not straightforward to efficiently generate transgenic lines when using BACs. As a consequence, many laboratories have avoided using BACs, even though the advantages of the approach are obvious and have been amply demonstrated in other systems, such as the mouse (Giraldo and Montoliu, 2001; Van Keuren et al., 2009).

Here, we present a method that allows the generation of zebrafish BACs, which, through recombineering, can be modified to contain a fluorophore of choice at any given position in the BAC (most commonly the first translated ATG of a particular gene). The BAC is also modified to contain the long terminal repeats (LTRs) of the Medaka Tol2 transposon (Kawakami, 2007; Suster et al., 2009b; Suster et al., 2009a), which enable single-copy integration of the BAC into the zebrafish genome when co-injected with mRNA encoding the Tol2 transposase into one-cell zygotes. The average transgenesis rate is higher than 10%, which is a considerable improvement over conventional methods using BAC DNA. We have generated 66 lines with 23 different BACs using this protocol. Importantly, in many cases the analysis of injected embryos is immediately informative, as even transient expression from injected BACs is widespread and faithfully recapitulates endogenous gene expression. This is not only of immediate use to the researcher and in many cases allows circumvention of two-color in situ hybridization, but also allows an informed and fast decision to be made as to whether the newly generated BAC will be useful in generating the desired transgenic line.

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MATERIALS AND METHODS

A step-by-step protocol with a summary of required materials, a guide to BAC clone identification and a trouble-shooting guide are available at http://www.hubrecht.eu/research/schulte-merker/protocols.html and we strongly recommend that these data are consulted in combination with the information provided below.

BAC recombineering overview

Various methods have been developed – collectively called recombineering – in which laboratory strains of *E. coli* [which are normally deficient in homology-directed repair (HDR)] are designed to inducibly express genes encoding the HDR machinery. In this protocol, we employed a commercially available plasmid-based recombineering setup (GeneBridges, Germany) in which an arabinose-inducible promoter regulates the expression of an operon of HDR genes. This setup depends on a single plasmid (pRedET), which means that HDR can be performed in bacteria that already contain the BAC, avoiding the need for BAC transformation into a specialized recombineering strain.

BAC DNA is stable using this system (see Fig. S1 in the supplementary material). Importantly, the efficiency of this recombineering setup is very high, which means small (2 ml) bacterial culture volumes can be used, allowing for processing of up to eight different constructs in parallel. The pRedET plasmid contains a temperature-sensitive origin of replication (SC101), which replicates at 30°C but not at 37°C. Therefore, after successful recombination, culturing at 37°C allows purification of BAC DNA without pRedET contamination.

The recombineering procedure we employed consists of three steps. First, BAC clones were transformed with the pRedET plasmid. Then, two Tol2 LTRs in opposing directions flanking an ampicillin resistance cassette (iTol2_amp) were inserted into the BAC vector backbone (Suster et al., 2009b). Finally, a reporter gene together with a kanamycin resistance cassette was inserted at the start ATG of the gene of interest. We have generated template plasmids encoding ten reporters: three different, bright and fast-maturing fluorophores [mCerulean (Rizzo et al., 2004)], Citrine (Griesbeck et al., 2001) and mCherry (Shaner et al., 2004), with cytoplasmic, nuclear or membrane localization. In addition, we cloned the transcriptional activator *Gal4FF* (Asakawa et al., 2008) for use with Gal4/UAS binary expression systems (see Fig. S2 in the supplementary material).

Isolated BAC DNA was co-injected with *tol2* transposase mRNA to create first transient and, subsequently, stable transgenic zebrafish lines. Transient expression already gave a very reliable indication of the final expression of the stable transgenic reporter line. Therefore, within 12-14 days after the start of the procedure, an estimate could be made of the usefulness of the transgenic reporter for visualizing the organ, cell or protein of interest. A key step was the selection for raising of at least 20 embryos that show high and widespread expression of the injected reporter BAC.

This protocol has been optimized for the parallel preparation of multiple (up to eight) BAC constructs. In principle, the recombineering pipeline could be scaled up further, as, for example, described by Sarov et al. (Sarov et al., 2006). However, the hands-on time spent on embryo injection, founder identification and analysis will ultimately limit the number of transgenic lines that can be generated by a single person (we have had success with one person being responsible for preparing the BAC constructs, which were then handed over to the individual requester).

BAC recombineering method

In silico BAC clone identification

Clone identification was performed using Ensembl (http://www.ensembl.org) or the UCSC genome browser (http://genome.ucsc.edu). Various established BAC libraries exist that contain zebrafish genomic DNA of 35 kb to 250 kb. The BAC end sequences of these libraries have been aligned to the zebrafish reference genome ('BAC end mapping') and these maps were used to identify clones that were likely to contain the gene(s) of interest. BAC clones were ordered from BACPAC resources (Oakland, CA, USA) or ImaGenes (Berlin, Germany). A step-by-step example of BAC clone identification can be found at http://www.hubrecht.eu/research/schulte-merker/protocols.html.

Primer design

Standard primers of ~70 nucleotides were used for targeting an iTol2_amp cassette to the BAC vector backbone; the choice of specific primers for this step depends on the sequence of the BAC vector (either pTarBAC derived or pIndigoBAC derived). Primer sequences for this step, including standard control primers to confirm correct recombination, are provided in Table S1 in the supplementary material.

Gene-specific primers were designed that contain 50 nucleotide homology arms around the start ATG of the gene of interest (positions –53 to –4 and +4 to +53) with ~20 nucleotide ends to amplify an XFP_kan/Gal4FF_kan cassette. Gene-specific primers and control primers were designed to amplify a 150-400 bp fragment upon correct recombination. Standard control primers within the XFP_kan/Gal4FF_kan cassettes are provided in Table S1 in the supplementary material.

A step-by-step example of gene-specific primer design can be found at http://www.hubrecht.eu/research/schulte-merker/protocols.html.

Generation of targeting PCR products

High-fidelity PCR reactions (100 μl) were set up to generate targeting PCR products (templates for both targeting steps were generated at the same time): 1 μl 5 ng/μl plasmid template, 20 μl 5× Phusion HF Buffer (NEB), 2 μl 10 mM dNTPs, 2.5 μl each of 10 μM (pTarBAC/pIndigoBAC)_HA1_iTol2_fw and (pTarBAC/pIndigoBAC)_HA2_iTol2_rev recombineering primers (first targeting) or GeneX_HA1_(XFP or Gal4)_fw and GeneX_HA2_kanR_rev recombineering primers (second targeting), 1 μl Phusion DNA polymerase (NEB) and 71 μl double-distilled (dd) H_2O. The following PCR conditions were used: initial denaturation at 98°C for 30 seconds; then 30 cycles of 98°C for 10 seconds, 58°C for 20 seconds, 72°C for 60 seconds; with a final extension at 72°C for 5 minutes.

Correct product sizes were confirmed on agarose gels. Then 2 μl *Dpn*I (20 units) were added directly to the PCR reaction and incubated for 1 hour at 37°C. PCR products were precipitated by adding 5 μl 5 M LiCl and 300 μl 100% ethanol, incubated at –20°C for 30 minutes and subjected to centrifugation in a cooled bench-top centrifuge at maximal speed (13,000 rpm, 16,000 ***g***) for 30 minutes at 4°C. The supernatant was discarded and the pellet washed with 70% ethanol. After 5 minutes centrifugation at maximal speed, the supernatant was discarded and the pellet dried at room temperature for 5-10 minutes. The pellets were resuspended in 21 μl ddH_2O and 1 μl was used to measure the DNA concentration; ddH_2O was then added to a final concentration of 500 ng/μl.

BAC confirmation and pRedET transformation

Ordered BACs were streaked on an LB agar plate containing 11.3 μg/ml chloramphenicol (Cm) and incubated overnight (o/n) at 37°C to obtain single colonies. One to three single colonies per BAC were picked and resuspended in 50 μl ddH_2O. Then 1 μl was used for colony PCR to identify BACs containing the targeting sites and to test the control primers. Multiple PCRs were set up in 96-well PCR plates by combining, per well, 1 μl suspended bacterial colony, 2 μl 10× PCR buffer, 0.4 μl 10 mM dNTPs, 0.4 μl each of 10 μM GeneX_HA1_control_fw and GeneX_HA2_control_rev primers, 0.1 μl Taq polymerase and 13.7 μl ddH_2O. PCR was performed as: initial denaturation at 95°C for 5 minutes; 30 cycles of 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 30 seconds; with a final extension at 72°C for 5 minutes. Orange G loading dye (2.5 μl) was added to the PCR reactions and product sizes analyzed on a 2% agarose gel. If product sizes were as predicted, 20 μl of the remaining bacterial colony suspension were added to 1 ml of LB medium containing Cm and cultured o/n at 37°C.

A glycerol stock was generated by adding 500 μl 50% glycerol to 500 μl of the o/n culture for storage at –80°C. Forty microliters of the o/n culture were transferred to 2 ml of fresh LB medium containing Cm and cultured for 3 hours at 37°C. Cultures were transferred to a 2 ml microcentrifuge tube and centrifuged at 5000 rpm (2300 ***g***) for 5 minutes. The supernatant was carefully removed and 1 ml of ice-cold ddH_2O was added. Bacteria were resuspended by pipetting up and down twice. The bacteria were then recentrifuged at 5000 rpm for 5 minutes and the pellet resuspended in 1 ml of ice-cold ddH_2O. After a final centrifugation, the supernatant was mostly removed, leaving ~50 μl in the tube. Then, 1 μl of

10 ng/µl pRedET plasmid was added and transferred to a prechilled 1-mm electroporation cuvette on ice (the transfer also served to mix and resuspend the bacterial pellet). Bacteria were electroporated using standard *E. coli* settings (1800 V, 25 µF, 200 Ω, time constant of 4.8-5.2 mseconds), and, immediately after electroporation, 1 ml of LB medium without antibiotics and containing 20 mM D-glucose was added. Bacteria were transferred to a fresh culture tube and cultured at 37°C for 60 minutes. Then, 100 µl of the cells were plated on LB plates containing Cm and 3.3 µg/ml tetracycline (Tet) and incubated at 30°C o/n. It could take up to 24 hours before colonies became visible (>0.5 mm in diameter). A single colony was picked and transferred to fresh LB medium containing Cm and Tet and incubated at 30°C o/n (maximum 16 hours).

Insertion of iTol2_amp into the vector backbone

Five-hundred microliters of the o/n culture were used to generate a glycerol stock (as above), and 150 µl of the o/n culture were transferred to a culture tube containing 1.8 ml of fresh LB medium with Cm and Tet and cultured for 2.5 hours at 30°C. Sixty-seven microliters of 10% (w/v) L-(+)-arabinose were added to induce the expression of the HDR genes, and culturing was continued for 1 hour at 37°C. The culture was transferred to a 2-ml centrifuge tube and processed for electroporation by washing twice in ice-cold ddH_2O in the same way as for the pRedET transformation. Before transferring to the electroporation cuvette, 1 µl of 500 ng/µl iTol2_amp targeting PCR product was added. After electroporation, bacteria were incubated in LB medium without antibiotics but containing 20 mM D-(+)-glucose for 45 minutes at 37°C. Bacteria were plated on LB plates containing Cm, Tet and 16.7 µg/ml ampicillin (Amp) and incubated at 30°C o/n. It could take up to 24 hours before colonies were visible (>0.5 mm).

Correct iTol2_amp insertions were identified by colony PCR using the same protocol as described above for the BAC clone confirmation. Two to six colonies were picked per BAC (>95% had correct recombination). Primers used were pTarBAC/pIndigoBAC_HA1_control_fw and amp_HA1_control_rev for the left homology arm and pTarBAC/pIndigoBAC_HA2_control_rev and amp_HA2_control_fw for the right homology arm. Product sizes were analyzed on a 2% agarose gel and only colonies in which both arms were correctly integrated were used. Products should be of the following sizes: pTarBAC_HA1_control_fw + amp_HA1_control_rev, 405 bp; pTarBAC_HA2_control_rev + amp_HA2_control_fw, 317 bp; pIndigoBAC_HA1_control_fw + amp_HA1_control_rev, 420 bp; pIndigoBAC_HA2_control_rev + amp_HA2_control_fw, 327 bp.

Twenty microliters of the remaining bacterial colony suspension were added to 1 ml of LB medium containing Cm, Tet and Amp and cultured o/n at 30°C (maximum 16 hours).

Insertion of XFP_kan or Gal4FF_kan at the start ATG of the gene of interest

Five-hundred microliters of the o/n culture were used to generate a glycerol stock (as above), and 150 µl of the o/n culture were transferred to a culture tube containing 1.8 ml of fresh LB medium with Cm, Tet and Amp and cultured for 2.5 hours at 30°C. Sixty-seven microliters of 10% (w/v) L-(+)-arabinose was added to induce the expression of the HDR genes, and the culture was continued for 1 hour at 37°C. The culture was transferred to a 2-ml centrifuge tube and processed for electroporation by washing twice in ice-cold ddH_2O in the same way as for the pRedET and iTol2_amp transformation. Before transfer to the electroporation cuvette, 1 µl of 500 ng/µl XFP_kan/Gal4FF_kan targeting PCR product was added. After electroporation, bacteria were incubated in LB medium without antibiotics and containing 20 mM D-(+)-glucose for 75 minutes at 37°C. Bacteria were plated on LB plates containing Cm, Amp and 16.7 µg/ml kanamycin (Kan) and incubated at 37°C o/n.

Correct XFP_kan/Gal4FF_kan insertions were identified by colony PCR using the same protocol as described above for the BAC clone confirmation. Two to six colonies were picked per BAC (>95% are expected to have correct recombination). Primers used were GeneX_HA1_control_fw and CFP/YFP/RFP/Gal4FF_HA1_control_rev for the left homology arm and CFP/YFP/RFP/Gal4FF_HA2_control_fw and GeneX_HA2_control_rev for the right homology arm. Products sizes were analyzed on a 2% agarose gel and only colonies in which both arms were correctly integrated were used. Product size depended on the relative positions of the control primers to the homology arms. Twenty microliters of the remaining bacterial colony suspension were added to 1 ml of LB medium containing Cm, Amp and Kan and cultured o/n at 37°C (maximum 16 hours).

BAC DNA preparation

Five-hundred microliters of the o/n culture were used to generate a glycerol stock (as above) and the remaining culture was used to inoculate 100 ml of LB medium containing Cm, Amp and Kan. The BAC DNA preparation was based on the HiPure Midiprep kit (Invitrogen), with modifications for BAC DNA isolation as described by the manufacturer. Precipitated DNA

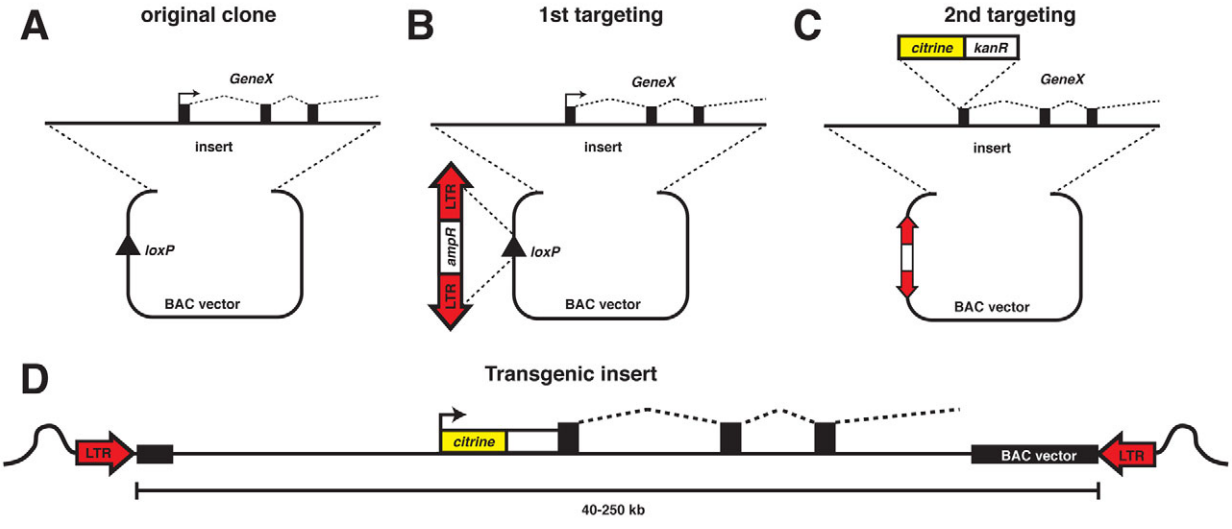

Fig. 1. Schematic representation of the BAC recombineering steps. (**A**,**B**) The unmodified BAC clone (A) is first modified with a PCR product containing inverted Tol2 LTRs (red arrows) and an ampicillin resistance gene (*ampR*), in the process deleting a loxP site from the vector backbone (B). (**C**) In the second recombineering step, a cassette containing a reporter gene (in this case *citrine*, yellow) and a kanamycin resistance gene (*kanR*) is inserted at the start codon of the gene of interest. (**D**) After *tol2*-mediated transgenesis, this results in the insertion of a 40-250 kb transgene into the zebrafish genome. The BAC vector sequences and Tol2 LTRs occupy the extremes of the transgenic inserts. The first and second targeting steps may be interchanged, but introducing the Tol2 LTRs first allows one to generate different reporter transgenes using the same BAC.

Table 1. Transgenesis rates and copy number estimations for selected BAC transgenes

Gene	BAC clone	Reporter	Adults screened	Founders identified	Copy number	Pattern	Insert (kb)
cxcl12b	CH73-291M8	YFP	26	2	1	Yes	97
entpd5	CH73-213B8	YFP	19	4	1	Yes	82
cathepsin K	CH73-114M20	YFP	13	2	2	Yes	78
cdh5	CH73-357K2	YFP	8	3	1	Yes	163
cyp26b1	DKEY-53O14	YFP	1	1	1	Yes	171
arid1b	CH73-334P10	YFP	18	2	1	Yes	104
collI	CH73- 184B14	RFP	46	8	1-2	Yes/No*	103
sox9b	CH1073-175H21	YFP	9	1	1	Yes	35
prox1	CH211-174N9	YFP	25	3	1	Yes	161
prox1b	CH73-247L15	YFP	10	3	1	Yes	116
Total			175	29			

Examples of ten different BAC transgenesis constructs. Adults screened refers to the number of adult putative founders screened by outcrossing. The copy number was estimated from the F1 transmission rate. Pattern refers to correlation of the reporter expression pattern with that of the endogenous gene (the asterisk indicates the one line that was an exception in this regard, see main text). Not all transgenic lines displayed in Fig. 2 are also listed here, where only those ten lines for which a complete set of parameters is known are shown.

was resuspended in 101 µl and 1 µl was used to measure the DNA concentration. ddH_2O was added to a final concentration of 200 ng/µl and DNA was stored at 4°C for up to 2 weeks.

Generation of transgenic lines

Zebrafish maintenance and transgenesis were performed in accordance with the animal research guidelines of KNAW.

tol2 transposase mRNA was prepared by in vitro transcription from *Xba*I-linearized *pDB600* (Balciunas et al., 2006) using the T3 mMessage mMachine kit (Ambion). RNA was purified using the RNeasy RNA purification kit (Qiagen), diluted to a final concentration of 100 ng/µl and divided into 5 µl aliquots and stored at –80°C.

Fish were set up on the day before injections. For Gal4FF reporter constructs, we used *Tg(UAS:GFP)^{nkuasgfp1a}*. On the morning of the injections, a 5 µl aliquot of BAC DNA (200 ng/µl) was prepared and a 5 µl aliquot of *tol2* transposase mRNA (100 ng/µl) was thawed on ice. Zebrafish eggs were collected and, immediately prior to injection, BAC DNA and *tol2* transposase mRNA were mixed and loaded into an injection needle. The tip was broken and 2 nl injected per embryo into the cytoplasm of 200 embryos at the 1- to 2-cell stage. A relatively large needle tip diameter and short injection pulses were used to limit shearing of the BAC DNA. Embryos were screened for malformations 8 and 24 hours after fertilization (hpf) and malformed or unfertilized embryos were discarded. Optimal transgenesis results were obtained when 30-50% of embryos were malformed at 24 hpf.

Embryos were screened for mosaic fluorescence in the expected tissues at 1-5 days post-fertilization (dpf) and the 10% first-tier (brightest) (n=10-20) and 40% second-tier (n=40-60) embryos were selected. These two batches were raised to adulthood.

Germline transmission was identified by mating sexually mature adult fish to wild-type fish and examining their progeny for fluorescence. Positive embryos were raised to adulthood and the line was maintained (and in some cases converted to a single-copy line) by outcrossing.

In situ hybridization

In situ hybridization was performed as previously described (Schulte-Merker, 2002; Bussmann et al., 2007). RNA probes used were for *cdh5* (Larson et al., 2004) and *cxcl12b* (Miyasaka et al., 2007). The antisense *gfp/yfp* probe was generated for 720 nucleotides of *yfp* (*citrine*) coding sequence, which has greater than 99% nucleotide sequence identity to *gfp* (*egfp*) and therefore could be used to detect both mRNAs.

RESULTS

We developed a protocol that is based on a recombination event that introduces an iTol2 cassette into a BAC of choice. The BAC was then further modified (Fig. 1), as described in detail in the Materials and methods. The recombineering and transgenesis pipeline outlined in this protocol was tested and successfully used in the generation of 66 transgenic lines in two different laboratories, with transgenesis efficiencies ranging between 5% and 100% (founders identified out of total adult fish screened). Table 1 shows data for those 29 transgenic lines for which we have a complete dataset available, including copy numbers. To date, all BACs generated have been successfully converted into stable transgenic lines. Although it is mostly single-copy insertions that are recovered [in marked contrast to plasmid-based transgenesis regimes (Stuart et al., 1988; Winkler et al., 1991)], in some cases two independent copies were found in single F1 fish (Table 1), which required additional out-crossing to obtain fish with single-copy insertions. Transgenesis efficiency was found to be independent of BAC insert size (Fig. 2) in the size ranges of the currently available BAC libraries (up to 200 kb). This effectively allows for greater flexibility in choosing which BAC can be used for generating a transgenic line. There was a strong correlation between known expression patterns and the expression of the respective reporters (Fig. 3A; see Fig. S3 in the supplementary material; see also below).

In contrast to smaller, promoter-based constructs, we found BAC transgenes to be highly resistant to position effects. Quantitative differences in fluorescence intensity between individual insertions of the same BAC were observed, however, potentially reflecting the chromatin status of the insertion sites. Only in one case among the 29 transgenic founders were qualitative differences in expression detected: here, the transgene showed only some of the expression domains that were expected based on prior knowledge of the endogenous mRNA expression pattern. However, seven

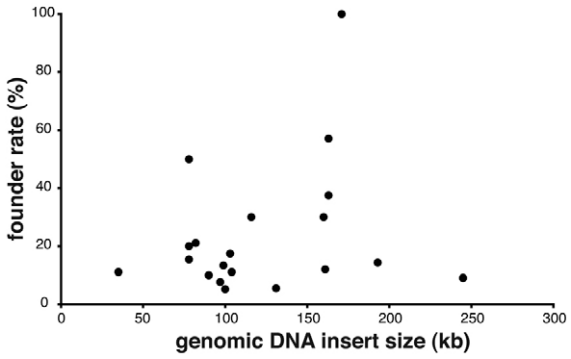

Fig. 2. BAC insert size does not affect transgenesis rate. Founder rates (percentage of germline founders among total adult fish screened) for constructs of different sizes (35-250 kb) indicate size independence of *tol2*-mediated BAC transgenesis within the typical size range of zebrafish BAC libraries.

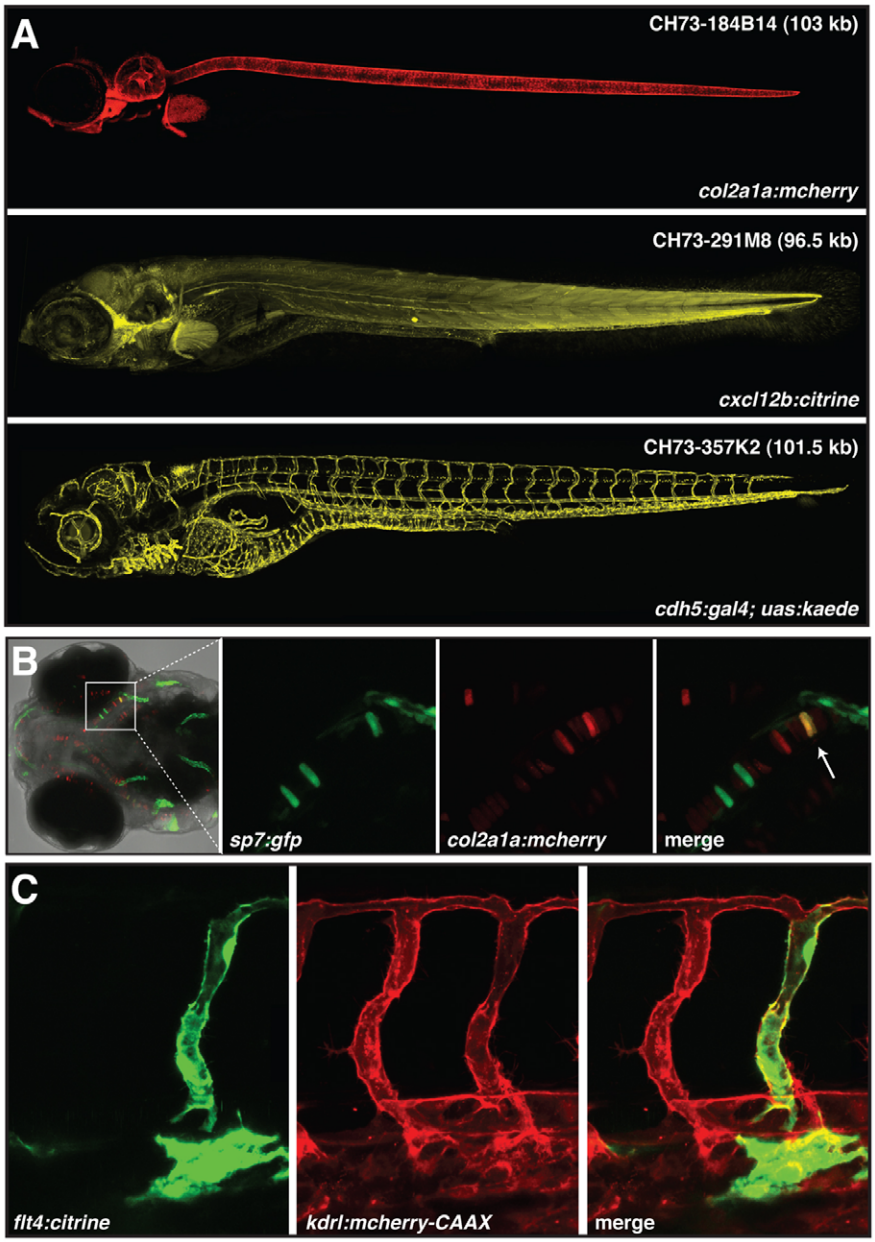

Fig. 3. Colocalization studies using recombineered BAC vectors. (**A**) Examples of stable, single-copy transgenic lines obtained using the described protocol. Lateral views of whole zebrafish embryos at 4-7 dpf. BAC clone IDs and predicted zebrafish genomic DNA insert sizes are indicated in the top right corner. (**B**) Dorsal view of the head of an *sp7:gfp* (Spoorendonk et al., 2008) transgenic zebrafish embryo at 5 dpf injected with *col2a1a:mCherry* BAC DNA and *tol2* transposase mRNA. Note the colocalization of GFP and mCherry in at least one cell (arrow). (**C**). Lateral view of the trunk of a *kdrl:mCherry-CAAX* (Hogan et al., 2009) transgenic embryo at 48 hpf injected with a *flt4:yfp* (*citrine*) BAC and *tol2* transposase mRNA. Individual YFP$^+$ endothelial cells (yellow) are detectable within the posterior caudal vein and intersegmental vessels.

other lines using the same (*col2a1a*) BAC showed identical patterns that were in full agreement with the in situ hybridization data. Since the transgenesis rate is high, we routinely aim to identify two or three independent founder lines in order to then isolate the strongest expression line.

DISCUSSION

In an attempt to establish a method that allows for scalable generation of recombineered BACs and transgenic lines, we have implemented a procedure comprising three distinct main phases: (1) in silico design of the experiment and selection of an appropriate BAC (see http://www.hubrecht.eu/research/schulte-merker/protocols.html); (2) BAC recombineering (Fig. 1); and (3) the actual generation of the transgenic line. In using this method, we were particularly interested in addressing whether there is a negative correlation between BAC size and the rate of transgenesis, whether the recombineered BACs show sufficiently widespread expression to make them immediately useful for transient experiments (without the need to generate a transgenic line), and whether position effects are observable among different lines generated from the same BAC.

In previous experiments (Spoorendonk et al., 2008; Hogan et al., 2009), we have used I-SceI-mediated transgenesis (Grabher et al., 2004; Kimura et al., 2006) to perform BAC integration. In the limited number of BACs tested, we obtained maximal founder rates of 5%, but also failed to identify founders in some cases. Therefore, the *tol2* system appears to be more robust in terms of transgenesis efficiency. We presume that the use of the *tol2* system in conjunction with BACs results in early integration of BAC DNA into the fish genome, which in turn leads to a high number of embryos exhibiting an informative GFP or RFP expression pattern. This is of particular importance as it allows for immediate use of the recombineered BACs. As exemplified in Fig. 3B,C, the injection of a BAC into embryos that are transgenic for a different reporter, with a different color of fluorescence, enables colocalization studies in vivo with single-cell resolution. This is an important aspect because other

available technologies used for colocalization studies are often technically challenging: the blue BCIP/NBT precipitate masks the red precipitate in those cases in which Fast Red is used, and if two different transcripts are to be detected in the same cell this poses a detection problem. Fluorescent in situ hybridization is an alternative here (Hauptmann and Gerster, 1994; Schulte-Merker, 2002; Bussmann et al., 2011), but this again poses technical problems. Furthermore, if late embryonic or larval stages are to be analyzed, and if the tissue in question is difficult for probes and antibodies to access (as is the case for cartilage and bone), in situ hybridizations are extremely difficult. In such cases, we have found BAC injection into an existing transgenic line to be a reliable alternative (Fig. 3B).

Although we have exclusively described the generation of zebrafish transcriptional reporter transgenic lines, with minor modifications many additional applications are possible. One example is the generation of C-terminal fusion proteins, which can be generated by changing the location of the homology arms from the 5′- to the 3′-terminus of the coding sequence of the gene of interest. Another example is the generation of BAC transgenes in the final stages of a positional cloning project. Here, a tiling path of BAC transgenes (modified with the iTol2_amp cassette and a promoter-reporter transgene in the BAC vector backbone to select for transgenic founders) can be generated. If injected into eggs from the mutant strain in question, within one generation founders can be identified that are heterozygous for the causative mutation as well as being carriers of a specific transgene; these individuals may then be used for complementation crosses, eventually identifying the BAC that contains the wild-type copy of the mutated gene. We have used this approach twice and were successful in both cases (J.B. and S.S.-M., unpublished).

In summary, the method described here provides a scalable procedure for generating recombineered BACs that give high transgenesis rates when used in conjunction with *tol2* mRNA injection and independently of BAC size. Furthermore, we have observed position effects in only one case, and demonstrate here that BACs can be of immediate use for in vivo colocalization studies. Finally, this BAC transgenesis system could be extended to all vertebrate model systems in which *tol2* transgenesis has been shown to function efficiently and for which well-annotated BAC libraries are available, such as *Xenopus*.

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Competing interests statement
The authors declare no competing financial interests.

Author contributions
J.B. designed the work and carried out and analyzed experiments. J.B. and S.S.-M. wrote the manuscript. S.S.-M. directed the work.

Supplementary material
Supplementary material for this article is available at
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