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Recombineering of BAC DNA for the Generation of Transgenic Mice

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1. Introduction

Bacterial Artificial Chromosomes (BAC) are low copy plasmids that stably maintain genomic DNA sequences hundreds of kilobases (Kb) in length. Thus, BAC plasmids usually contain the entire locus of one or more genes, enabling their use for genetic engineering and for the creation of genomic libraries for large-scale gene sequencing projects.

The use of BAC plasmids for transgenic gene expression is also gaining popularity over traditional proximal promoter driven transgene expression because the BAC typically contain most, if not all, of the important regulatory elements required to recapitulate endogenous gene expression (Giraldo and Montoliu 2001). Maintaining the coding sequence within its regulatory locus insulates the transgene from integration position dependent effects on expression enforced by nearby enhancers and heterochromatin (Wilson, Bellen et al. 1990).

Harnessing the power of BAC plasmids requires developing and optimizing methods for manipulation of the gene loci within the BAC, generally referred to as "Recombineering". This chapter will discuss the use of such technology to modify BAC DNA, specifically for the introduction of a fluorescent reporter to mark the expression of a gene of interest. We will also discuss the characterization of BAC transgenic mice and their experimental utility.

2. Overview of recombineering technology

Traditional cloning approaches rely on the presence of unique restriction enzyme sites for modification of plasmid DNA via a series of digestions and ligations to incorporate or remove desired DNA sequences. Unfortunately, most restriction enzyme sequences are not unique or conveniently located within the genomic sequence. Thus, the availability/use of restriction sites is often a limiting factor when attempting to modify plasmid DNA using such approaches.

Recombineering technology achieves DNA modification using a phage homologous recombination system, which uses linear DNA as template. Thus, an investigator can use linear targeting vectors containing 5' and 3' arms with homology to a target locus to introduce new DNA sequence. Subtle changes can now be achieved, including single point

mutations. Since the modification to the locus is based solely on sequence present in the target and not restriction enzymes, DNA can be introduced to the target wherever needed. Thus, recombineering technology has opened up an unlimited number of possibilities for genetic modifications.

Prior to the use of lambda phage in Recombineering, the study of homologous recombination in *E. coli* laid the groundwork for the use of this technology. Homologous recombination via linear DNA is suppressed in *E. coli* by the recBCD enzyme complex. In the recBCD model, the enzyme complex moves destructively along double strand breaks. The recB and recC subunits operate as helicases while recD operates as an exonuclease. Thus, in recBCD wild type *E. coli* strains, recombination does not occur through linear DNA, as reviewed elsewhere (Myers and Stahl 1994; Yeeles and Dillingham 2010).

The recBCD complex moves destructively along linear DNA until it encounters a DNA motif called a chi site. The chi site motif is a “recombination hotspot” that facilitates homologous recombination by ejecting the recD subunit responsible for the exonuclease activity of the complex, but does not affect the helicase activity. The helicase activity results in single strand DNA that serves as substrate for homologous recombination. This molecular reaction was exploited by incorporation of chi sites into targeting vectors to enable homologous recombination of target genes.

Cloning by homologous recombination was also studied in recBCD deficient strains. These experiments used recBCD mutants in an attempt to modify bacterial chromosomal and plasmid DNA with a linear DNA targeting construct (Jasin and Schimmel 1984; Oliner, Kinzler et al. 1993). The success of these studies demonstrated the effectiveness of cloning by homologous recombination, but was dependent on the use of specialized bacterial strains with constitutively active recombination enzymes. This enzyme activity resulted in unwanted intramolecular rearrangements in the modified plasmid (Copeland, Jenkins et al. 2001). Therefore, the recBCD mutant strains were limited in their use in cloning by homologous recombination.

The Recombineering technology used today is based on the lambda phage Red double strand break repair system, which uses the phage proteins *exo*, *bet*, and *gam*. This system is initiated when the 5' to 3' exonuclease *exo* digests linear double stranded (ds) DNA leaving a 3' overhang of single stranded (ss) DNA. The resultant 3' ssDNA is coated by the *bet* protein, which facilitates its annealing to a complementary strand of DNA. Once the homologous DNA is annealed, the 3'OH becomes a priming site for DNA replication resulting in double strand break repair.

The activities of *exo*, *bet*, and *gam* have been adapted for BAC cloning by homologous recombination. The dsDNA substrate for *exo* is a linear targeting construct with 5' and 3' homology to the target locus. The linear targeting construct can be generated by PCR or excised from a plasmid. The resultant 3' ssDNA is coated by the *bet* protein and facilitates the annealing of the 3' ssDNA of the targeting construct to the targeted sequence on the BAC containing the gene locus. The linear targeting construct is unaffected by recBCD activity due to the presence of the *gam* protein, which inhibits recBCD binding to the dsDNA targeting construct (Murphy 2007).

Studies that employed the introduction of lambda phage Red double strand break repair into *E. coli* demonstrated that it was an efficient system for cloning by homologous

recombination (Murphy 1998). Other studies demonstrated the use of homology arms as short as 27nt in length facilitated cloning by homologous recombination, with increasing efficiency with increased homology length. This study also investigated insert length between homologous arms and found it useful for inserts from 0-3100bp in length (Zhang, Buchholz et al. 1998). It was with these findings that lambda phage Red double strand break repair was adopted for cloning by homologous recombination.

3. Recombineering materials for BAC modification

There are currently two different resources for phage based Recombineering tools, Genebridges and NCI Frederick Systems. The major differences between the systems are how the phage genes are introduced into bacteria and what types of gene modifications they are capable of.

3.1 Genebridges plasmid based system

Genebridges (<http://www.genebridges.com/>) has adopted a plasmid based system to introduce the phage recombination proteins under a pBAD promoter (Noll, Hampp et al. 2009). In this system, expression of the lambda phage genes is repressed by araC dimer. Gene expression is induced when the araC dimer is released from the pBAD promoter in the presence of L-arabinose. This system introduces the recombineering apparatus into the E. coli strain containing the BAC and does not require specialized bacterial strains that endogenously express the recombination proteins.

3.2 NCI Frederick bacterial based system

The National Cancer Institute [http:// web.ncifcrf.gov/ research/ brb/ recombineering Information.aspx](http://web.ncifcrf.gov/research/brb/recombineeringInformation.aspx) offers E. coli strains that contain the phage recombination proteins stably integrated into the genome. The proteins are under transcriptional control of the λ PI promoter in concert with the temperature sensitive cl857 repressor. Transcription of the lambda phage proteins is repressed at 32°C. Repression of the λ PI promoter is released by incubating cells at 42°C for 15 minutes. Placing the stably integrated phage proteins under tight transcriptional control circumvents the problems of unwanted recombination associated with constitutive expression (Warming, Costantino et al. 2005). NCI offers strains with an L-arabinose inducible Cre or Flpe expression, which are useful for excision of the selectable antibiotic markers used for positive selection of recombinants.

In addition, NCI has developed plasmids to facilitate the cloning of sequence from BAC DNA into high copy plasmids for downstream use as targeting vectors (Liu, Jenkins et al. 2003). Plasmids for epitope tagging new proteins of interest have also been developed, allowing the investigator to localize and purify proteins. This application speeds the functional characterization of proteins by eliminating the lengthy process of generating antibodies that would serve similar functions (Poser, Sarov et al. 2008).

4. Recombineering methods and applications for BAC modification

In this section, we will discuss the protocols and approaches used, in conjunction with NCI recombineering E. coli strains, for the modification of a BAC plasmid, which was used for the creation of a new mouse reporter line.

4.1 Experimental design

In our studies, we were interested in generating a reporter mouse line to monitor the expression of Smooth Muscle- α -Actin (SMA) *in vivo* and track the fate of cells expressing this gene. Transcriptional control of the SMA locus had been well defined in previous studies (Mack and Owens 1999; Mack, Thompson et al. 2000). Using that information, we decided to replace exon 2 (the first coding exon of SMA) and 50bp distal of the 3' end of exon 2, with a myristoylated mCherry fluorescent reporter (Shaner, Campbell et al. 2004). Our rationale for this design was to a) insert the reporter in a manner where the first codon of the reporter replaced the first codon of SMA, and b) to avoid generating a fusion protein by removing the exon 2 splice site donor (Fig. 1a).

Our targeting construct consisted of a mCherry expression sequence with a myristoylation sequence upstream of a PGK-neomycin resistance cassette (Fig. 1a). The PGK-neomycin resistance cassette was flanked at the 5' and 3' end by FRT recombination sites. Inclusion of the PGK-neomycin resistance cassette facilitated selection of positive recombinant clones by kanamycin (Fig. 1b). Inclusion of the FRT recombination sites allowed for subsequent excision of the PGK-neomycin resistance cassette by Flpe recombinase, thus eliminating any unwanted transcription effects of the PGK promoter on the reporter (Fig. 1c). The targeting construct ended with a SV40 polyadenylation sequence to enable efficient transcription (Fig. 1a).

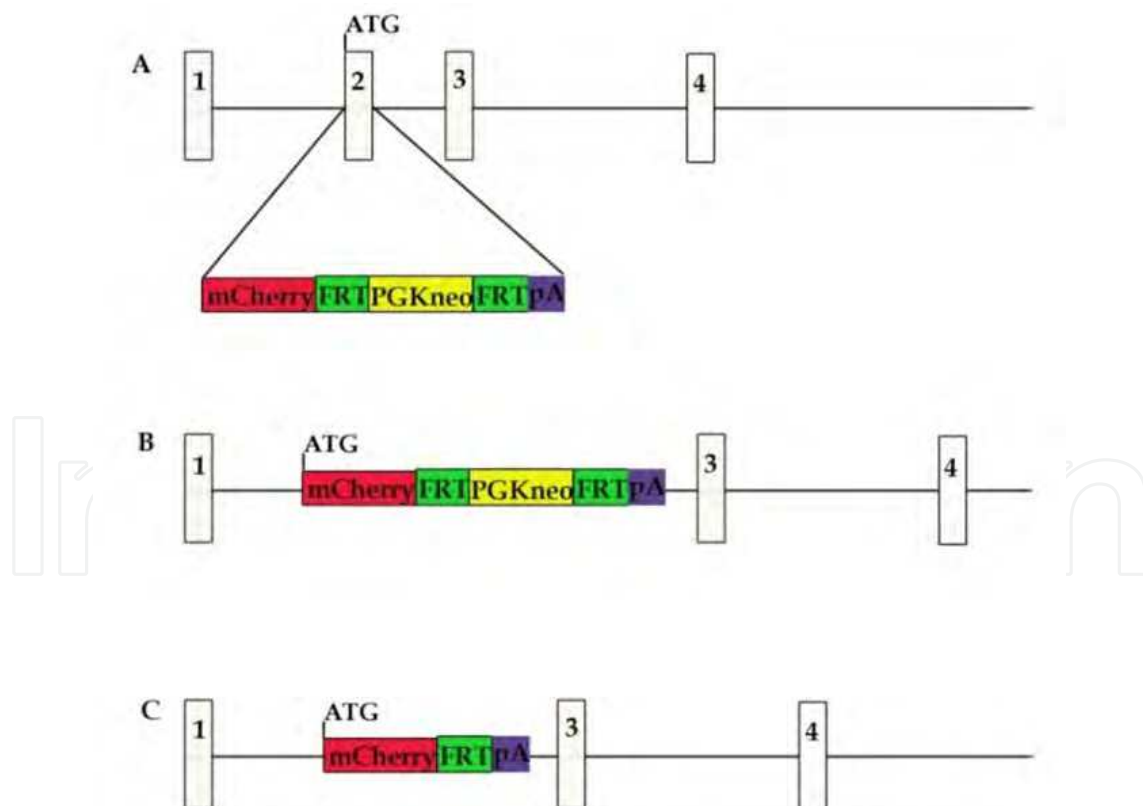


Fig. 1. Experimental design for the generation of a Smooth-Muscle- α -Actin (SMA) reporter construct. (a) The endogenous SMA locus and linear targeting construct. (b) The SMA locus correctly targeted by recombineering. (c) The final reporter construct following excision of PGK-neomycin selection cassette.

Our immunohistochemistry on cultured cells selected for mCherry expression by Fluorescent Activated Cell Sorting (FACS) showed a distinctly different membrane localization of our mCherry reporter compared to the cytoskeletal staining pattern of SMA indicating we had avoided generating a SMA-mCherry fusion (Armstrong, Larina et al. 2010).

4.2 Isolation of BAC DNA

Isolation of BAC DNA can be achieved using a standard alkaline lysis phenol/chloroform extraction followed by alcohol precipitation. However, these types of BAC DNA preparations are subject to contamination by genomic DNA. Column purification of BAC DNA, such as that achieved using NucleoBond BAC 100 kit by Clontech, will yield more pure and less degraded BAC DNA. With either procedure, the removal of fragmented linear genomic DNA can be achieved using ExoV exonuclease or increasing lysis of the bacterial wall using lysozyme, as needed. However, large BAC DNA sequences are susceptible to shearing, so one must take care during preparation so as not to degrade the intact BAC DNA.

Quick BAC prep

Although there is a considerable genomic DNA contamination using this Quick BAC Prep procedure, we found it to be sufficient for isolating BAC DNA for introduction into the SW105 recombineering bacterial strain.

1. Pick colony and place in 5ml LB with appropriate selection reagent and place in shaking incubator at proper temperature (30°C for SW bacterial series; 37°C for DH10 bacteria) overnight.
2. Pellet bacteria by centrifugation at 4,000xg for 15min.
3. Resuspend pellet in 1000ul of P1 buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA) with 100ug/ml RNaseA.
4. Add 1000ul P2 buffer (200 mM NaOH, 1% SDS) for lysis reaction for 5 min at RT.
5. Add 1000ul P3 buffer (3.0 M potassium acetate, pH 5.5 adjusted with glacial acetic acid) for cell debris precipitation. Do not vortex! Vortexing BAC DNA will destroy it. Swirl to mix, and then incubate on ice for 5 min.
6. Centrifuge the prep at 4,000xg for 45 min at 4°C. Decant supernatant.
7. Precipitate BAC DNA with 2 ml 2-propanol, incubated at -20°C for 30min or more.
8. Centrifuge at 4,000xg for 30 min at 4°C to pellet DNA.
9. Decant supernatant and wash with 1 ml 70% ethanol. Centrifuge 4,000xg for 15 min at 4°C.
10. Decant supernatant and dry DNA pellet, but do not allow to completely dry. BAC DNA that has completely dried is difficult to resuspend.
11. Resuspend pellet in 200 ul TE at 4°C overnight. This prep should yield 500 ng-1 ug/ul DNA with approximately 50-75% genomic contamination.

4.3 Electroporation of BAC DNA into bacteria

Electroporation of bacteria is usually performed with an exponential decay electroporation instrument, although square wave instruments can be adapted for bacterial electroporation.

We found that for the initial electroporation of BAC DNA into recombinering bacterial strains, a “dirty” prep of BAC DNA containing genomic DNA prepared by alkaline lysis alcohol precipitation was sufficient.

With “Quick Prep” BAC DNA (described above), electroporation can be performed at 1.8KV with a time constant of 5 μ s. Cells are then resuspended in 1ml LB media and incubated for 1hr at 30°C. As with standard practice, 100ul cells are streaked on selection plates and incubated at 30°C overnight. To the remaining electroporated cells, 4ml LB medium are added and incubated overnight at 30°C. The following day, cells were plated on selection plates and transformed cells are obtained from this selection.

4.4 Preparation of induced electrocompetent cells

Electrocompetent cells are prepared by inoculation of 50 ml LB plus appropriate reagent to select for the BAC clone of interest at 1:50 dilution from an overnight culture. Cells are incubated at 30°C until the culture achieves an OD600 of 0.50, which is usually 3-4 hrs. Cells are then divided into two aliquots, one for induction of lambda phage proteins and one un-induced control.

To induce the lambda phage proteins, cells are incubated at 42°C for 15 minutes, and then harvested by centrifugation at 5000xg for 15 minutes at 4°C. Cells are then washed 3 times in 25ml ice-cold 0.2 μ m-filtered ddH₂O containing 10% glycerol, resuspended in 30 μ l 10% glycerol in ddH₂O, and then transferred to cooled cuvettes for electroporation.

4.5 Generating a targeting vector

Recombineering modifies the genomic locus using a linear targeting construct. The linear targeting construct can be generated either by PCR or excision from plasmid DNA, as discussed below.

4.5.1 PCR based approach

A convenient method for generating linear targeting vectors is via a PCR based approach in which primers are designed against two important sequence elements. The 5' end of the primer contains sequence homology to the targeted locus while the 3' end contains sequence for PCR amplification of the targeting construct. When these primers are used in a PCR reaction, the product produced contains both homology to the target and the targeting construct. Using a PCR based approach allows an investigator to use the same template to target an alternative locus. By changing the 5' homology of the primers to match an alternative locus, a new linear targeting construct can be generated. Therefore, the template used for generating the linear targeting construct is modular.

4.5.2 Plasmid based approach

Cloning longer (100-500 bp) homology arms into the targeting construct is an alternative approach to generating a linear targeting vector. Using this type of construct is also useful for cloning sequence from BAC DNA into high copy plasmids for subsequent modification and use for in vitro cell targeting (i.e. embryonic stem cells). The linear

targeting construct is released from the vector by restriction enzyme digest and gel purified. This approach reportedly reduces base changes that are sometimes generated during PCR.

4.6 Preparation and electroporation of targeting vector for BAC modification

In our studies, we generated a linear targeting vector by PCR. Following PCR, the reaction is DpnI digested to remove the plasmid template. This step is incorporated in an attempt to reduce background and false positive clones that would result from electroporation of plasmid template. The PCR product is then gel purified and used to electroporate cells containing the BAC clone. Cells are electroporated with 1ug of linear targeting vector using the parameters previously described in section 4.3. Recombinants are obtained by plating of the overnight culture, as described above.

4.7 Selection of positive recombinants

Selection of positive recombinants can be facilitated by inclusion of a PGK-neomycin selection cassette in the targeting vector that confers kanamycin resistance to recombinant clones. Cells are plated on LB agar plates containing 15ug/ml kanamycin and grown at 30°C overnight, as described above. Following antibiotic selection, clones are screened for correct targeting using DNA isolated from kanamycin resistant clones in a PCR reaction using two different sets of primers that would amplify a product across the 5' and 3' recombination sites. PCR reactions are then analyzed on 1% agarose gel to correctly targeted clones. The PCR products are then sequenced to ensure the recombination had taken place as expected. Southern Blot analysis is another method for identifying the inclusion of the targeting construct into the BAC. While Southern Blot is sensitive, it gives no information on the sequence and requires additional time and labeling reagents (i.e. radioactive nucleotides).

4.7.1 Identifying false positive recombinants

In most cases, the template DNA used in the PCR reaction generating the linear targeting vector is eliminated by a DpnI digest, followed by gel purification. However, despite using DpnI to digest methylated DNA used as template in the generation of linear targeting vector by PCR, some plasmid template may be introduced into bacteria during the electroporation (Fig. 2). Identification of this contamination can be facilitated by gel electrophoresis of uncut BAC DNA isolated from kanamycin resistant clones. Even though the linear targeting construct is gel purified, the plasmid template can transform kanamycin resistant clones. For example, the undigested supercoiled plasmid DNA and the linear targeting vector can run at approximately the same molecular weight, thus contaminating the linear targeting vector during gel purification, as occurred in our studies.

5. Characterization of BAC transgenic mice

5.1 BAC reporter expression recapitulates endogenous expression

To verify that the transgenic BAC reporter is expressed in the same temporal and spatial pattern as the protein of interest, immunohistochemistry is employed to evaluate tissue

and cellular specificity. Not only is it important to demonstrate that the reporter and the protein of interest are co-expressed, but it is also important to demonstrate that the reporter does not exhibit ectopic expression, beyond endogenous expression. In situ hybridization can also be utilized to characterize the co-expression of the reporter and targeted gene of interest.

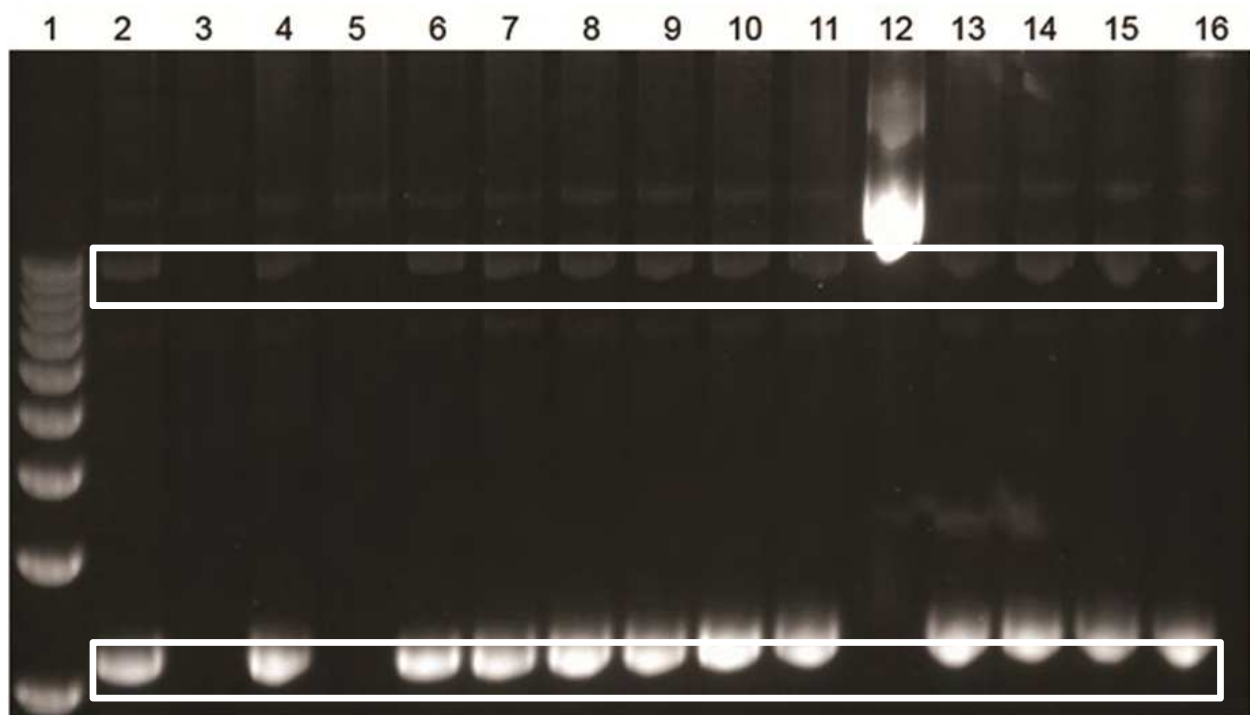


Fig. 2. Identification of PCR template contamination in selected BAC clones. Uncut BAC DNA preps run on 1% agarose gel. BAC DNA is in the upper box, and contaminating PCR template in the lower box. Lane 1 - Hi Mark Ladder. Lanes 3, 4, and 6 are true positive recombinants without contaminating PCR template used to generate the linear targeting construct.

5.2 BAC transgene copy number

An assay was developed by Chandler and coworkers (Chandler, Chandler et al. 2007) to determine the number of copies of BAC transgene integrated into the genome. We adopted this method to measure copy number in our SMA-mCherry transgenic lines (Armstrong, Larina et al. 2010), and found that the level of expression of the reporter correlated with BAC copy number, which is useful for the comparative evaluation of the mouse lines.

5.3 Integration site of BAC transgene

Chandler and coworkers were also able to identify multiple integration sites in BAC reporters. This was achieved by employing the BAC transgene copy number assay described above and analyzing outcrossed F2 generations for the number of transgene copies (Chandler, Chandler et al. 2007). Since the level of reporter expression is dependent on transgene copy number, it is useful to identify founders that transmit a single integration site, ensuring consistent reporter expression in future progeny.

6. Utility of BAC transgenics

6.1 FACS analysis and isolation of live cells based on intracellular protein expression

One advantage of introducing a reporter into the gene locus of an intracellular protein is that one can then use the reporter mouse or cell line to identify and isolate live cells by Fluorescence Activated Cell Sorting (FACS) for in vitro applications. For example, we used the SMA-mCherry mice that we developed to isolate live cells expressing SMA from tissues during embryonic development (Fig. 3a), and demonstrated that the cells retain reporter expression after culture in vitro (Fig. 3b).

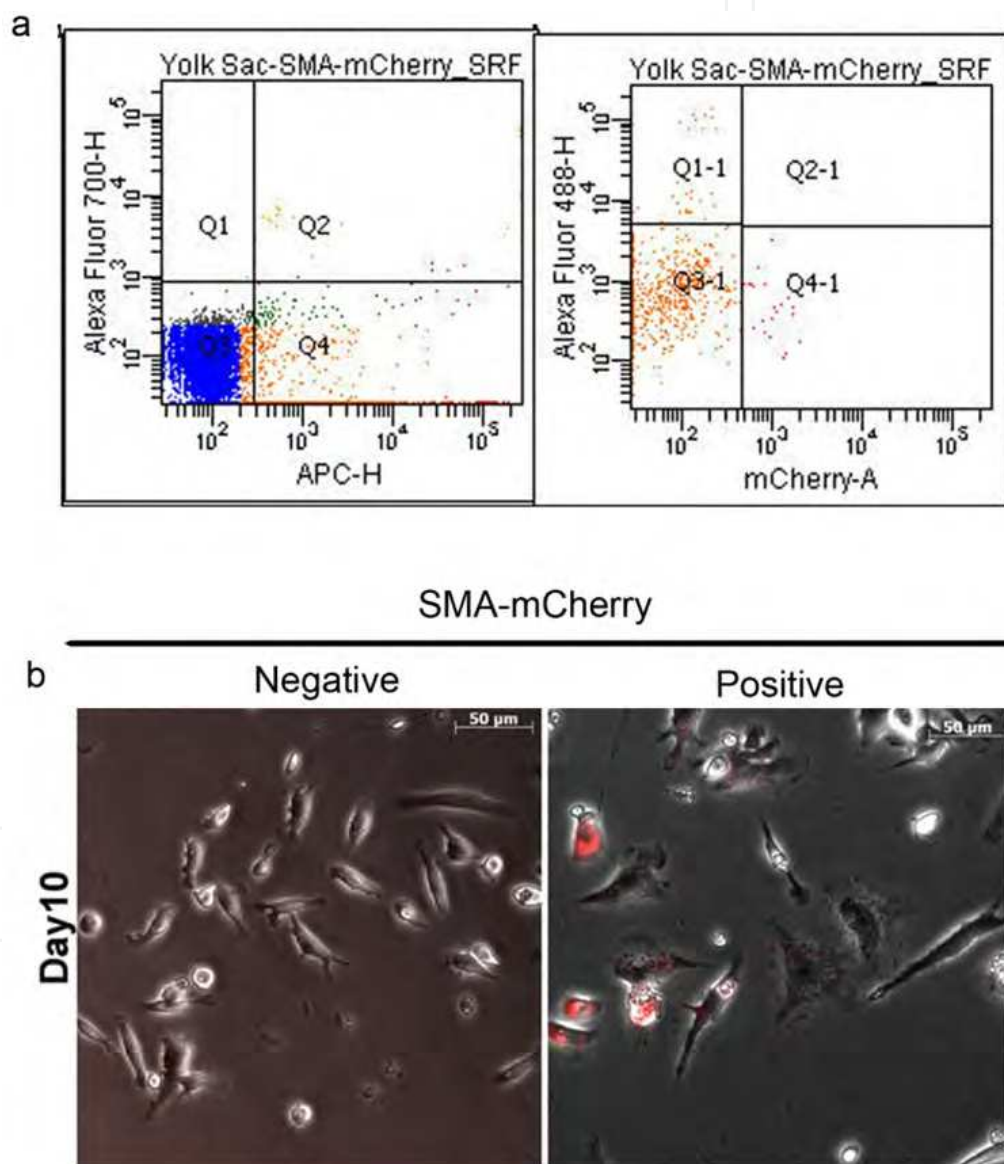


Fig. 3. Isolation of SMA positive cells by flow cytometry. (a) FACS analysis of E9.5 yolk sac endothelium reveals that a subset of endothelium expressed the mesenchymal marker SMA. (b) Expression of SMA was retained when the cells were cultured in vitro.

6.2 *In vivo* imaging of embryonic vascular development

BAC transgenic mice expressing reporter constructs can also be used to monitor and measure the dynamic emergence and fate of distinct cell types. For example, in our studies, we adopted an embryo culture system previously used for *in vivo* imaging of the cardiovascular system and hemodynamics (Garcia, Udan et al. 2011; Garcia, Udan et al. 2011). We used the SMA-mCherry reporter mice, crossed to an endothelial specific Flk1-YFP reporter mouse line, to monitor endothelial-mesenchymal interactions during vascular development (Fraser, Hadjantonakis et al. 2005). Still images of a time-lapse experiment are shown in Figure 4. At early time points in the experiment (~E8.5), only (YFP+) endothelial cells are present within the developing yolk sac (Fig. 4a,d). As vascular development progresses during embryo culture (~E9.0), SMA expressing cells appear (Fig. 4b,e, arrowhead); their migration within the tissue can be monitored over time (Fig. 4c,f, arrowhead). Heart development and function can also be monitored and measured using these mice, as previously reported (Armstrong et al. 2010).

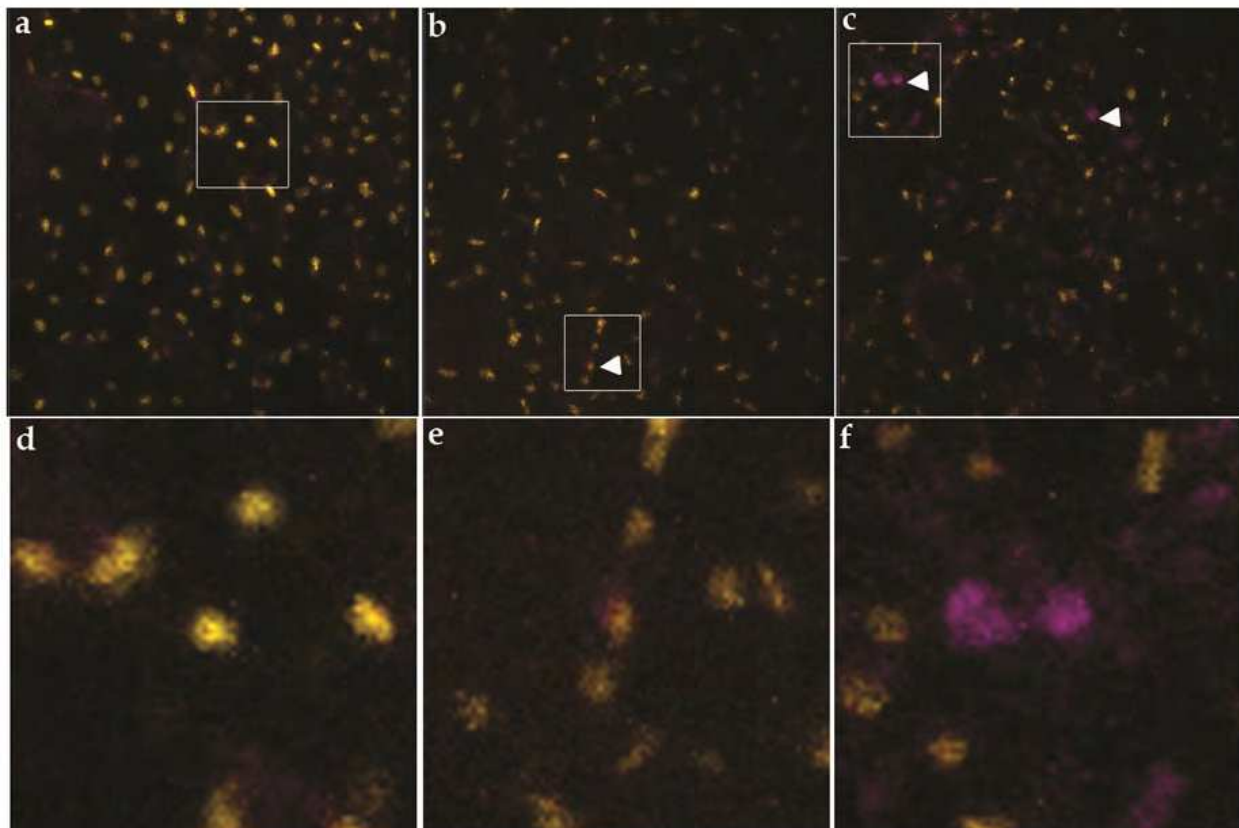


Fig. 4. Still series from *in vivo* imaging. (a,d) YFP+ endothelial cells prior to the emergence of SMA+ cells. At later time points, SMA-expressing cells beginning to appear (b,e), and their movement within the tissue can be monitored over time (c,d).

7. Conclusions

In summary, BAC clones provide a stable source of starting material for gene targeting. BAC clones, used in conjunction with Recombineering technology, provide investigators with numerous gene modifications approaches such as those needed to create transgenic reporter mouse lines. In addition to BAC transgenics, Recombineering technology can be employed for the construction of conditional alleles, point mutations, insertions and deletions. Thus, this technology is versatile and powerful.

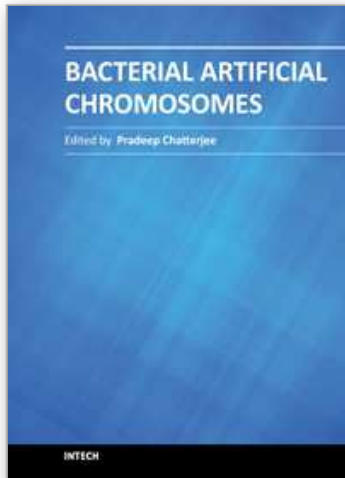
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Bacterial Artificial Chromosomes

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This book focuses on the numerous applications of Bacterial Artificial Chromosomes (BACs) in a variety of studies. The topics reviewed range from using BAC libraries as resources for marsupial and monotreme gene mapping and comparative genomic studies, to using BACs as vehicles for maintaining the large infectious DNA genomes of viruses. The large size of the insert DNA in BACs and the ease of engineering mutations in that DNA within the bacterial host, allowed manipulating the BAC-viral DNA of Varicella-Zoster Virus. Other reviews include the maintenance and suitable expression of foreign genes from a Baculovirus genome, including protein complexes, from the BAC-viral DNA and generating vaccines from BAC-viral DNA genomes of Marek's disease virus. Production of multi-purpose BAC clones in the novel *Bacillus subtilis* host is described, along with chapters that illustrate the use of BAC transgenic animals to address important issues of gene regulation in vertebrates, such as functionally identifying novel cis-acting distal gene regulatory sequences.

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