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Techniques for analyzing gene expression using BAC-based reporter constructs

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Abstract

To characterize the complex regulatory control of gene expression using fluorescent protein reporters, it is often necessary to analyze large genomic regions. Bacteria artificial chromosome (BAC) vectors, which are able to support DNA fragments of up to 300 kb, provide stable platforms for experimental manipulation. Using phage-based systems of homologous recombination, BACs can be efficiently engineered for a variety of aims. These include expressing fluorescent proteins to delineate gene expression boundaries using high-resolution, *in vivo* microscopy, tracing cell lineages using stable fluorescent proteins, perturbing endogenous protein function by expressing dominant negative forms, interfering with development by mis-expressing transcription factors, and identifying regulatory regions through deletion analysis. Here, we present a series of protocols for identifying BAC clones that contain genes of interest, modifying BACs for use as reporter constructs, and preparing BAC DNA for microinjection into fertilized eggs. Although the protocols here are tailored for use in echinoderm embryonic and larval stages, these methods are easily adaptable for use in other transgenic systems. As fluorescent protein technology continues to expand, so do the potential applications for recombinant BACs.

1 Introduction

During the course of development, temporal and spatial boundaries of gene expression are tightly controlled by an array of regulatory elements encoded within the genome. Although these regulatory elements are often located in close proximity to the start of transcription, they can also act over large genomic distances (Spitz, 2016). Gene regulation may also be more complex, such as suites of tandemly arrayed genes that are co-regulated by a single locus control region (Cao & Moi, 2002). Thus, experimentally characterizing the transcriptional control of specific genes may require working with large regions of genomic DNA.

Echinoderm embryos have been important experimental models for regulatory biology (Davidson, 2010; Davidson et al., 2002). This is due in part to the experimental tractability of many echinoderm species (particularly sea urchins and sea stars). Fertilized eggs can be microinjected to efficiently produce transgenic animals and the embryonic and larval stages are transparent, allowing for high-resolution in vivo imaging. Together, these traits allow researchers to use fluorescent protein reporter constructs that recapitulate endogenous gene

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expression to precisely define spatiotemporal expression patterns and to infer regulatory connections based on perturbation and *cis*-regulatory analysis experiments. For many of these experiments, plasmid vectors containing small regions of DNA (3–5 kb) have proven to be useful. This strategy is limited, however, by the size constraints of the cloning vector. Many such reporter constructs result in either ectopic expression or transcript levels that differ substantially from the endogenous genes. In contrast, by using reporter constructs that are generated in the context of bacterial artificial chromosomes (BAC), it is possible to capture the complexity of regulatory information that may span large genomic regions (Buckley, Ho, et al., 2017).

BAC vectors, which are based on sequences from the P1 bacteriophage (P1-derived artificial chromosomes) or the functional fertility plasmid (F-plasmid) from *Escherichia coli*, were originally developed to maintain large fragments of genomic DNA (Monaco & Larin, 1994; Shizuya et al., 1992). These vectors are capable of supporting inserts of up to 350 kb, and are straightforward to isolate and manipulate. From an experimental standpoint, BAC vectors provide a stable genetic background for perturbation studies. Many non-model organisms exhibit high levels of heterozygosity among individuals. BACs thus provide a consistent genetic environment for experimental studies. Using phage-based systems of homologous recombination (e.g., Thomason, Sawitzke, Li, Costantino, & Court, 2014), BACs can be used to efficiently generate reporter constructs.

Echinoderm BAC libraries were initially constructed as part of the Sea Urchin Genome Project (Cameron et al., 2000), and played a central role in the assembly of the *Strongylocentrotus purpuratus* genome sequence (Sodergren et al., 2006). Since then, however, BAC clones have continued to serve as invaluable experimental resources in echinoderm biology. As reporter constructs, echinoderm BACs have been used for lineage tracing (Barsi, Tu, & Davidson, 2014), *cis*-regulatory analysis (Solek et al., 2013), analysis of alternative transcript expression (Schrankel, Solek, Buckley, Anderson, & Rast, 2016), and precisely defining expression patterns at the level of individual cells using *in vivo* imaging (Buckley, Dong, Cameron, & Rast, 2017). Additional strategies that rely on BAC reporter constructs are outlined in (Buckley, Ho, et al., 2017). Here, we outline protocols for working with BACs, including strategies to identify a clone containing the gene of interest, using homologous recombination to generate a reporter construct, and preparing the BACs for microinjection.

2 Identifying a BAC of interest

The first step in generating a recombinant BAC is identifying a BAC clone that encompasses the gene of interest. Echinobase maintains arrayed BAC libraries from several species of echinoderms and hemichordates, as well as recombinant BAC reporter constructs for many transcripts (see www.echinobase.org; reviewed in Buckley, Dong, et al., 2017). These BACs can be obtained by contacting Echinobase directly. If a BAC clone containing the gene of interest is not known, two strategies are available: (1) computationally identifying a BAC using Echinobase; and (2) screening arrayed BAC libraries. Details for using the *S. purpuratus* genome browser to identify an appropriate BAC are found in Fig. 1. See also accompanying volume 150 (Adams, Heyland, Runft, & Foltz). A hybridization-based

protocol for identifying clones of interest from BAC libraries arrayed onto filters is outlined below.

2.1 Materials

Probe template

α-^{32}P-labeled deoxyadenosine triphosphate (dATP) or deoxycytidine triphosphate (dCTP)

Kit for random oligonucleotide-labeling DNA (e.g., Prime-It II Random Primer Labeling Kit, Agilent Technologies)

Nitex sheets: 150–200μm mesh, slightly larger than the filters (22 × 22cm) Rotating hybridization oven and glass bottles Solid pin plate replicator

Nitrocellulose or nylon transfer membranes (e.g., Protran BA85 nitrocellulose membrane circles; Sigma Aldrich)

Solutions

20 × SSPE: 3.0MNaCl, 0.2MNaH_2PO_4, 0.02MEDTA, pH 7.4. Autoclave prior to use.

20 × SSC: 3 M NaCl, 300mM Trisodium Citrate, pH 7.0

0.5% (w/v) SDS

Hybridization solution: 5 × SSPE, 0.1% sodium pyrophosphate, 5% (w/v) SDS

Wash buffers:

2 × SSPE, 0.1% (w/v) SDS

1 × SSPE, 0.1% (w/v) SDS

0.1 × SSPE, 0.1% (w/v) SDS

Stripping buffer: 0.4M NaOH, 20mM EDTA.

Colony blot solutions:

Lysis buffer: 10% SDS

Denaturation buffer: 0.5N NaOH, 1.5M NaCl

Neutralization buffer: 0.5M Tris-Cl, 1.5M NaCl, pH 7.4

Crosslinking buffer: 2× SSC

2.2 Methods

2.2.1 Preparing probes—BAC libraries can be screened using several types of probes, including radiolabeled RNA fragments (e.g., Buckley & Smith, 2007) or single-stranded oligonucleotides (e.g., Ross, LaBrie, McPherson, & Stanton, 1999). Here, we describe a method using double-stranded DNA as a template. Random oligonucleotides anneal to the

template, forming a binding site for DNA polymerase I (Klenow fragment). As this enzyme synthesizes new DNA fragments, it incorporates a radiolabeled nucleotide (α-^{32}P-dATP or -dCTP). We have successfully screened libraries using templates 200–1500 nucleotides in length. If the probes are amplified from cDNA, purify the products by gel extraction to reduce background contamination from the template. Because the radiolabeled DNA fragments are randomly primed, probe templates may span multiple exons. Highly repetitive sequences should be avoided. Finally, the pBACe3.6 vector contains T7 and Sp6 binding sites. If the probe templates contain these sequences, the probes will hybridize to all of the BACs. Consult manufacturers' instructions from the labeling kit for additional details.

2.2.2 Hybridizing library filters—The hybridization temperatures below are optimized for high-stringency conditions. If lower stringency is required (e.g., if the probes and filters are from different species), reduce the temperature; typically, 55 °C is appropriate for species within the same class. Additionally, the final wash (0.1 × SSPE) can be eliminated to reduce stringency. The protocol below is slightly modified from (Cameron, Rast, & Brown, 2004).

1. If the filters have not been screened previously, use the stripping protocol below prior to hybridization.
2. After hybridization, positive clones appear as pairs of spots on either developed films or phosphorimaging screens. To correlate these spots to positions in the 384-well library plates, it is necessary to register the film with the filters. The clone pattern may be slightly distorted relative to the edges of the filters, because the filters are not always straight and typically stretch slightly during printing. Thus, while the filters are still dry, it is useful to mark the corners of the printed grid. The most straightforward way to approach this is to place the filters under a dissecting microscope. Direct the light onto the filters at an angle until the printing impressions are apparent. Using a pencil, carefully mark each of the four corners.
3. Stack the filters on top of one another, placing a Nitex sheet between each filter. Transfer the stack to a glass bottle that fits in the hybridization oven. Place the bottle in a rotating hybridization oven.
4. Add 50 mL of hybridization buffer to the bottle and prehybridize for 30 min at 65 °C. This volume may need to be adjusted depending on the dryness of the filters and Nitex sheets.
5. Denature the double-stranded DNA probes by boiling for 5 min.
6. Pour off excess hybridization buffer. When the bottle is standing on end, there should be ~1 in. of liquid in the bottom.
7. Transfer the remaining hybridization buffer to a tube (e.g., a 50mL conical tube). Add the denatured probes. Mix well, then add the probe/buffer mixture back to the filters in the glass bottle.
8. Incubate the filters in a rotating hybridization oven for at least 12h at 65 °C.

9. Following hybridization, wash the filters with increasing stringency as follows:
    a. Pre-heat all wash buffers to 65 °C.
    b. Pour out the hybridization buffer into a container for radioactive waste.
    c. Wash the filters three times by adding ~100mL of 2 × SSPE directly to the bottle for each wash. Shake the bottle and then transfer the buffer to radioactive waste.
    d. Remove the filters from the bottle and place them in a deep dish that is large enough to hold the filters without folding. Remove the Nitex sheets, which may damage filters.
    e. In a shaking, heated water bath (65 °C), wash filters two times in 2 × SSPE buffer for 15 min.
    f. In a shaking, heated water bath (65 °C), wash filters two times in 1 × SSPE buffer for 15 min.
    g. In a shaking, heated water bath (65 °C), wash filters two times in 0.1 × SSPE buffer for 15 min.
10. Transfer the filters to cassettes for autoradiography. A used sheet of film can serve as a surface on which to place filters. Unwrap a piece of plastic wrap (still attached to the roll) onto the film. From the final wash, pick up a filter and allow it to dry on Whatman paper for ~10s to remove excess moisture. Place the filter, DNA side up on the plastic wrap. Filters are labeled in the upper right-hand corner; the pencil marks in the grid corners (step 2) should also be visible. Carefully roll plastic wrap on top of the filter, avoiding bubbles. Perform autoradiography.
11. Importantly, do not allow the filters to dry until they are stripped (see below).

2.2.3 Stripping and storing filters—After hybridization, strip the filters to remove labeled probe. This should be done while the filters are still slightly wet. Even after the radioactive signal decays, the presence of probe may prevent hybridization in future screenings.

1. Bring 0.5% SDS to a boil
2. Pour on the filters and allow to cool to room temperature.
3. For short-term storage: Sandwich the filter between two sheets of Whatman paper pre-wetted with Stripping Buffer. Wrap the papers and the membrane with plastic wrap. Store at 4 °C until use.
4. For long-term storage: assess that the stripping is complete by exposing the filter to film for at least 12h. If no spots are evident, place the filter between two sheets of dry Whatman paper and incubate at room temperature for at least 24h (until the filters are completely dry). If the stripping is incomplete, repeat steps 1 and 2 above.

2.2.4 Using colony blots to deconvolve multiplexed probes—

Although screening a set of arrayed filters using the protocol above is fairly straightforward, the most efficient (higher throughput) approach is to hybridize using several probes and subsequently deconvolve the signal generated by the individual probes. Although this can be done using PCR-based approaches, we find that results from PCR assays are sometimes difficult to interpret. A less labor-intensive approach uses the radiolabeled probes generated for the original library screen. From 250 μCi of α-^{32}P-labeled dATP, up to six radioactive probes can be generated. During the library hybridization (Section 2.2.2, step 7), multiplex the probes using 90% of the reaction. Store the remaining 10% at 4 °C for use in the following deconvolution protocol. This assay also serves as a secondary line of evidence that the BAC clone contains the region of interest.

1. Inoculate 1 mL LB cultures with the appropriate antibiotics from glycerol stocks of the clones of interest. Culture overnight at 37 °C, 225 rpm.
2. Transfer 100μL of each culture to either 96-well plate or PCR strip tubes.

   In this format, 48 clones can be grown on a LB/agar plate (100mm). Larger or smaller plates may be appropriate depending on the number of potential clones.
3. Using a multi-pin plate replicator or multichannel pipette, replicate the colonies on LB/agar plates containing appropriate antibiotics. One plate should be used for each probe. It may be helpful to either omit or add positive controls (e.g., the probe template) to specific wells to aid the plate orientation in subsequent steps.
4. Using forceps, carefully place a nitrocellulose or nylon filter onto each plate. Circular filters that correspond to Petri dish diameters are commercially available. Alternatively, larger membranes can be cut to fit inside the plates. Mark the filters for orientation; asymmetric notches are often useful here.
5. Incubate the plates for 5–7h 37 °C.
6. Pre-wet a few sheets of blotting paper in each of the four colony blot buffers. Place in clean trays or dishes. The sheets should be damp but not soaking wet.
7. Using forceps, carefully remove the filters from the plates. At this point, the plates can be returned to 37 °C; colonies should be apparent following an overnight incubation. Place each filter, DNA-side up on the blotting paper and incubate as follows:
   a. Lysis buffer: 3 min
   b. Denaturation buffer: 5 min
   c. Neutralization buffer: 5 min
   d. Crosslinking buffer: 5 min

      It may be necessary to re-wet the papers using the colony blot buffers as the filters are processed.
8. Place the filters on Whatman paper and dry at room temperature for 30 min.

9. Crosslink the DNA to the filters by using a UV crosslinker (e.g., Stratalinker UV Crosslinker) or by wrapping the filters in aluminum foil and baking at 80 °Cfor 2h.

10. Screen as outlined in Section 2.2.2, but segregate the filters into individual tubes with single probes for the hybridization step. During the washing steps, filters can be combined in a single dish.

11. Carry out autoradiography. At this step, the clones are present in much higher numbers than the original screen. Consequently, the exposure times can be reduced considerably.

3 BAC recombineering

The most straightforward strategy to generate a fluorescent protein reporter in the context of a BAC clone is to use homologous recombination (Court, Sawitzke, & Thomason, 2002; Sharan, Thomason, Kuznetsov, & Court, 2009; Yang, Model, & Heintz, 1997; Zhang, Muyrers, Testa, & Stewart, 2000). When applied to BACs, this process is known as “recombination-based engineering” or, more simply, “recombineering.” This technique relies on specific strains of bacteria that have been genetically engineered to express enzymes capable of mediating homologous recombination in response to specific environmental cues. Many variations on this method have been described (Chan et al., 2007; Ohtsuka et al., 2007; Sarov et al., 2006). Here, we present a straightforward technique that has been used to generate reporter constructs for echinoderm larvae (outlined in Fig. 2; Buckley, Dong, et al., 2017; Buckley, Ho, et al., 2017; Schrankel et al., 2016; Solek et al., 2013). The bacterial strains and cloning vectors used to generate the recombination cassette are available from Echinobase (www.echinobase.org).

3.1 Materials for BAC recombineering

Cell lines—Several cell lines are available for performing homologous recombination (Warming, Costantino, Court, Jenkins, & Copeland, 2005). We most commonly use EL250 cells, as described in the procedure below, but other strains may offer specific experimental advantages.

DH10B. Commercially available, electrocompetent DH10B cells can be transformed efficiently. Alternatively, electrocompetent DH10B cells can be generated from a glycerol stock as outlined below.

EL250. A DH10B-derived strain that contains a λ prophage with the recombination genes *exo, bet*, and *gam*. These genes are repressed by the temperature-sensitive repressor *cI857* (Yu et al., 2000).

Media and antibiotics—Kanamycin. Prepare a stock solution in water (25 μg/μL). Filter sterilize and store at −20 °C. Dilute 1:1000 in media for a working concentration of 25 μg/mL. Chloramphenicol: Prepare a stock solution in ethanol (25 μg/μL). Filter sterilize and store at −20 °C. Dilute 1:2000 in media for a working concentration of 12.5 μg/mL.

Luria-Bertani Broth: Combine the following in distilled water: 1% tryptone,

0.5% yeast extract, 10% sodium chloride. Adjust pH to 7.5 with NaOH and sterilize by autoclaving. Alternatively, liquid or powder forms can be obtained commercially (e.g., LB Broth [Miller]; Sigma-Aldrich).

SOC Media: Combine the following in distilled water: 0.5% yeast extract, 2% tryptone, 10mM sodium chloride, 2.5mM potassium chloride, 10mM magnesium chloride and 10mM magnesium sulfate. Sterilize by autoclaving. Prior to use, add filter-sterilized glucose to a final concentration of 20mM. Alternatively, this media can be obtained commercially (e.g., SOC Media; Thermo-Fisher).

Pulsed-field gel electrophoresis (PFGE) apparatus. After each stage in the recombination procedure, PFGE is used to assess the quality of the DNA isolation and to ensure the integrity of the BAC insert. These large DNA fragments cannot be analyzed using standard agarose gel electrophoresis (see Fig. 3).

Electroporator and electroporation cuvettes (0.1 mm gap). Unlike plasmid DNA, transforming bacteria with BACs requires electroporation. Chemical transformations do not work.

10% L-(+)-arabinose.

Gel extraction kit (e.g., QIAquick Gel Extraction Kit, Qiagen).

High fidelity DNA polymerase (e.g., Phusion® High-Fidelity DNA Polymerase, New England Biolabs).

DpnI (New England Biolabs).

3M NaOAc (pH 5.2).

3.2 Method

3.2.1 Working with BAC DNA—The efficiency of each of the following steps relies on high-quality BAC DNA. These large DNA molecules (which can easily fragment) must be handled with more care than smaller, plasmid DNA. BACs should never be vortexed. When isolating BAC DNA, do not resuspend bacterial pellets by vortexing. For restriction digests, resuspension of pelleted BAC DNA, or phenol:chloroform extractions, gently flick the tubes or mix by inversion rather than vortexing. Additionally, freezing BAC DNA breaks the constructs into small pieces. This may be acceptable if the BAC is to be used as template for PCR, but for injections or the transformation steps described below, BACs should be maintained at 4 °C.

3.2.2 Preparing the recombination cassette—During homologous recombination, short stretches of DNA are used to target the recombinase proteins to the region of interest on the BAC. These sequences are known as “recombination arms” (Fig. 2). On the wild-type BAC, the arms flank the target region and precisely define the boundaries of the sequence

that is replaced during recombineering. A linear, double-stranded DNA construct, known as the “recombination cassette,” is generated in which identical arms surround the fragment of exogenous DNA that is inserted into the BAC during recombination (e.g., the coding sequence for a fluorescent protein, and antibiotic resistance).

The locations of the recombination arms depend on the intended experiments. To characterize spatiotemporal patterns of gene expression, fluorescent protein sequence is often introduced into the BAC at either the transcription or translation start site. To assess alternative splicing, fluorescent protein sequence(s) can be inserted into individual exons (Schrankel et al., 2016). This may generate fusion proteins; in such cases it is important to ensure that the reading frame of the protein is maintained through the reporter sequence. In some cases, fusion proteins may exhibit dominant-negative functions. Finally, reporter BACs can be constructed that are either resistant or susceptible to perturbation by morpholino antisense oligonucleotides (MASOs), by modifying the DNA sequences that encode the MASO binding site. In designing the recombination arms, detailed knowledge of gene structure is invaluable. Transcription and translation start sites may be accurately identified using RACE PCR, RNA-seq data or phylogenetic comparisons. It is not uncommon for echinoderm genes to include alternative transcription start sites or a first exon that primarily encodes untranslated sequence (K.M.B., personal observation).

To mediate recombination, the arms should be at least 50 nucleotides in length (Court et al., 2002), although longer arms may be more practical. We typically design arms that are 120–250 nucleotides in length. Many strategies are available for generating a recombination cassette, including cloning, overlap PCR, or commercial DNA synthesis, which we will not detail here (e.g., Livi & Davidson, 2006; Solek et al., 2013). Recombination cassette vectors encoding several fluorescent proteins that are suitable for cloning arms are available from Echinobase upon request (www.echinobase.org). The final product should be a double-stranded, linear DNA fragment. Because the recombination frequency is relatively low (10^{-4}–10^{-5}; Sharan et al., 2009), contamination with plasmid DNA may pose a significant barrier. The plasmid DNA contains the antibiotic resistance marker used to identify recombinant BACs. Consequently, antibiotic resistance indicates one of the following two transformation outcomes: (1) recombinases successfully generated a recombinant BAC in which the antibiotic resistance is encoded on the BAC (the desired result); or (2) an individual bacterial cell was transformed with plasmid DNA (background contamination). A protocol for amplifying the recombination cassette from plasmid DNA and subsequently removing this source of contamination is below.

1. Amplify the recombination cassette.

   Use high fidelity DNA polymerase to amplify the recombination cassette with both arms (amplify from the 5′ end of the 5′ arm to the 3′ end of the 3′ arm) from plasmid DNA. Minimize the amount of plasmid DNA used in PCR. Set up enough reactions to produce 200ng of cleaned DNA. Run the product on a 0.8% agarose gel and purify the fragment using a commercial gel extraction kit.

2. Treat the recombination cassette with DpnI.

The restriction enzyme *DpnI* recognizes a four-nucleotide sequence and only cleaves methylated DNA. Here, *DpnI* digests the template plasmid, but not the amplified PCR product, and thus reduces background during the recombination step.

a. Treat the amplified recombination cassette with 5U *DpnI* in the appropriate buffer.

b. Incubate the reaction at 37 °C for 1h.

c. To inactivate the enzyme, heat the reaction at 65 °C for 15 min.

d. Precipitate the recombination cassette by adding 0.1 volume sodium acetate (3M, pH 5.2) and 2 volumes cold ethanol (100%).

e. Incubate at −20 °C at least 2h to overnight.

f. Pellet the DNA by centrifugation (maximum speed, 4 °C, 30 min).

g. Wash the pellet with 70% ethanol, dry briefly and resuspend in H_2O.

3.2.3 Transforming EL250 cells with the BAC

1. Prepare electrocompetent EL250 cells.

EL250 cells lack antibiotic resistance. Use sterile techniques when handling these cells to avoid contamination in the absence of antibiotics. Additionally, this strain contains the temperature-sensitive lambda prophage recombination genes *exo, bet*, and *gam*, which are de-repressed at high temperatures (Lee et al., 2001). Culturing these bacteria at 37°C is typically lethal; always grow EL250 cells at temperatures lower than 32 °C.

a. Inoculate a starter culture of EL250 cells in 2mL LB. Incubate overnight (30 °C, 225 rpm).

b. Dilute the culture 1:35 in LB (e.g., 0.7mL starter culture in 50mL LB). Incubate (30 °C; 225 rpm) until the bacteria reach log phase ($O.D._{600}$ is between 0.8 and 1.0; typically about 5h).

c. From this step onward, keep bacteria on ice at all times to improve electroporation efficiency. Pre-chill all tubes and buffers on ice prior to use.

d. Pellet bacteria in a pre-chilled tube by centrifuging at 4 °C for 10 min at 1750 × g.

e. Wash the bacteria twice in 50 mL of ice cold dH_2O (centrifuge at 4 °C, 10min, 1750 × g). During these washes, resuspend the bacteria gently, by adding 1–2mL dH_2O and briskly swirling the tube. Once the cell suspension is homogeneous, add the remaining volume and mix by inversion.

f. Resuspend the washed bacteria in 1 mL ice cold dH_2O and transfer to a chilled microcentrifuge tube. Centrifuge to pellet the bacteria (4°C,

2min, 14,000 × g). Wash the EL250 cells three additional times in 1 mL dH_2O. After the last wash, remove as much of the supernatant as possible and store the electrocompetent bacteria on ice. Aliquots of these cells can be stored at −80 ° C in glycerol (final concentration = 15%), although this drastically reduces transformation efficiency.

2. Transform the EL250 cells with the BAC of interest.

Two factors greatly affect transformation efficiency: the condition of the reagents and temperature. Optimal transformation occurs when using freshly washed EL250 cells and newly isolated BAC DNA. The following steps should be performed quickly and on ice.

a. Prepare electrocompetent EL250 cells (outlined above).

b. Miniprep BAC DNA (see Section 4).

c. In a chilled microcentrifuge tube, combine 200ng BAC DNA (or 1/3 of a miniprep), 10 μL electrocompetent EL250 cells, and 10 μL dH_2O.

d. Transfer the mixture to a cold electroporation cuvette (0.1 mm)

e. Electroporate (1.4–1.7kV/cm) and immediately add 1 mL SOC to the cuvette. In contrast to plasmid DNA, BACs are more readily transformed at slightly lower voltages; as the size of the BAC clone increases, decrease the voltage. Generally, for BACs with an insert size of >50kb, we have had success electroporating at 1.4 kV/cm.

f. Transfer the cells to a culture tube and incubate at 30 °C (225 rpm) for 1 h. Spread the entire 1 mL on LB/chloramphenicol (chl) agar plates and incubate overnight at 30 °C. The EL250 cells containing BACs grow slowly at 30 °C. Colonies may be small after overnight growth and may require up to 24h to be visible.

g. Select several colonies to inoculate 2mL cultures of LB/chl (30 °C, 225 rpm).

h. Identify successful transformants by isolating BAC DNA using the alkaline lysis miniprep protocol (Section 4). Verify that the insert size of BACs isolated from the EL250 cells equals that of the wild-type BAC by restriction digest and PFGE analysis (Fig. 3).

i. For long-term storage, make glycerol stocks (final glycerol concentration = 15%) of EL250-BAC clones and store at −80°C.

3.2.4 Recombineering GFP in BAC DNA

a. Inoculate a starter culture of the EL250-BAC cells in 2mL LB/chl and incubate overnight at 30 °C (225rpm).

b. Dilute the culture 1:50 in 50mL LB/chl. Incubate at 30°C (225 rpm) until the bacterial growth reaches log phase ($O.D._{600}$ is 0.8–1.0).

c. To de-repress the recombinase genes, transfer the culture to a pre-warmed (42 °C) shaking water bath and incubate for 15 min.

d. Rapidly chill the culture by swirling in a slurry of ice and water for 10 min.

e. Prepare the bacteria for electroporation by washing the cells as described in Section 3.2.2 (Prepare Electrocompetent EL250 Cells).

f. In a chilled microcentrifuge tube, combine: 200ng of recombination cassette, 10 μL electrocompetent EL250-BAC cells and 10 μL dH_2O. Transfer to a chilled electroporation cuvette (0.1 mm).

g. Electroporate at 1.8kV/cm and immediately add 1 mL SOC media.

h. Incubate at 30°C for 1h (225 rpm).

i. Spread 50 μL and 500 μL of the transformed bacteria on LB/chl/kan plates and incubate for 24 h at 30°C.

j. EL250 cells with recombinant BACs often grow slower than cells that contain free recombination cassettes. Thus, it is advisable to select colonies that are visible after 24h of incubation rather than overnight.

k. Confirm the recombination using restriction digest and PFGE analysis or PCR (Fig. 3).

l. Store the recombinant strains (EL250-BAC^R) as glycerol stocks (final glycerol concentration = 15%) at −80 °C.

3.2.5 Removing the kanamycin cassette—Although this step is not required prior to injection, removing the kanamycin resistance cassette generates a nearly “seamless” recombinant BAC in which the fluorescent protein sequence is located in a genomic context that is almost identical to the endogenous gene of interest. Removing the kanamycin cassette relies on expression *of flippase (flp*), a recombinase enzyme that recognizes short flippase recombination target (FRT) sites (Schlake & Bode, 1994).

a. Inoculate a 2mL LB/chl/kan culture of EL250-BAC^R cells. Incubate overnight until turbid (30 °C, 225 rpm).

b. Dilute the culture 1:50 in 25mL of LB/chl. Grow the culture (30°C, 225 rpm) until the bacteria enter log phase growth ($O.D._{600}$ = 0.5).

c. To induce *flp* expression, add 250 μL of 10% L-(+)-arabinose. Incubate for 1 h at 30 °C (225 rpm).

d. Dilute the activated culture 1:10 in LB/chl media. Grow for 1 h at 30 °C, 225 rpm.

e. Streak 2 μL of this culture on an LB/chl agar plate. Incubate the plate overnight at 30 °C.

f. Identify colonies in which the kanamycin resistance cassette was removed (EL250-$BAC^{R/kan–}$) by replicate streaking individual clones on LB/chl and LB/chl/kan agar plates and growing overnight (30 °C). Select strains that are

resistant to chloramphenicol but cannot grow the presence of kanamycin. This step is highly efficient; in most cases, kanamycin resistance is lost in 100% of clones.

g. Store the flipped, recombinant strains (EL250-BAC$^{R/kan-}$) as glycerol stocks (final glycerol concentration = 15%) at −80 °C.

3.2.6 Transferring the recombinant BAC to DH10B cells—In the EL250 cells, genes encoding the recombination machinery are expressed at temperatures >32 °C. To avoid off-target recombination with the BAC, or the endogenous *E. coli* genome, it is best to avoid activating these enzymes. This temperature restriction limits bacterial growth rate, interfering with the ability to grow large numbers of bacteria for BAC isolation. Thus, the EL250 cells should only be used during the recombineering process. Once the EL250-BAC$^{R/kan-}$ has been generated, this construct should be transferred to the more stable DH10B *E. coli*. Miniprep the BAC (Section 4) and use DNA to electroporate competent DH10B cells. Commercially available aliquots of DH10B *E. coli* exhibit high efficiency and simplify this step, but electrocompetent cells can be made using the same procedure as for EL250 cells (Section 3.2.3).

At this point, it is advisable to analyze the BAC insert a final time using restriction digestion and PFGE analysis (Fig. 3). The original, wild-type BAC and the EL250-BAC$^{R/kan-}$ should be used to confirm that the insert size has not been altered during the recombination process. Store the DH10B-BAC$^{R/kan-}$ as a glycerol stock at −80 °C.

4 Isolating BAC DNA: Alkaline lysis miniprep

4.1 Materials

Buffer P1: 15mM Tris, pH 8, 10mM EDTA, pH 8, 100μg/mL RNase A. Buffer should be made without the RNAse and stored at 4 °C. Add RNaseA just prior to use.

Buffer P2: 0.2N NaOH, 1% SDS. This solution precipitates easily and should be made freshly before each use.

Buffer P3: 3M KAc pH 5.5, autoclave. Chill on ice before use.

100% isopropanol.

70% ethanol.

TE Buffer: 10mM Tris, pH 8, 1 mM EDTA, pH 8.

4.2 Method

1. Inoculate a 2mL culture in LB media containing the appropriate antibiotics from a single colony grown on an agar plate or a glycerol stock of bacteria containing the BAC of interest. Grow overnight (<16h) at 37 °C, 250 rpm.

2. Pellet bacteria by centrifuging 2mL of culture at maximum speed for 2min. Discard the supernatant.

3. Carefully resuspend the bacterial pellet in 0.3mL of P1, using a wide bore pipette tip. Avoid vortexing as this disrupts the large fragments of BAC DNA.
4. Add 0.3mL of P2 and invert tubes to mix. The suspension should become translucent.
5. Add 0.3mL of cold P3 and gently invert tubes to mix. A thick white precipitate will form.
6. Incubate the tubes on ice for 5 min.
7. Pellet the precipitate by centrifuging for 10min at maximum speed, room temperature.
8. Transfer the clear supernatant (~800μL) to a clean microcentrifuge tube.
9. Add 0.8mL ice cold isopropanol to the supernatant. Mix well by inverting several times.
10. Pellet BAC DNA by centrifuging at maximum speed for 20min, 4 °C.
11. Carefully remove the supernatant and discard. Wash the pellet 2 × by adding 0.5mL of ice cold 70% ethanol. Centrifuge at maximum speed for 5min, 4°C.
12. Gently remove the supernatant, taking care not to dislodge the pellet. The easiest way to do this is to remove most of the supernatant using a P1000 micropipette, quickly microcentrifuge the tubes to collect the liquid, and then use a P200 micropipette to remove the remaining ~50μL of ethanol.
13. Quickly dry the pellet at room temperature and gently resuspend in 30μL TE.
14. To analyze the isolated DNA, digest 6μL of the resuspended BAC and analyze using PFGE, or dilute BAC DNA 1:20 in TE and analyze by PCR.

5 Preparing BACs for microinjection

For routine analysis of BAC DNA, the alkaline lysis miniprep protocol described above should be used. However, BACs isolated with this procedure are typically contaminated with a significant proportion of bacterial DNA. To purify BAC DNA that is suitable for microinjection into fertilized echinoderm eggs, other methods should be used. If researchers have the necessary equipment and expertise, cesium chloride/ ethidium bromide gradients can be used to obtain large quantities of clean BAC DNA (Humphreys, Willshaw, & Anderson, 1975). We have had considerable success using the NucleoBond BAC 100 Kit (Clontech).

Using either of these methods, researchers can expect large yields (we routinely obtain several hundred micrograms). For long-term storage, resuspend BACs in TE buffer (10mM Tris, pH 7.4, 70mM EDTA). This high EDTA concentration prevents nuclease activity, but also interferes with other enzymatic reactions (e.g., restriction digestion or PCR). Prior to use in these applications, ethanol-precipitate an aliquot of the purified BAC and resuspend in a buffer with less EDTA.

To analyze spatiotemporal gene expression in sea urchins, BACs must be linearized prior to microinjection. Notably, this step is not required when generating transgenic *Asterina miniata* embryos (K.M.B., unpublished observations). The pBACe3.6 vector has a single *AscI* site that can be used to linearize most BACs. However, if an *AscI* site is present within the insert, this enzyme may not be optimal. An alternative enzyme is *NotI*: two *NotI* recognition sites within the vector flank the insert sequence. Additional, rare-cutting enzymes (i.e., enzymes that recognize eight nucleotide sequences or GC-rich targets) may also provide alternatives. When selecting an enzyme, the most important criterion is ensuring that the regulation of the reporter gene is not disrupted.

5.1 Method

1. Combine the following in a microcentrifuge tube: 10μg BAC DNA, 20U restriction enzyme and the appropriate digestion buffer.
2. Incubate at the optimal temperature for the enzyme (usually 37 °C) for 2h.
3. Add 20U of enzyme and incubate for an additional hour.
4. Confirm that that digestion reaction is complete by analyzing an aliquot of the reaction using PFGE.
5. Purify the linearized BAC DNA by phenol:chloroform extraction. Add 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1) to the digestion. Mix well by carefully inverting the tube.
6. Separate the aqueous and organic phases by centrifugation (3 min, maximum speed).
7. Transfer the aqueous (top) phase to a new microcentrifuge tube.
8. Add 1 volume of chloroform. Mix and centrifuge as in steps 5–7.
9. Repeat step 8. From this step onward, ensure that reagents are clean and as free of contaminants to optimize microinjection.
10. Precipitate the BAC DNA by adding 0.1 volume sodium acetate (3M, pH 5.2), and 2 volumes of ethanol (100%). Mix well and incubate at −20 °C for at least 3h to overnight.
11. Pellet the DNA by centrifugation (30 min, maximum speed, 4 °C).
12. Remove the supernatant and wash the pellet using 1mL 70% EtOH.
13. Air dry the pellet 2–3min (be careful not to over dry)
14. Gently remove the supernatant, taking not to dislodge the pellet. The easiest way to do this is to remove most of the supernatant using a P1000 micropipette, quickly microcentrifuge the tubes to collect the liquid, and then use a P200 micropipette to remove the remaining ~50 μL of ethanol.
15. Resuspend in 50μL TE (10mM Tris pH 7.4, 0.5mM EDTA). This lower EDTA concentration reduces the toxicity of microinjection.

16. Store at 4 °C.

6 Conclusions

BAC provides a powerful strategy for precisely analyzing gene expression in echinoderm embryos and larvae. Reporter constructs that using fluorescent proteins to recapitulate endogenous gene expression allow researchers to delineate the spatial boundaries of gene expression at single-cell resolution. As described here, BAC clones are straightforward to identify, purify, modify, and microinject to generate transgenic echinoderm embryos. However, using homologous recombination, BAC clones can be constructed to serve much broader applications. With improvements in fluorescent protein technology, BACs can be generated to express an array of fluorescent wavelengths, as well as biosensors (e.g., Ca^{2+}, reactive oxygen species) or timer proteins. Photoconvertible or photo-switchable proteins may provide insight into lineage tracing experiments as genes are re-used during development. Beyond fluorescent proteins, recombinant BACs can be used to drive tissue- or stage-specific expression of dominant negatives, or tagged versions of proteins to facilitate isolation. Finally, BACs could be constructed to control expression of components of the CRISPR/Cas9 system for genome editing. By providing a stable genetic background, BACs will continue to serve as valuable experimental resources for outbred organisms even as genome editing technology improves. Using the highly controlled homologous recombination strategy described here, BAC clones afford vast experimental potential and will continue to shed light on fundamental questions in developmental and evolutionary biology.

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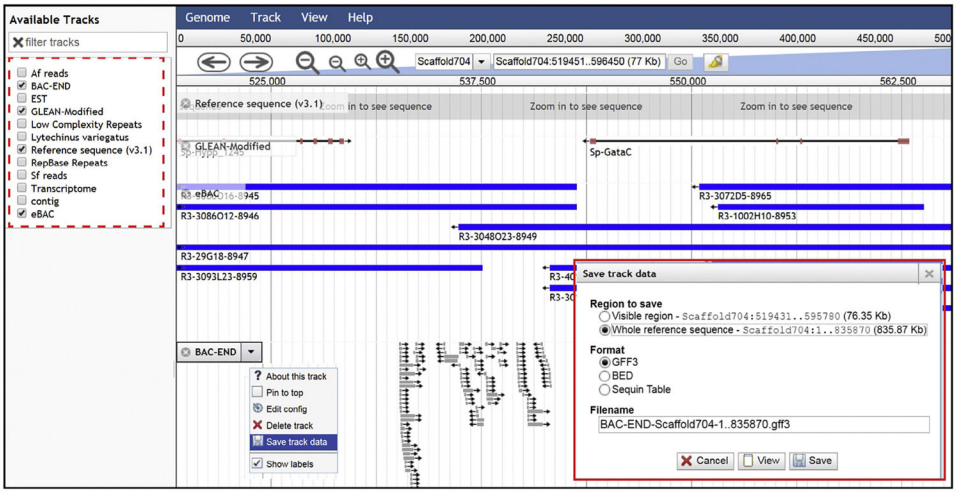

FIG. 1.

Using Echinobase to computationally identify a BAC containing the gene of interest. In the course of the *S. purpuratus* genome project, ~8000 BAC clones were sequenced and assembled using a clone-array pooled shotgun sequencing (CAPSS; Cai, Chen, Gibbs, & Bradley, 2001). Additionally, 76,020 BAC clones were end-sequenced (Cameron et al., 2000). These data served as a framework for assembling the complete genome sequence of *S. purpuratus* (Sodergren et al., 2006), and, importantly for the protocols described here, are included in the *S. purpuratus* genome browser on Echinobase. To find a BAC that encompasses a specific gene or region of the genome, go to the JBrowse section of Echinobase (www.echinobase.org; Cary, Cameron, & Hinman, 2018). Navigate to the appropriate genomic region using the search bar in the center of the page. To view the available BAC sequences, check the “BAC-END” and “eBAC” boxes on the left-hand side of the browser (highlighted with a dashed red line). The “GLEAN-modified” track is also useful to visualize the gene of interest, although it is advisable to confirm the starts of transcription and translation using other lines of evidence. The eBAC track shows complete BAC sequences obtained using the CAPSS assay. The BAC coordinates (beginning with R3-) are indicated under each feature. The BAC-END data are single Sanger sequencing reactions using the T7/Sp6 primer sites within the pBACe3.6 vector. The most efficient way to analyze these data is to download all of the track data by clicking on the down arrow next to the BAC-END track name. Selecting “Save track data” brings up the window shown in the red box. Select “whole reference sequence” and download the data as a GFF file, which can be read as a text file or in a spreadsheet program. The ideal BAC will have sequence on both sides of the gene of interest.

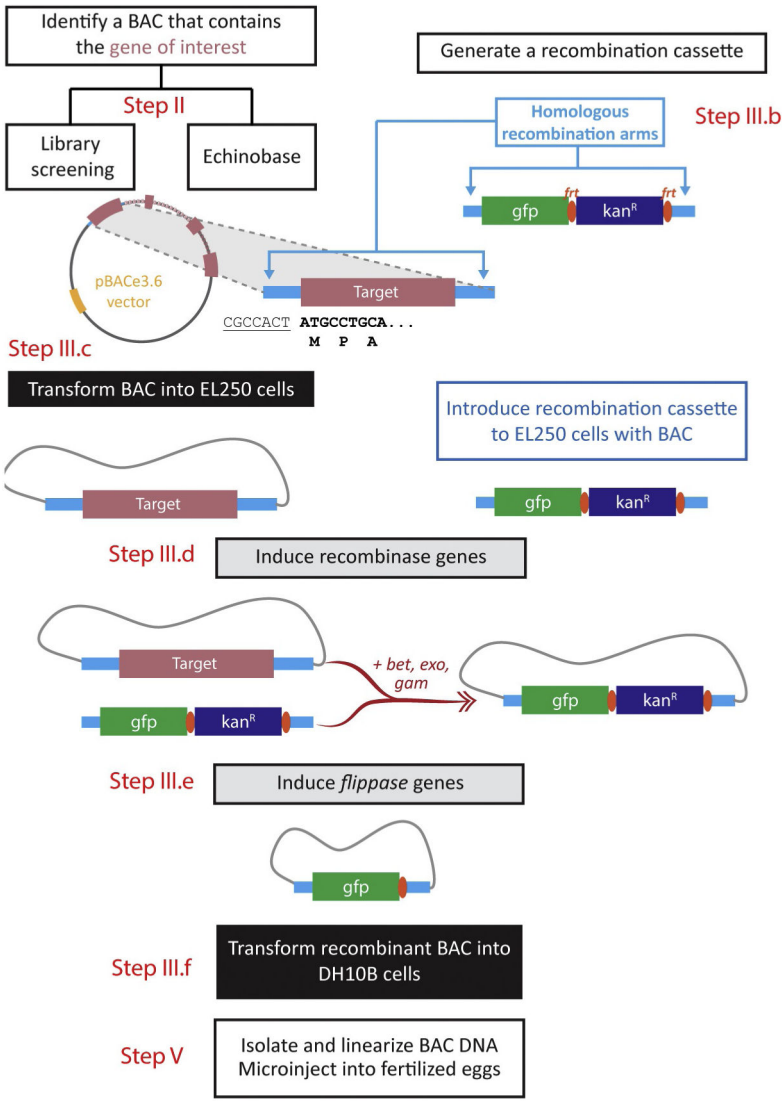

FIG. 2.

Using homologous recombination to generate reporter constructs. An overview of the protocols described here is shown. The relevant steps are indicated in red text. In this example, the recombination cassette consists of GFP (indicated by a green box), although the sequence encoding any protein can be used (e.g., fluorescent proteins, dominant negative forms of proteins, or transcription factors). Here, the recombination cassette is introduced at the translation start site of the target exon (pink; hypothetical nucleotide and translated amino acid sequences are shown below). The underlined sequence indicates the recommended location for the 5′ homology arm (blue). To ensure that the recombination arms are identical in sequence to those on the BAC, we recommend amplifying these regions directly from BAC DNA. Even a few polymorphisms in the arms can reduce recombination efficiency. Flippase is a DNA transposase that recognizes flippase recognition target (*frt*; ovals) sequences. DNA between these sites is removed, and a single frt remains in the recombinant BAC.

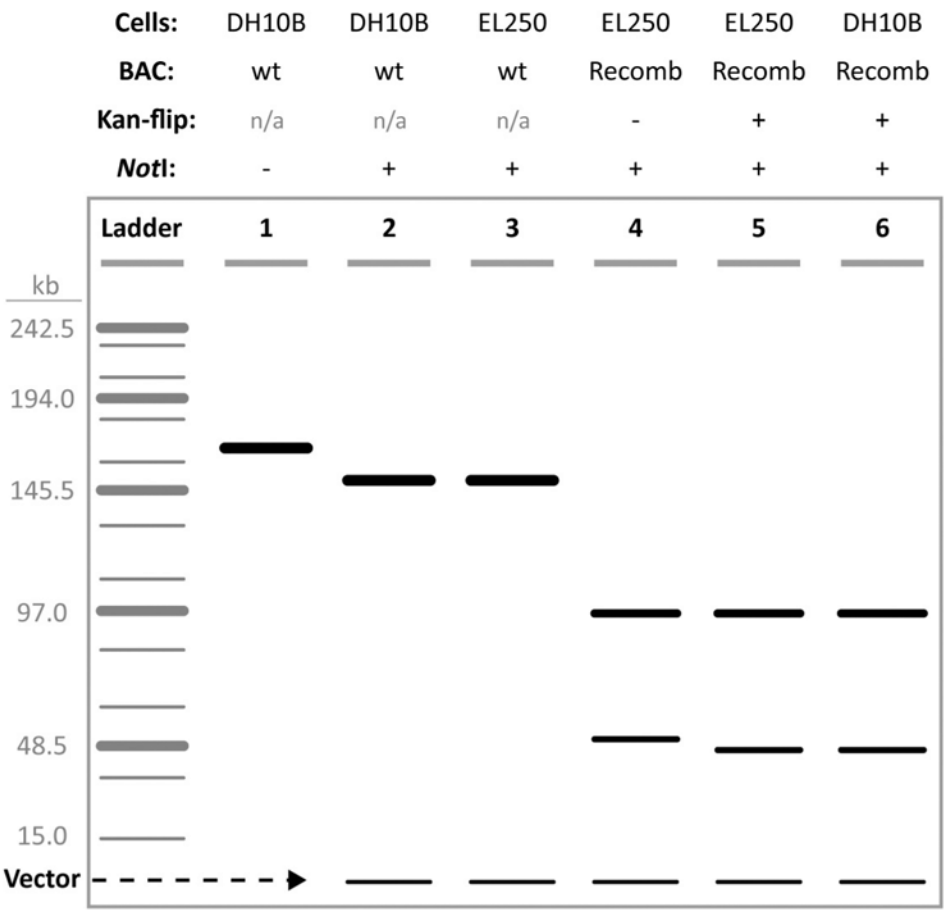

FIG. 3.

Using pulsed-field gel electrophoresis (PFGE) to analyze recombinant BACs. During homologous recombination, short regions of homology within the BAC insert may result in large deletions. This is particularly true for tandemly arrayed gene paralogs. After each step of the BAC recombineering, PFGE should be used to confirm the insert size. A hypothetical example of PFGE analysis of BACs at each stage of recombineering is shown to illustrate expected results. The pBACe3.6 vector contains *NotI* sites on each side of the insert. Consequently, digestion with this enzyme linearizes the BAC DNA by releasing the insert from the vector sequence. Several ladders are available for PFGE analysis, but we have had considerable success with the MidRange PFG Marker (New England Biolabs). To analyze BAC DNA, use the following parameters: 1% agarose; buffer, 0.5 × TBE; temperature, 14°C; voltage, 6V/cm; switch times, 60–120s; run time, 16h. *Lane 1*: Uncut, wild-type BAC DNA. *Lane 2*: Wild-type BAC DNA linearized with *NotI*. In this example, the insert size is ~150kb. Note the 9kb vector fragment at the bottom of the gel. This should be consistent in all digested BAC samples. *Lane 3*: Wild-type BAC DNA isolated from EL250 cells (Section 3.2.3). After transformation, select 10–20 colonies, miniprep the DNA (Section 4), and digest with *NotI*. Run the original BAC DNA as a control. The insert size of the EL250-BAC DNA should be identical to that of the original. *Lane 4*: Recombinant BAC DNA. In this case, the recombination cassette includes a diagnostic *NotI* restriction site that cleaves the original 150kb fragment into two fragments (~100 and ~50kb). The sum of the smaller

fragments should equal the size of the original insert. *Lane 5*: Recombinant BAC DNA without kanamycin resistance. After activating the *flp* recombinase (Section 3.2.5), plate cells and identify strains unable to grow in the presence of kanamycin. Miniprep the DNA and digest with *NotI*. If the fragments are small, the loss of the 2kb kanamycin resistance cassette is evident on the gel (note the slightly smaller lower band relative to Lane 4). *Lane 6*: Recombinant BAC DNA (EL250-BAC$^{R/kan-}$) in DH10B cells. This is the last step in the BAC recombineering protocol (Section 3.2.6). Here, the BAC DNA isolated from DH10B cells should appear identical to that isolated from EL250 cells (Lane 5).