Generation of cell lines with attP sites and serum-free insect cell culture

Daniel Mariyappa*, Arthur Luhur*, Kris Klueg*, Danielle Overton, Johnny Roberts, Andrew Zelhof,

Drosophila Genomics Resource Center (DGRC), Department of Biology, Indiana University, Bloomington, IN

*equal contribution

dgrc@indiana.edu

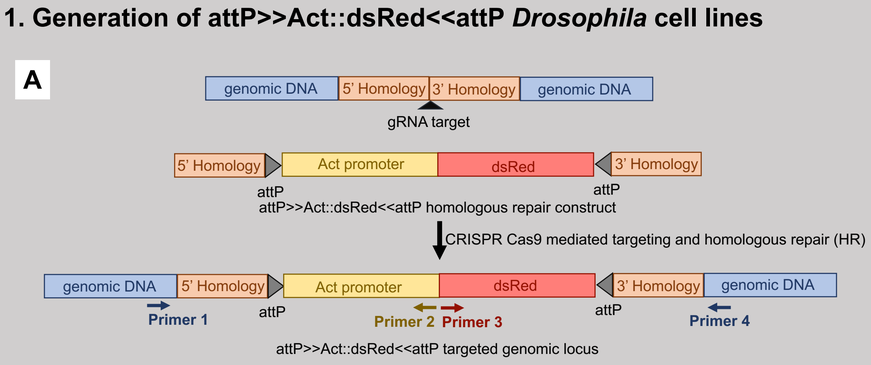
Abstract 1

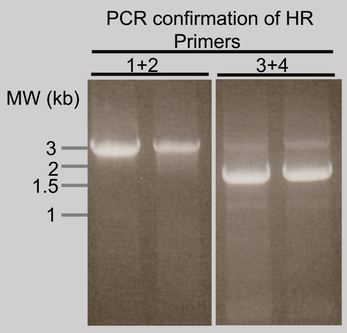
Here we report two new sets of resources for the research community. The first are a set of *Drosophila* cell lines with attP sites at specific genetic loci (Figures 1 and 2). Several *Drosophila* lines with attP sites are now available for researchers to generate transgenic flies. Nevertheless, replicating *in vivo* experiments in *Drosophila* cell lines currently is limited to transient transfection with the gene of interest. To provide a more controlled experimental setup, we are creating clonal *Drosophila* cell lines with attP integration sites using CRISPR/Cas9 gene editing. The insert has attP sites flanking Act::dsRed, enabling the positive selection of clones with attP sites. The genomic loci 25C6 (2L) and 99F8 (3R) were chosen based on: 1) The transgenic efficiency in flies, 2) non-coding loci and 3) the usage of the loci amongst researchers. We present data outlining the creation and verification of the attP sites at these loci in S2R+, S2-DGRC, Kc167, DmBG2-c2 and DmBG3-c2 cell lines. To test the functionality of the inserted attP sites, we have also created two GFP expressing constructs flanked by attB. Successful cassette exchange was achieved with one of the attB cassettes. In the long-term, we aim to generate S2R+, S2-DGRC, Kc167, DmBG2-c2, DmBG3-c2 and OSS cell lines carrying the attP>>Act::dsRed<<attP cassette at these two genetic loci, functionally test and distribute them along with the exchange cassettes.

Abstract 2

The second set includes cell lines that don't require fetal calf serum (FCS) for growth (Figures 3 and 4). Historically, the inability to generate large amounts of insect hemolymph led researchers to supplement insect cell culture media with FCS. While *Drosophila* cells grow well with FBS supplemented culture, it is not physiological, is expensive and prone to batch variations. To circumvent these issues, we investigated the possibility of serum-free *Drosophila* cell culture by supplementing the media with fly extract (FEX, an extract from Oregon R adult flies), a more physiologically relevant source to support insect cell growth. Cells (S2R+, S2-DRSC and Kc167) were step-wise adapted to M3 media containing decreasing amounts of FCS with concomitant increase in FEX. We report our results on adapting *Drosophila* cells to serum-free growth conditions, and suitability for transient transfections, in conjunction with transcriptomic analyses.

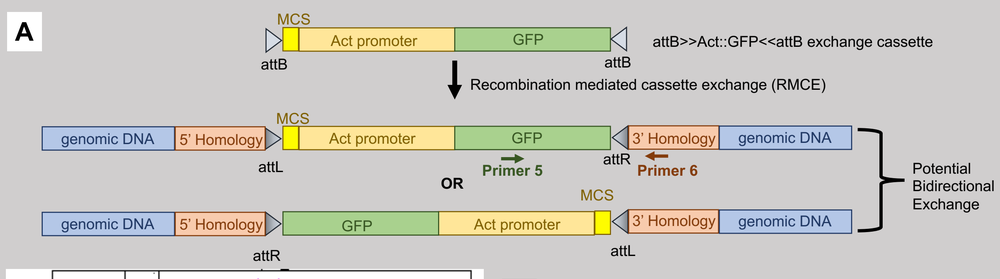
1. Generation of attP>>Act::dsRed<<attP *Drosophila* cell lines

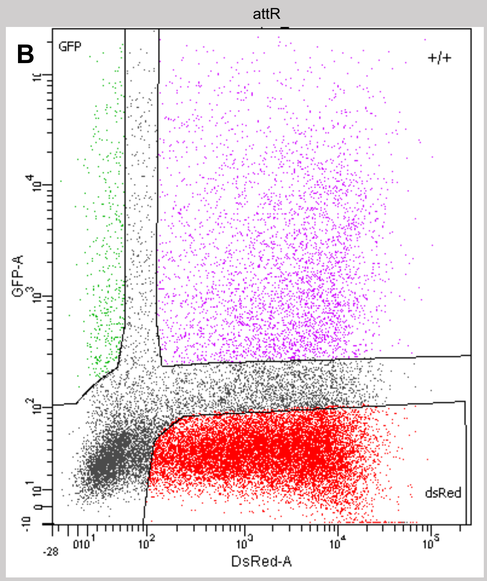


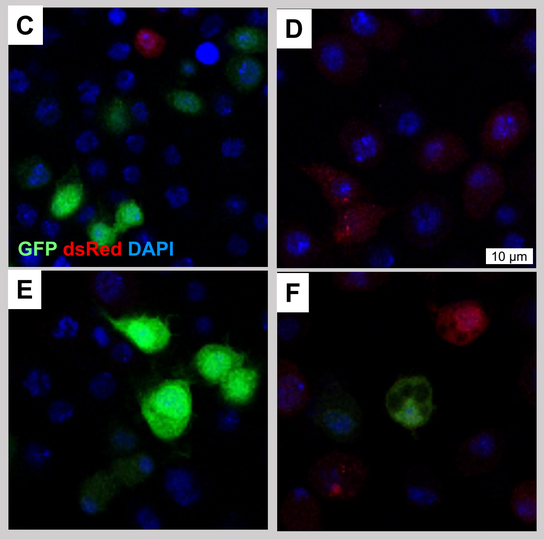


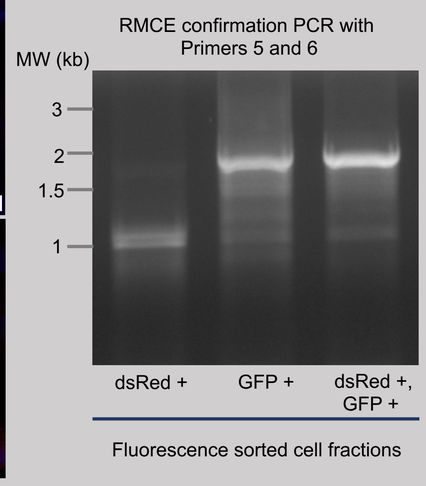
Schematic for generation of S2R+ 99F8 attP>>Act::dsRed<<attP cell line (A). Transfections with the Act::Cas9, gRNA and attP>>Act::dsRed<<attP homologous repair (HR) constructs were carried out to generate S2R+ 99F8 attP>>Act::dsRed<<attP cell line. To confirm HR, genomic DNA from a single cell clone obtained by fluorescent cell sorting was subjected to PCR with primer pairs 1+2 and 3+4, respectively (B). Both the reactions were run in duplicate. The amplicons were sequence verified to ascertain the presence of the inserted DNA.

2. Demonstration of the cassette exchange functionality of the generated attP sites in *Drosophila* cells









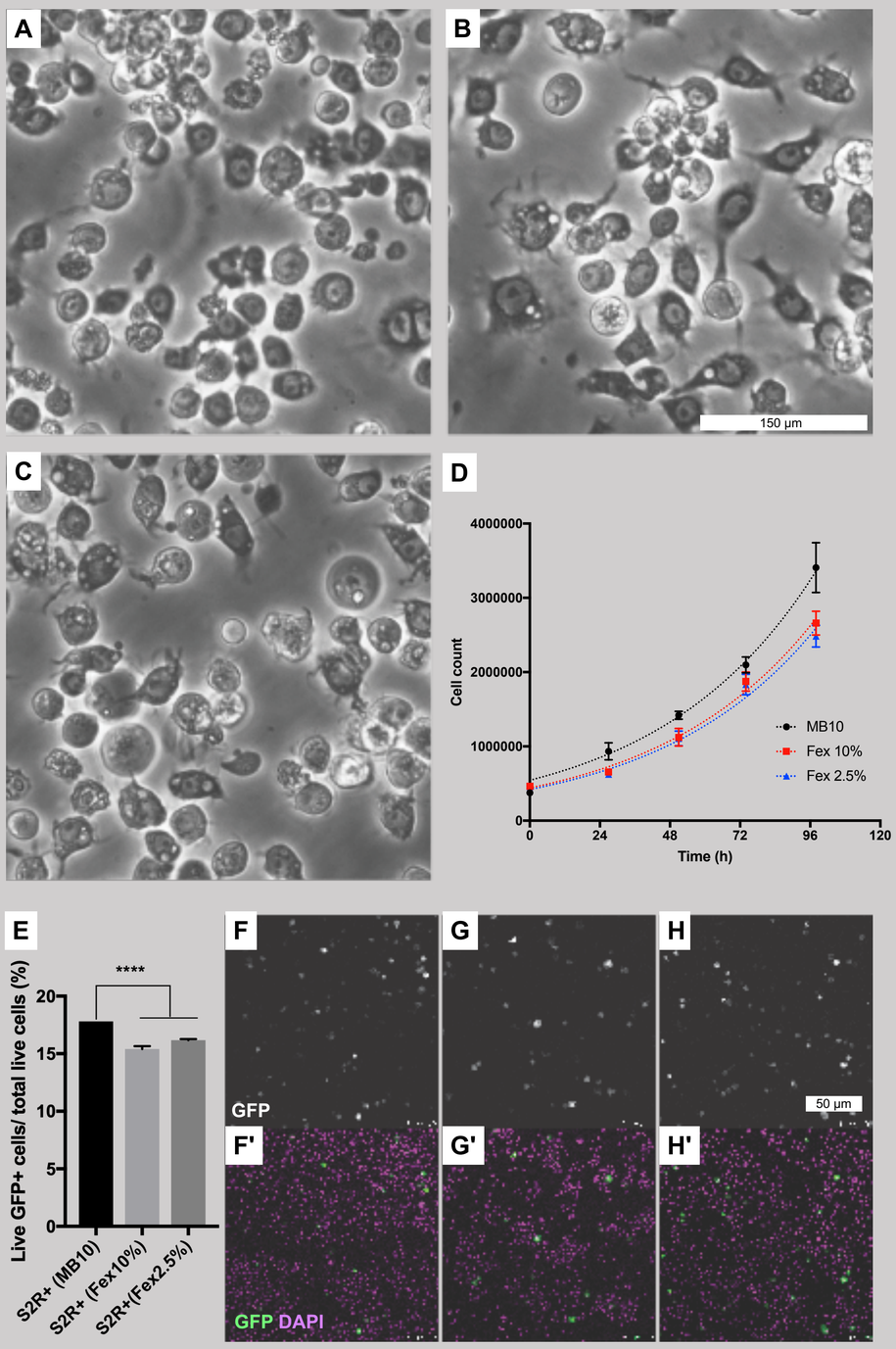
Schematic to generate recombination mediated cassette exchange (RMCE) of S2R+ 99F8 attP>>Act::dsRed<<attP single cell clones (A, generated as per Fig. 1). RMCE with the attB>>Act::GFP<<attB construct can potentially lead to exchange in either orientation. S2R+ 99F8 attP>>Act::dsRed<<attP single cell clones cells were transfected with attB>>Act::GFP<<attB construct. The transfected cells (C) were fluorescent sorted (B) and the dsRed positive (D), GFP positive (E), dsRed, GFP double positive (F) sorted fractions were imaged after immunostaining with GFP and dsRed antibodies. To confirm RMCE, genomic DNA from the fractions (as indicated) were subjected to PCR with primers 5 and 6 (Fig. 2A). The amplicons were sequence verified to ascertain the cassette exchange. MCS: Multiple Cloning Site.

Cell line	2L 25C6		3R 99F8	
	dsRed attP SCC	RMCE	dsRed attP SCC	RMCE
Dm-BG2-c2	Confirmed	To confirm	NA	NA
Dm-BG3-c2	Confirmed	To confirm	Confirmed	To confirm
Kc167	Confirmed	Confirmed	Confirmed	Confirmed
S2 DGRC	Confirmed	Confirmed	NA	NA
S2R+	NA	NA	Confirmed	Confirmed

Summary of attP cell lines generated so far

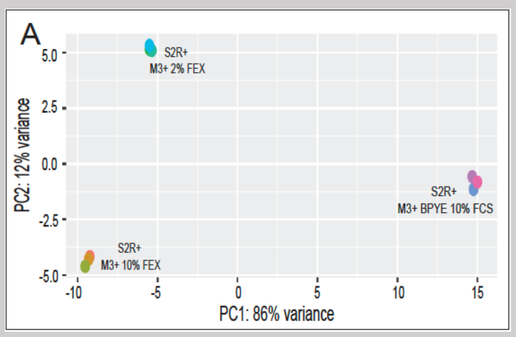
attP lines at the indicated genomic loci have been generated for the listed cell lines. Single cell clones (SCC) were confirmed by PCR + sequencing from genomic DNA of dsRed positive clones derived by fluorescent activated cell sorting (FACS). Recombination Mediated Cassette exchange (RMCE) was confirmed by FACS followed by PCR of the various fractions generated (Fig. 2G). Presence of GFP, indicating successful RMCE was confirmed by sequencing the PCR products. NA: Not available

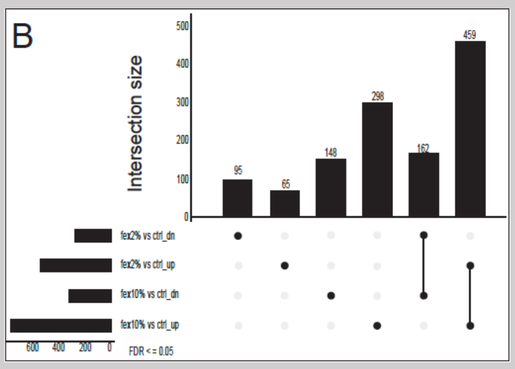
3. Absence of serum does not alter the transfection efficiency of S2R+

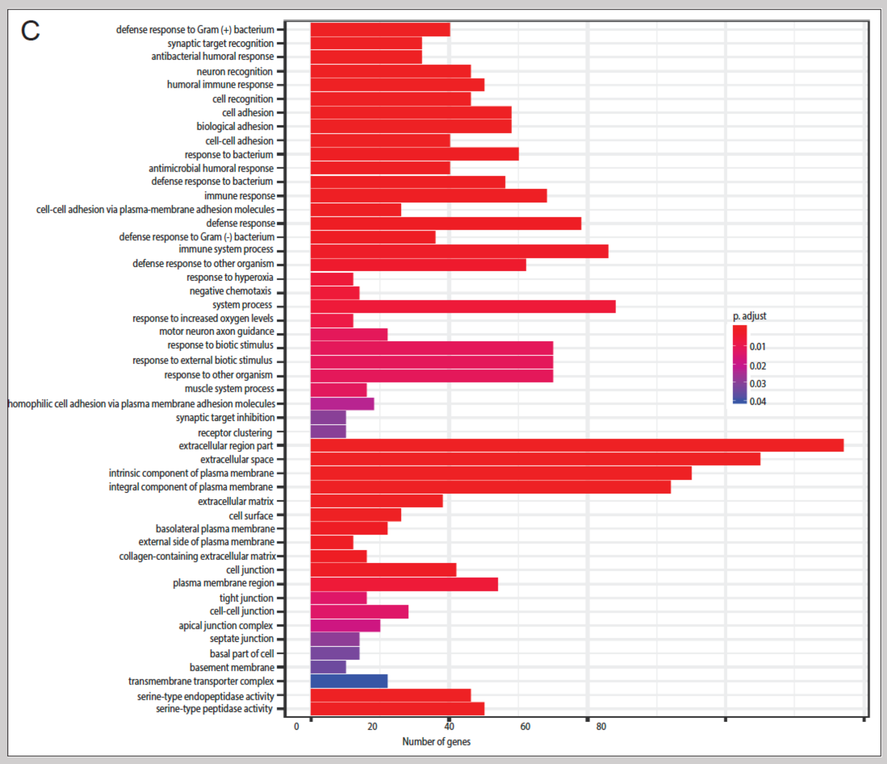


The morphology of S2R+ cells grown in M3+BPYE+10% FCS (MB10, A), M3+10% FEX (B) and M3+2.5% FEX (C) is comparable. The growth curves of S2R+ grown in three distinct media as indicated (D). Transfection efficiency of S2R+ cells grown in three distinct media as determined by flow cytometry (E). Micrographs of S2R+ cells transfected with tub-GAL4 and UAS-GFP grown in M3+BPYE+10% FBS (F,F'), M3+10% FEX (G,G') and M3 + 2.5% FEX (H,H'). (**** denotes p <0.001).

4. Transcriptomic analysis reveals minimal disruption of intracellular signaling







(A)The Principal Component Analysis (PCA) score plots of RNA-sequencing data show that the transcriptome of S2R+ cells cultured in Fly extract (FEX) supplemented media is significantly different from that of the Fetal Calf Serum (FCS) supplemented control cells. (B) An UpSet plot displaying the intersection sizes of the number of differentially regulated genes across multiple conditions. Each column represents a set and each row corresponds to one segment in a Venn diagram. Light gray circles indicate that the set is not part of an intersection. Filled circles indicate that the set is participating in the intersection. (C) GO enrichment analysis (biological function) of transcripts significantly upregulated in cells cultured in FEX supplemented media.

Acknowledgments

- **Chris Hemmerich – web support**
- **DGRC Advisory Board Members**
- **IUB FCCF – Christiane Hassel – Flow Facility**
- **IU Light Microscopy Imaging Facility**
- **Bloomington *Drosophila* Stock Center**
- **Flybase**
- **All the donors and the *Drosophila* community**

Interested in authenticating your *Drosophila* cell line?

We are currently **developing a cell authentication method** for *Drosophila melanogaster* cell lines. The aim of developing this cell authentication method will be to provide a standard/platform for the *Drosophila* research community to authenticate the cell lines as per guidelines from NIH and other funding agencies across the world. In order to validate our cell authentication pipeline, we are at this point acquiring blinded *D. melanogaster* cell line genomic DNA. We are specifically looking for *D. melanogaster* cell lines that were either obtained from a source other than DGRC or obtained from DGRC at least 5 years ago. If you would like to contribute to this endeavor or for any other interests/queries, get in touch: dgrc@indiana.edu

Funding

National Institutes of Health
Turning Discovery Into Health

DGRC is supported by NIH grant 2P40OD010949