and the locomotor activity that occurs from one frame to the next in rows. In the standard 28 minute videos at 10 FPS collected in my lab, 16,800 jpgs are generated, which results in 16,799 rows × 20 columns outputted by the script. A third perl script is then used to bin the data outputted from the "quantify630" script into more manageable chunks. Baseline activity, area under the curve scores, and other descriptive data are then calculated from these extracted data. The data outputted by the quantify630 script can also be copied and pasted directly into a spreadsheet for analysis, although this operation can be extremely unwieldy if a large number of frames is analyzed.

The second method uses the "MTrack3" plugin for ImageJ. Given a sequence of frames, this plugin detects all objects (subjects) above a user-defined threshold and assembles tracks for each object across frames as a series of time-stamped x-y coordinates. Similar to the commercially available DIAS, empirically-derived values are used to specify the size of the objects to be tracked, the minimum number of frames a single object must be tracked, and the maximum distance that a single object can move from frame to frame and still be counted as the same object. We run MTrack3 in a macro that imports a user-defined number of frames according to a user-defined array of time points (*e.g.*, frames 1-200, 201-400, 401-600). This macro analyzes all of the experiments contained in a directory and outputs the data into separate directories corresponding to the names of the experiments. These data are analyzed using an Awk script, which groups individual object tracks by tube, calculates the total distance moved by all of the flies in a tube at each time point, and divides the total distance by the number of objects and frames that each object was tracked.

While these two methods of analysis produce comparable results under most conditions, the underlying methods of analysis are different and can produce different results. We have found that the nested_window script can produce unexpected results when comparing subjects that differ in body size, such as can occur when comparing strains or male and female flies. This occurs because larger flies will necessarily produce more white pixels from frame to frame than will smaller flies, resulting in larger flies showing more apparent movement than smaller flies between any two frames. To correct this, observed distances traveled can be corrected for body weights by division or analysis of covariance. In contrast, the Mtrack3 plugin calculates movement as the location of the centroid of an object in one frame compared to the location of the same centroid in the next frame. Consequently, this method seems to be less sensitive to differences in body size of the subjects. The scripts and macros used for either of these analyses are available upon request.

Acknowledgments: I would like to acknowledge Tom Kliethermes for modifying the quantify630 script to work with large numbers of files, as well as for writing the script to extract binned activity data generated by this script. And for just being generally swell.

References: Ramazani, R.B., H.R. Krishnan, S.E. Bergeson, and N.S. Atkinson 2007, J. Neurosci. Methods 162: 171–179; Wolf, F.W., A.R. Rodan, L.T.-Y. Tsai, and U. Heberlein 2002, J. Neurosci. 22: 11035–11044.

Co-injected ΦC31 transgenes frequently produce multiple independent germline transformation events in a single *D. melanogaster* embryo.

Albanus, Ricardo D'Oliveira[1]*, David S. Lorberbaum[1,2]*, Andrea I. Ramos[1,2], and Scott Barolo[1,3]. [1]Department of Cell & Developmental Biology and [2]Program in Cellular and Molecular Biology, University of Michigan Medical School, Ann Arbor, MI 48109, USA. [3]Corresponding author: sbarolo@umich.edu. *These authors contributed equally.

Introduction

Injecting *Drosophila melanogaster* embryos with P element or ΦC31 plasmid vectors to generate single-copy transgenic fly lines is a common and well-established procedure (*e.g.*, Rubin and Spradling, 1982; Bischof *et al.*, 2007). Today, many labs employ outside injection services, which charge by the construct, to

generate transgenic fly lines. In standard transformation protocols, transgenic constructs are injected one at a time. However, given the generally high efficiency of ΦC31 transformation, multiple plasmids can be mixed and co-injected, reducing time, effort, and expense compared to multiple single-construct injections. Here, we demonstrate that single embryos injected with a mixture of three ΦC31 vectors can, at a high frequency, produce individual offspring bearing each of the three transgenes. In other words, at least three independent lines, carrying different transgene insertions, can easily be established from the progeny of a single embryo via multiplex injection.

Methods

Three short synthetic DNA constructs of equal length (186 bp) were Gateway-cloned into the 11-kb ΦC31 transformation vector pHPdest-eGFP (Boy *et al.*, 2010) as previously described (Swanson *et al.*, 2008; Ramos and Barolo, 2013). The DNA sequences of the three transgenic constructs, referred to here as A, B, and C, differed from one another by four base pairs. A mixture containing an equal amount (16 µg) of each construct was sent to the commercial service provided by Rainbow Transgenic Flies (Camarillo, CA) for microinjection into the ΦC31 landing site 86Fb on chromosome arm 3R (Bischof *et al.*, 2007). Injection stock: *y w* M{eGFP.vas-int.Dm}ZH-2A; +/+; M{RFP.attP}ZH-86Fb.

Injected females were crossed to w^{1118} males, and the progeny were screened for red eye color, indicating expression of the *mini-white* marker gene from the integrated transgenic vector. We selected three red-eyed male progeny from each of these crosses and individually crossed them to w^{1118} virgin females to establish stocks. After several days, we recovered each red-eyed male, extracted its genomic DNA (Gloor *et al.*, 1993), and amplified the insert sequences with primers anchored in vector DNA (primer sequences are available on request). We then sequenced the PCR products using the same primers (University of Michigan Sequencing Core) and analyzed the sequence with Lasergene software.

Table 1. Transgene sequencing results. Each row shows the identity of the transgene in three male progeny derived from a female embryo co-injected with transgenes A, B, and C.

Injected female	Transformed F_1 #1	Transformed F_1 #2	Transformed F_1 #3
1	**B**	**C**	**A**
2	**A**	**A**	**C**
3	**B**	**B**	**B**
4	**B**	**C**	**C**
5	**A**	**B**	**C**
6	**B**	**C**	**A**

Results

Of 21 viable *y w* females co-injected with transgenes A, B, and C and then crossed to *w* males, 12 produced one or more transformant (red-eyed) progeny. Injected males were not examined in this analysis. Sequencing of PCR-amplified transgenes from genomic DNA (see Methods) revealed the identity of the transgene integrated into the progeny of injected flies. Three red-eyed offspring derived from each of six injected females were genotyped (Table 1). Of the six injected females followed in this analysis, three (#1, 5, 6) produced three males bearing three different transgenes. Two (#2, 4) produced two males with one transgene and one with another, while one female (#3) produced three males carrying the same transgene. The observed frequencies did not significantly deviate from a null assumption of no bias in transgene frequencies derived from a given injected embryo (chi-square test, $p > 0.995$).

Progeny from two additional females, who only produced two red-eyed offspring each, were also genotyped. In each case, both of the offspring carried the same transgene (not shown). This result is consistent with the possibility that these injected embryos, which gave rise to fewer red-eyed offspring, may have undergone transformation in only one germline cell. If so, a relatively high transformation rate is likely necessary in order to achieve the high frequency of "co-transformation" observed here.

Discussion

Our results demonstrate that individual germline cells within an injected embryo can be transformed with different ΦC31 transgenes at a very high frequency, and thus that multiple independent transgenic lines

can be derived from a single injected embryo. Injection of a mixture of transgenic plasmids therefore provides a fast and cheap method of generating multiple transformed fly lines with a relatively small number of microinjections.

The co-injection method requires that transformed progeny be individually genotyped, but this does not slow down the crosses, as red-eyed flies are genotyped after mating. More importantly, the transgenes of all transformed lines should be sequenced, to rule out human error and acquired mutations, regardless of the method of injection. For example, of the 18 flies genotyped here, one showed evidence of mutations in the "A" transgene that was not present in the injected "A" DNA, or in any other fly bearing the same transgene. This may be the result of a PCR amplification error, but alternatively it may reflect a DNA mutation occurring before or after transgene insertion. Mutations aside, without genotyping it is impossible to rule out the possibility that the DNA or the flies could have been mislabeled, either in the lab or by the injection company.

The results presented here almost certainly underestimate the frequency of independent transformation events in different germline cells within a multiplex-injected embryo, for two reasons. First, only three progeny were selected for genotyping; sequencing of additional red-eyed progeny could only have increased the count of embryos giving rise to progeny bearing all three transgenes. Second, only three transgenic vectors were co-injected; it is possible that, for example, the three "B" progeny of injected embryo #3 represent three independent transformation events within that embryo. Taking this into account, it is possible that the number of co-injected plasmids could be increased significantly, further reducing the number of embryos to be injected.

References: Bischof, J., R.K. Maeda, M. Hediger, F. Karch, and K. Basler 2007, Proc. Natl. Acad. Sci. U.S.A. 104: 3312–3317; Boy A.L., Z. Zhai, A. Habring-Müller, Y. Kussler-Schneider, P. Kaspar, and I. Lohmann 2010, Genesis 48: 452–456; Gloor, G.B., C.R. Preston, D.M. Johnson-Schlitz, N.A. Nassif, R.W. Phillis, W.K. Benz, H.M. Robertson, and W.R. Engels 1993, Genetics 135: 81–95; Ramos, A.I., and S. Barolo 2013, Philos. Trans. R. Soc. Lond. B 368: 2013-18; Rubin, G., and A. Spradling 1982, Science 218: 348–353; Swanson, C.I., T. Hinrichs, L.A. Johnson, Y. Zhao, and S. Barolo 2008, Gene 408: 180-186.

An efficient and cheap entomological aspirator to collect mycophylic and anthophilic adult Drosophilidae flies.

Machado, Stela[1], João Pedro Junges dos Santos[1], Lizandra Jaqueline Robe[1,2], and Elgion Lucio da Silva Loreto[1]. [1]Universidade Federal de Santa Maria (UFSM), Programa de Pós Graduação em Biodiversidade Animal (PPGBA), Avenida Roraima, 1000, CEP 97105-900 - Santa Maria, RS - Brazil, Phone: +55 55 3220-8912; [2]Universidade Federal do Rio Grande (FURG), Programa de Pós-Graduação em Biologia de Ambientes Aquáticos Continentais (PPGBAC), Instituto de Ciências Biológicas – ICB, Av. Itália, km 08, Campus Carreiros, CEP 96203-900 - Rio Grande, RS – Brazil, Phone: +55 53 3293-5128; Corresponding author: Lizandra Jaqueline Robe, lizbiogen@gmail.com.

Introduction

Traditionally, the methodology used to collect drosophilids in Brazil relies on flies' attraction to traps baited with resources, principally fermented fruits like banana (Tidon and Sene, 1988; Medeiros and Klaczko, 1999). However, this collection method attracts mainly frugivorous species of the genus *Drosophila* (Gottschalk *et al.*, 2008), providing a biased sample of subjacent biodiversity, once species with other feeding preferences are rarely recorded. In fact, *Drosophila* encompasses almost 60% of the 304 reported Brazilian drosophilid species, being followed by far by the mycophylic *Zygothrica* (with 54 species) and *Hirtodrosophila* genera (with only 16 species) (Gottschalk *et al.*, 2008).

Species of *Hirtodrosophila*, *Mycodrosophila*, *Paraliodrosophila*, and *Zygothrica* encompass the putatively monophyletic *Zygothrica* genus group (Grimaldi, 1990), which presents different degrees of association with macroscopic fungi. As only part of these species use fungi as resources for feeding or