Tools Allowing Independent Visualization and Genetic Manipulation of Drosophila melanogaster Macrophages and Surrounding Tissues

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ABSTRACT Drosophila melanogaster plasmatocytes, the phagocytic cells among hemocytes, are essential for immune responses, but also play key roles from early development to death through their interactions with other cell types. They regulate homeostasis and signaling during development, stem cell proliferation, metabolism, cancer, wound responses, and aging, displaying intriguing molecular and functional conservation with vertebrate macrophages. Given the relative ease of genetics in Drosophila compared to vertebrates, tools permitting visualization and genetic manipulation of plasmatocytes and surrounding tissues independently at all stages would greatly aid a fuller understanding of these processes, but are lacking. Here, we describe a comprehensive set of transgenic lines that allow this. These include extremely brightly fluorescing mCherry-based lines that allow GAL4-independent visualization of plasmatocyte nuclei, the cytoplasm, or the actin cytoskeleton from embryonic stage 8 through adulthood in both live and fixed samples even as heterozygotes, greatly facilitating screening. These lines allow live visualization and tracking of embryonic plasmatocytes, as well as larval plasmatocytes residing at the body wall or flowing with the surrounding hemolymph. With confocal imaging, interactions of plasmatocytes and inner tissues can be seen in live or fixed embryos, larvae, and adults. They permit efficient GAL4-independent Fluorescence-Activated Cell Sorting (FACS) analysis/sorting of plasmatocytes throughout life. To facilitate genetic studies of reciprocal signaling, we have also made a plasmatocyte-expressing QF2 line that, in combination with extant GAL4 drivers, allows independent genetic manipulation of both plasmatocytes and surrounding tissues, and GAL80 lines that block GAL4 drivers from affecting plasmatocytes, all of which function from the early embryo to the adult.

KEYWORDS macrophage plasmatocyte hemocyte FACS imaging Genetics of Immunity

Drosophila plasmatocytes are well known for their immune functions in combating bacteria, fungi, and viruses through phagocytosis and siRNA production (Braun et al. 1998; Elrod-Erickson et al. 2000; Lemaitre and Hoffmann 2007; Tassetto et al. 2017). Yet recent years have revealed the many ways in which they also play crucial roles in development and homeostasis, contacting and exchanging signals with surrounding cells. This has expanded the repertoire of functions that plasmatocytes are known to carry out to protect the organism; their patrolling serves not only to detect and destroy foreign invaders, but also to assess defects in endogenous cell states and stimulate corrective cellular responses. Many of the processes they affect and the molecular pathways they use to do so are conserved with vertebrate macrophages, making Drosophila plasmatocytes an excellent model system (Wynn et al. 2013; Ratheesh et al. 2015).

Plasmatocytes influence development in several different ways. They migrate widely in the embryo to phagocytose and thus clear cells that have undergone programmed cell death (Tepass et al. 1994; Zhou et al.
1995; Franc et al. 1996). As they move, plasmatocytes secrete extracellular matrix (ECM) components, which assemble into a stable basal lamina whose presence affects later steps in development (Fessler and Fessler 1989; Olofsson and Page 2005; Martinek et al. 2008; Matsubayashi et al. 2017). This effect can occur by the ECM providing a substrate for cell movement or by binding Dpp, a BMP family member, and influencing its signaling (Olofsson and Page 2005; Bunt et al. 2010; Van De Bor et al. 2015). These developmental functions are conserved in vertebrates. Vertebrate macrophages also engulf apoptotic cells during development (Gouon-Evans et al. 2000; Leers et al. 2002), and show molecular conservation with Drosophila in some of the receptors they use to recognize dying cells (Franc et al. 1996; Fadok et al. 1998; Manaka et al. 2002; Greenberg et al. 2006; Kurucz et al. 2007; Wu et al. 2009). Vertebrate macrophages secrete the ECM component collagen (Schnoor et al. 2008), which can bind BMP family members (Vukicevic et al. 1994; Sieron et al. 2002).

Plasmatocytes are also crucial for maintaining the organism after it has formed. They alter responses to damage in the gut, regulating stem cell proliferation by secreting stimulatory factors (Ayyaz et al. 2015; Chakrabarti et al. 2016). Plasmatocytes kill tumor cells by expressing TNFα (Parisi et al. 2014), or stimulate their invasion if tumors also express activated Ras, through MMP1 induction by TNFα-induced JNK signaling (Cordero et al. 2010; Pérez et al. 2017). Plasmatocytes can even alter metabolism and aging: upon engulfing lipids, they induce JAK-STAT signaling in surrounding tissues, which modulates insulin sensitivity, hyperglycemia, fat storage, and lifespan (Woodcock et al. 2015). Conservation with vertebrates is seen for these processes as well. Vertebrate macrophages alter gut stem cell proliferation to promote regeneration; they may also use BMP to do so as BMP2 inducible kinase is upregulated in responding gut tissues (Pull et al. 2005). Vertebrate macrophages can promote tumor induction of MMPs and invasion by secreting TNFα (Hagemann et al. 2005). Finally, vertebrate macrophages also participate in an inflammatory response to obesity that leads to insulin insensitivity (Weisberg et al. 2003; Xu et al. 2003; Patsouris et al. 2008; Pollard 2009), as is seen in the Drosophila response to lipid ingestion (Woodcock et al. 2015). Thus, conservation is seen between vertebrates and Drosophila in the ways in which immune cells and surrounding tissues affect one another and the molecular pathways they use to do so.

The genetic power of Drosophila melanogaster can help elucidate how plasmatocytes regulate organismal development and homeostasis, and how tissues signal their state to plasmatocytes to induce responses. Yet such studies require tools that are lacking, ones that permit the live imaging or manipulation of plasmatocyte behavior along with the modulation and visualization of other cells. Here, we describe a set of tools designed to facilitate these approaches and demonstrate that they function at all stages of the Drosophila life cycle. These lines will thus greatly aid investigations of the manifold interactions of Drosophila plasmatocytes with other tissues from birth to death, enabling insights that can be relevant for vertebrate systems.

**MATERIALS AND METHODS**

**Cloning**

Standard molecular biology methods were used, and all constructs were first tested for functionality by transfection into the plasmatocyte-like S2 cell line (Schneider 1972; Woodcock et al. 2015) (a gift from Frederico Mauri of the Knoblich laboratory at IMBA, Vienna) before injection into flies. Restriction enzymes BsuRI, PstI, and AscI were obtained from New England Biolabs (Frankfurt, Germany); XbaI and EcoRI were from Promega (Mannheim, Germany). PCR amplifications were performed with CloneAmp HiFi PCR Premix from Clontech’s European distributor Takara Bio Europe (Saint-Germaine en Laye, France) using a peqSTAR 2x PCR machine from PEQLAB (Erlangen, Germany).

All Infusion cloning was conducted using an Infusion HD Cloning kit obtained from Clontech’s European distributor (see above); relevant oligos were chosen using the Infusion primer tool at the Clontech website http://bioinfo.clontech.com/convertPcrPrimersinit.do.

**Construction of srpHemo-3XmCherry**

A 2.5 kb XbaI-EcoRI fragment, which contains three repeats of mCherry, was cloned from pHH1295 (Bakota et al. 2012; Evans et al. 2014) (a gift from Jürgen Heinisch, Addgene plasmid #36914), into the multiple cloning site of pCaSpeR4 (a gift from Leonie Ringrose, IMBA, Vienna). Subsequently, a 4.3 kb fragment of the srp promoter was amplified from plasmid srpHemoA (Brückner et al. 2004) (a gift from K. Brückner) by PCR with the following primers:

- 5’-CGAGGTCGACTCTAGAAGAATTTGATGTTTTTAATATAGCT TATCGAGCAATGCCA-3’.
- 5’-ACGAGCTTCTCTAGATATGCGATCGTGGGATGTCGAC-3’.

This fragment was cloned upstream of the 3xmCherry fragment at the XbaI site by Infusion cloning to create DSPL172.

**Construction of srpHemo-H2A::3XmCherry**

A 458 bp fragment containing the first 124 amino acids from histone H2A was amplified from pKS23b, a gift from Kristen Senti and Julius Brennecke at IMBA, using the following primers:

- 5’-AGAGAAGGCTTGTAGACGATGTAGGGAAGAAAG-3’.
- 5’-GCAGCTTCGACGCTACGGGTACGGCGCGCCCTCTAGACACTT-3’.

This fragment was placed by Infusion cloning at the BsiWI site by Infusion cloning to create DSPL172, downstream of the srp promoter and upstream of the 3XmCherry fragment, with the linker sequence SGGGRRTRTLQV to create DSPL216.

**Construction of srpHemo-moe::3XmCherry**

An 869 bp fragment from the Moesin cDNA SD10366 (DGRC) (Rubin et al. 2000) containing amino acids 370–646, and thus the ERM domain of the protein, was amplified by PCR using the following primers:

- 5’-AGAGAAGGCTTCTAGGATGCGACCATACGATGTCGAC-3’.
- 5’-GCAGCTTCGACGCTACGGGTACGGCGCGCCCTCTAGACACTT-3’.

This fragment was cloned as above at the BsiWI site in DSPL172, downstream of the srp promoter and upstream of the 3xmCherry fragment, with the linker MRTLQVD.

**Construction of srpHemo-QF2**

A 4.3 kb fragment containing the srpHemo promoter was amplified from the srpHemoA plasmid (Brückner et al. 2004) (a gift from K. Brückner) using the following primers:

- 5’-TTATGCTAGGGATCCAAATTTGATGTTTTTAATATAGCTTAT CAGCAAT GCGCA-3’.
- 5’-TGGCAGTGTGGAATCTCTATGGGGATGTCGTCGTTGGGATGTCGAC-3’.

This fragment was used to replace the synaptobrevin promoter in the nsys-QF2 plasmid (Riabinina et al. 2015) (a gift from C. Potter). The synaptobrevin promoter was released by a digest with BamHI and EcoRI and replaced by srpHemo using Infusion cloning.
Construction of srpHemo-GAL80

A 4.3 kb fragment of the srp promoter was amplified from plasmid srpHemoA (Brückner et al. 2004) (a gift from K. Brückner) by PCR with the following primers:

\[ 5'-GCAATGGAGACCTGGAGAAATTGATGTGGTTTTAAATAGTCITATCAGCACATTGC\AA-3' \]

\[ 5'-CTTCCTGCAAGGCCTGCCCAATCAATGATTACAACAAAAAGGAG-3' \]

\[ 5'-CGGTGCCTAGGCGCGCCTACCGGTAGGACAATAACAAAAGAGTACATGATAAGATAAATTGCAG-3' \]

This fragment was cloned by infusion into the (w+) attB plasmid (a gift from Jeff Sekelsky, Addgene plasmid #30326) at the XhoI site to create DSPL237.

A 1307 bp fragment containing GAL80 was amplified with the following primers from pAC-GAL80 (Potter et al. 2010; a gift from Liqun Luo, Addgene plasmid #24346):

\[ 5'-CTCCTGCAAGGCCTGCCCAATCAATGATTACAACAAAAAGGAG-3' \]

\[ 5'-CGGTGCCTAGGCGCGCCTACCGGTAGGACAATAACAAAAGAGTACATGATAAGATAAATTGCAG-3' \]

This fragment was introduced into DSPL237, downstream of the srp fragment at the Ascl site, using Infusion cloning to create DSPL322.

Drosophila melanogaster stocks

Flies were raised on standard agar, cornmeal, molasses, and yeast food containing 1.5% Nipagin bought from IMBA (Vienna, Austria). Adults were placed in cages in a Percival DR 36VL incubator maintained at 21°C and 65% humidity, and embryos were collected on standard plates prepared in house from apple juice, sugar, agar, and Nipagin, and treated with yeast from Lesaffre (Marcq, France). This applies to all fly husbandry conditions as described below. repo-GALA and QUAS-CDB:GFP were obtained from the Bloomington Drosophila Stock Center, UAS-moc:mCherry from P. Martin (Millard and Martin 2008), hml-dsRed from K. Brückner, and srp-GALA UAS-2xGFP from R. Reuter.

Embryo immunohistochemistry

Embryos were fixed with a standard 18.5% formaldehde/heptane fix for 20 min followed by methanol devitellinization. mCherry embryos were visualized directly after fixation, rehydration, and mounting.

srp-moc:GFP embryos were rehydrated and underwent antibody staining, using standard protocols and overnight incubation with a 1:500 dilution of GFP antibody (Aves Labs, Tigard, OR), followed (after washing) by incubation for 2 hr with a 1:500 dilution of Goat anti-Chicken Alexa Fluor 488 secondary (Invitrogen, Carlsbad, CA). Embryos to be stained by incubation for 2 hr with a 1:500 dilution of Goat anti-Chicken Alexa Fluor 488 secondary antibody (Invitrogen). After washing, they were mounted in Vectashield Mounting Medium (Vector Labs, Burlingame) on 76 x 26 mm slides from Glasfabrik Karl Hecht (Sondheim, Germany) with 22 x 40 mm coverslips, No. 1 thickness (VWR International, Radnor, PA).

Microscopy

Embryo images were taken with an Inverted LSM700 Confocal Microscope from Zeiss (Jena, Germany), using a Plain-Apochromat 20x/NA 0.8 Air Objective. Larvae and adult flies were imaged with a Leica M205 FA Stereo Microscope, a Leica Planapo 2.0x objective, and a Leica DFC3000G camera (Wetzlar, Germany). Larvae were anesthetized for 10-15 sec with a FlyNap Anesthetic Kit (ArtNr 173010, Carolina Biological Supply Company, Burlington, NJ), rinsed 2x in water, then examined under the stereomicroscope. Adult flies were anesthetized for 3 min in FlyNap, and then immediately examined under the stereomicroscope. For imaging on the confocal, larvae and adults were prepared as described, and then mounted in Halocarbon 200 oil (CatNr: 25073-100, Polysciences Inc., Warrington, PA) in a sandwich of a plastic frame, a YSI 5685 Membrane Kit 002 (ArtNr: 1518-9862, Yellow Springs Instrument Co., Yellow Springs, OH) and a cover glass (CatNr: 631-014724X50 mm, thickness 1.5, VWR) immediately prior to visualization.

Macrophage cell counts

Embryos were analyzed at stage 15–16 for total plasmacyte number using Imaris (Bitplane) by detecting all the plasmacyte nuclei as spots.

Time-lapse imaging

For the srpHemo-H2A::3xmCherry time-lapse movies, embryos were dechorionated in 50% bleach for 4 min, washed with water, and mounted in halocarbon oil 27 (Sigma) between a coverslip and an oxygen-permeable membrane (YSI), as described above. The anterior dorsolateral region of the embryo was imaged on an inverted multi-photon microscope (TrimScope II, LaVision) equipped with a W Plan-Apochromat 40x/1.4 oil immersion objective (Olympus), mCherry was imaged at 1100 nm excitation wavelengths, using a Ti-Sapphire femtosecond laser system (Coherent Chameleon Ultra) combined with optical parametric oscillator technology (Coherent Chameleon Compact OPO). Excitation intensity profiles were adjusted to tissue penetration depth and Z-sectioning for imaging was set at 1 μm for tracking. For long-term imaging, movies were acquired for 180-200 min with a frame rate of 40 sec. All embryos were imaged with a temperature control unit set to 28°C.

For the srp-QF2 QUAS-mCD8::GFP repo-GALA UAS-moc:mCherry time-lapse movies, flies were left to lay eggs on grape juice/agar plates overnight at 25°C. Embryos were dechorionated in bleach. Stage 15 embryos of the appropriate genotype were identified based on the absence of balancer chromosomes expressing fluorescent markers, and mounted in 10S Voltatef oil (VWR) between a glass coverslip and a gas-permeable Lumox culture dish (Greiner), as described previously (Milchanowski et al. 2004; Evans et al. 2010). Movie images were taken at room temperature every 15 min on an Ultraview spinning disk microscope (PerkinElmer) equipped with a 20x NA 0.5 Plan-Neofluar air objective. Maximum projection images were made from ~40 μm of Z stacks taken every 3 μm. Image processing was done by using ImageJ.

For the srpHemo-moc::3xmCherry time-lapse movies, embryos were dechorionated in bleach for 1:15 min, and stage 15 embryos were identified and mounted in a slide covered with a double-sided sticky tape, oriented ventrally, and covered with 105 Voltatef oil (VWR) and a glass coverslip, as described in Stramer et al. (2010). Movie images were taken at room temperature every 5 sec on a Zeiss LSM 880 microscope, using Airyscan and a 63x/1.40 Oil DIC objective. Maximum projection images were made from ~17 μm of Z stacks taken every 1 μm. Image processing was done by using ImageJ.

Transgenic line production

The srpHemo-GAL80 construct was injected into lines y¹ M{vas-int.Dm}ZH-2A w¹ M[3xP3-RFP.attP]ZH-51D (BL 24483) and y¹ M{vas-int.Dm}ZH-2A w¹ M[3xP3-RFP.attP]ZH-865F (BL 24749), obtained from Peter Duchen of IMBA, to produce inserts on the second and third chromosomes through C31-mediated integration (Bischof et al. 2007). Our srpHemo-QF2 driver was injected into yw; p[w³, y+, attP16a (Okulski et al. 2011) to produce an insert on the second chromosome. After injection, all male survivors were crossed to w; Sp/CyO, PrDr/TM3Ser
80% of circulating P, hemese P, lateral glia St 10 glial cells missing peroxidasin hemolectin croquemort P in adult, internal tissues St 12 Rpl32: 5

Table 1 GAL4 Driver lines previously utilized for Plasmatocyte expression

| Promoter Source | Tissue Expression of Reporter | Time of UAS-Reporter Expression in Plasmatocytes | References for Creation and Expression |
|-----------------|------------------------------|-----------------------------------------------|----------------------------------------|
| serpent (srpHemo) | P, LG in larva and adult PC | St 10-17 L1+, L2+/−, L3+/− | Brückner et al. (2004), Zaidman-Rémy et al. (2012) |
| serpent (srp) | P, LG, FB, embryonic midgut, amnioserosa, larval and adult PC, larval SG | St 10-17 L1+, L2+/−, L3+/− | Crozatier et al. (2004), Milchanowski et al. (2004), Avet-Rochex et al. (2010) |
| croquemort | P in adult, internal tissues | St 12-17 | Olofsson and Page (2005) (embryo) Clark et al. (2011) (adult) |
| peroxidasin | P, LG from L2 on, in larva and adult PC, weak FB in L3 | St 12-17 L1-L3 ++ | Stramer et al. (2005) (embryo) Stofanko et al. (2008) (larva) Ghosh et al. (2015) (adult) |
| glial cells missing | P, lateral glia | St 10-17 | Bernardoni et al. (1997), Olofsson and Page (2005), Avet-Rochex et al. (2010) |
| hemese | 80% of circulating P, sessile P, sections of midgut, SG | — | — |
| hemolectin | P, LG | L2, L3 ++ | Avet-Rochex et al. (2010), Sinenko and Mathey-Prevot (2004), Woodcock et al. (2015) (adult) |
| collagen | P, LG cortical zone, and FB at all stages | St 13-17 L1-L3 | Asha et al. (2003), Avet-Rochex et al. (2010) |
| singed | P | St 11-17 | — |
| eater | P, LG | L3 | — |
| | | | Zettervall et al. (2004) |

UAS, upstream activating sequence; P, Plasmatocytes; LG, lymph gland; PC, Pericardial cells; St, stage; FB, Fat Body; SG, salivary gland.

virgins. After hatching, we screened for transformants based on eye color and crossed them again to w; Sp/Cyo, PrDr/TM3Ser virgins to get rid of the integrase inserted on the X chromosome. We kept three transformants/landing site.

All other vectors were co-injected into w1118 (BL-3605) using standard injection methods, along with a helper plasmid Δ2-3 (Robertson et al. 1988) that permits P′ element transposase expression. w′ transformants were selected and double balanced.

qPCR

RNA was isolated from ~50,000 mCherry-positive or mCherry-negative cells using an RNeasy Plus Micro Kit (Qiagen), following the manufacturer’s protocol. Of the resulting RNA, 50 ng was used for cDNA synthesis using the Sensiscript RT Kit (Qiagen) and oligo dT primers. A Takyon qPCR Kit (Eurogentec) was used to mix qPCR reactions based on the provided protocol, using the following primers:

mCherry: 5′-ACATCCCGGACTACTTTGAAGC-3′ and 5′-ACCTTGTA GTGAACTCGCCG-3′

which were designed using Primer-BLAST (https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastDescAd).

Pvr: 5′-TGACTTTTGTCTGGTCCTG-3′ and 5′-GTTCACAGCCGCAGC-3′
Rhod: 5′-CTGAGCTATCCAGTACCAA-3′ and 5′-ACACCTTGTCC GTAATACGGTT-3′
Drpr: 5′-TCCAACCTATCGATTAAACACC-3′ and 5′-ACAGTCCCT CACAATACGGTT-3′
Rpl32: 5′-AGCATACAGCGCCCAAGATCG-3′ and 5′-TGTTGTCGA TACCCCTTGGGC-3′.

These four sets of primers were obtained from FlyPrimerBank (http://www.flyrai.org/FlyPrimerBank). qPCR was run on a LightCycler 480 (Roche) and data were analyzed using LightCycler 480 Software and Prism.

FACS analysis

Embryos were collected for 1 hr from adult w−; srpHemo-3xmCherry flies and aged for an additional 4 hr, all at 29°. Embryos collected from w− flies were processed in parallel and served as a negative control. Embryos were dissociated using a procedure adapted from Estrada and Michelson (2008). Embryos were dechorionated with fresh 50% bleach for 5 min, thoroughly rinsed with water, and blotted on a dry towel. Next, 30 mg of embryos were transferred with a paintbrush into a dounce homogenizer. Subsequent procedures were carried out at 4° or on ice, and all the solutions were cooled. Homogenizers were filled with 10 ml of SeccoF saline (6 mM Na2HPO4, 3.67 mM KH2PO4, 106 mM NaCl, 26.8 mM KCl, 6.4 mM MgCl2, and 2.25 mM CaCl2 at a pH of 6.8) and embryos were homogenized with 10 vertical strokes. The resulting suspensions from three homogenizers were collected into a 50 ml Falcon tube (Corning, NY) and centrifuged at 1250 rpm for 4 min to separate out the dead cells that remained in the upper phase after centrifugation. The resulting cell pellet was resuspended in 1-2 ml of Schneider’s media and aged for an additional 4 hr, all at 29°. Embryos collected from w− flies were processed in parallel and served as a negative control. Embryos were dissociated using a procedure adapted from Estrada and Michelson (2008). Embryos were dechorionated with fresh 50% bleach for 5 min, thoroughly rinsed with water, and blotted on a dry towel. Next, 30 mg of embryos were transferred with a paintbrush into a dounce homogenizer. Subsequent procedures were carried out at 4° or on ice, and all the solutions were cooled. Homogenizers were filled with 10 ml of SeccoF saline (6 mM Na2HPO4, 3.67 mM KH2PO4, 106 mM NaCl, 26.8 mM KCl, 6.4 mM MgCl2, and 2.25 mM CaCl2 at a pH of 6.8) and embryos were homogenized with 10 vertical strokes. The resulting suspensions from three homogenizers were collected into a 50 ml Falcon tube (Corning, NY) and centrifuged at 1250 rpm for 4 min to separate out the dead cells that remained in the upper phase after centrifugation. The resulting cell pellet was resuspended in 1-2 ml of Schneider’s media with 25% (0.2 μM filtered) heat-inactivated FBS and 2 mM EDTA (to reduce calcium dependent adhesion and thus the formation of clumps). The cell suspension was filtered to remove cell clumps using a Falcon 75 mm Polystyrene tube with a cell strainer cap containing a 35 μm nylon mesh. The cells were analyzed or sorted using a FACS
**RESULTS**

Direct fusion lines visualize plasmatocyte nuclei, the cytoplasm, and the cytoskeleton in the embryo

Visualizing plasmatocytes in fixed and live specimens is essential for understanding how these cells interact with surrounding tissues. Previous studies have placed plasmatocytes by using various GAL4 drivers to activate UAS-reporters (Table 1; Evans et al. 2014). However, this approach prevents the simultaneous use of other GAL4 drivers to independently affect or image separate tissues. Direct fusions have been made of plasmatocyte-specific promoters to fluorescent proteins (Table 2; Evans et al. 2014), but none of these expressed at all stages of the life cycle. Additionally, the expression that many displayed was weak and, in some lines, was also present in large extraneous tissues, making live plasmatocyte detection and FACS analysis challenging. Therefore, we fused the srpHemo promoter that guides specific plasmatocyte expression in the embryo (Brückner et al. 2004) to three copies of mCherry (Shaner et al. 2004; Bakota et al. 2012), a red fluorescent monomer with a rapid maturation time, low photobleaching, and the ability to survive fixation with fluorescence intact. We also fused the first 124 amino acids of Histone H2A to mCherry, concentrating the signal in the nucleus to facilitate cell counting and tracking. There is little autofluorescence in the embryo in the red spectrum, and thus these srpHemo-H2A::3xmCherry lines displayed extremely brightly fluorescing plasmatocytes with little background starting at embryonic stage 8 and continuing through stage 17; the signal was still strongly visible after fixation with heat, formaldehyde, and paraformaldehyde—utilizing methanol, ethanol, or a hand-held needle to devitellinize—without any antibody staining required (formaldehyde/methanol is shown in Figure 1A). In contrast, the plasmatocyte fluorescence in the previously constructed srp-moc::GFP (Moreira et al. 2010) does not survive fixation (data not shown) and is weak when viewed live [Supplemental Material, Figure S1, A and B; asterisks in A show autofluorescent yolk granules as plasmatocytes only become evident live at stage 10 (data not shown)].

Upon staining with an antibody against GFP, plasmatocytes can be observed starting at stage 8 but are accompanied by strong extraneous expression in the amnioserosa (arrow in Figure S1, C and D), which is also seen live (arrow in Figure S1B) but not observed in live or fixed negative controls (data not shown). Thus, utilizing three copies of mCherry fused directly to the srpHemo promoter produces a plasmatocyte marker that is brightly visible in live or fixed embryos without antibody staining from early embryonic stages onwards.

We demonstrated the effectiveness of our srpHemo-H2A::3xmCherry nuclear line for tracking in live embryos by making two-photon movies of plasmatocyte migration from the head into the germband in stage 10–12 embryos (File S1) (Figure 1B). There was much less auto-fluorescence at the 1100 wavelength used for mCherry than at the 980 nm used for eGFP in the yolk and, particularly usefully, in the vitelline membrane, where absorption of laser energy through auto-fluorescence at 980 nm frequently leads to membrane rupture and death of the embryo during movie acquisition. The brightness of the mCherry signal also permitted the use of low laser power for effective imaging and thus less photobleaching. Analysis of plasmatocyte displacement based on tracking the nuclei with Imaris software revealed distinct paths of migration within the anterior, corresponding to the different directions ultimately chosen (Figure 1C). Efficient localization
of srpHemo-H2A::3xmCherry to the nucleus (Figure 1F) also permitted easy determination of total plasmatocyte cell counts from confocal images using Imaris; we detected 592 ± 48 cells (n = 24) by analyzing wild-type stage 16 embryos, somewhat less than the 700 previously counted at stage 11 based on an antibody marker (Tepass et al. 1994). Thus, the nuclear-localized mCherry permits automated plasmatocyte tracking and counting, and eliminates many of the problems that occur with live two-photon imaging of GFP.
We assessed if our srpHemo-H2A::3xmCherry line also directs expression in crystal cells. These cells are born along with plasmatocytes from the mesoderm, migrate to a location around the proventriculus, and remain there during embryogenesis (Lebestky et al. 2000). In larvae and adults, they mobilize to enhance melanization in response to wounds or wasp egg infection (Galko and Krasnow 2004; Dudzic et al. 2015). We used an antibody recognizing Lozenge, a crystal cell marker, and observed colocalization with srpHemo-H2A::3xmCherry (Figure 1, D–D”) through much of embryogenesis, but by stage 15 (Figure 1, E–E”) no mCherry colocalization was detected. We also observed extraneous expression in the stomatogastric nervous system starting at stage 16/17 (data not shown). Given that there are 35 crystal cells (Milchanowski et al. 2004) and that we detect ~600 total cells, we conclude that 94% of all embryonic cells labeled with srpHemo-H2A::3xmCherry before stage 15 are plasmatocytes.

We further created srpHemo-3xmCherry lines to produce plasmatocytes with a labeled cytoplasm (Figure 1G and Figure S1, E and E’), which are useful for studies examining direct contact of plasmatocytes with other tissues as well as their phagocytosis of pathogens and apoptotic cells. To visualize polymerized actin in plasmatocytes during studies of migration, we fused the mCherry with the C-terminal part of moesin that had been previously used to detect actin (Edwards et al. 1997). In these actin-binding srpHemo-moe::3xmCherry lines, we could detect filopodial and lamellipodial extensions within the plasmatocytes in live and fixed embryos (Figure 1H and Figure S1, F and F’), and could make time-lapse movies of plasmatocyte actin dynamics (File S2). Although the expression is weaker than in the cytoplasmic version, plasmatocytes can still be easily seen from all of these lines in fixed heterozygous embryos (Figure S1, E–G’), which allows analysis of the heterozygous progeny that arise, for example, during RNAi crosses. Heterozygotes can also be used in live imaging (nuclear line is shown in File S3, movie stills in Figure S1, H and H’). These lines were inserted at random positions on the second and third chromosomes, and are viable as homozygous embryos. Thus, our lines fusing the srpHemo promoter to 3xmCherry, either on its own or combined with other protein domains, permitted easy visualization of either the cytoplasm, nuclei, or actin cytoskeleton of plasmatocytes in the embryo in multiple contexts.

Direct fusion lines visualize plasmatocyte nuclei, the cytoplasm, and the cytoskeleton in larvae and adults

These lines also permitted clear visualization of individual plasmatocytes in larvae and adults. In larvae, the characteristic pattern of resident plasmatocytes sitting in the body wall pockets (Makhljani et al. 2011) was most easily evident live through a stereomicroscope for the cytoplasmic 3xmCherry (Figure 2A), although it was also visible in the nuclear- and actin-localized forms (Figure S2, A and B). Individual plasmatocytes from the srpHemo-3xmCherry lines were also visible in these conditions floating in the hemolymph (Figure 2B and File S4), thereby allowing detection of the fluid flow. We frequently observed clusters of floating plasmatocytes adhering to a darker nonfluorescing droplet (most visible in File S4 when examining the cells indicated with an arrow in Figure 2B). The cortical zone of the third-instar larval lymph gland was labeled by mCherry (Figure 2C) along with 40 peri-cardial cells, pairs of large (50 μM) oval cells in a repeating pattern along the dorsal vessel that allow the heartbeat to be easily visualized (Figure S2C and File S5) (Das et al. 2007). We also observed this pericardial fluorescence in two other lines that visualize plasmatocytes, srpHemo-GALA UAS-GFP (Brückner et al. 2004) and pxn-GALA UAS-GFP.
but not in \textit{hml-DsRed} \citep{Makhijani2011} (data not shown). Expression was also seen in the stomatogastric nervous system during larval stages (Figure S2D). In fixed or live larvae, plasmatocytes were also visible deep within the body, at depths of \( \leq 130 \) \( \mu \)m with confocal imaging (Figure S2, E and E’). At adult stages, cytoplasmic- (Figure 2, D and E), nuclear-, and actin-targeted \textit{mCherry}-labeled plasmatocytes (Figure S2, G–I) were visible in the head, thorax, and legs using a stereomicroscope. Confocal images of live \textit{srpHemo-3xmCherry} adults detected plasmatocytes within the body, at depths up to 94 \( \mu \)m (Figure 2, F and F’). The discovery of plasmatocytes encircling cells in the fat body (Figure 2G) is particularly interesting given recent results demonstrating their role in regulating metabolism \citep{Woodcock2015}. While in larvae the \textit{srpHemo-3xmCherry} signal is similar to that seen in third-instar \textit{hml-DsRed} larvae (data not shown), in the adult the \textit{srpHemo-3xmCherry} signal is much brighter (compare Figure S2, F’ and G’). This allows direct detection of the presence of the chromosome in adults, greatly facilitating crosses. 

\textit{srp-moe::GFP}, the other direct fusion line expressed beyond a single stage (Table 1), is much weaker than \textit{srpHemo-3xmCherry} in second- and early third-instar larvae (Figure S3, A–C), and not detectible in late third-instar larvae and adults even with a confocal microscope (Figure S3, D–K). Thus, the \textit{srpHemo-3xmCherry} lines permit visualization of...
plasmatocytes in live and fixed samples without antibody staining from the embryo to the adult.

**FACS sorting from the embryo to the adult using the direct fusion cytoplasmic line**

The srpHemo-3xmCherry line also facilitated purification of plasmatocytes by FACS. In stage 11 embryos, 2% of total cells from this line were mCherry-positive (Figure 3, A and B and Figure S4A). These cells were enriched for plasmatocyte markers such as Per, Papilin, and Rhol (Cho et al. 2002; Kramerova et al. 2003; Siekhaus et al. 2010), as assessed by qPCR (Figure 3B), but not for the broadly expressed gene Notch (Hartley et al. 1987), thus identifying the mCherry+ cells as plasmatocytes. We compared this line to the other extant direct fusion line in the red spectrum, hml-DsRed, which turns on in second-instar larvae. According to modENCODE data on FlyBase (http://flybase.org/reports/FBgn0029167.html), hemolectin is moderately expressed in L3, almost absent in pupa, and shows low expression in adults, particularly females. We did not analyze srp-moc::GFP as it had strong extraneous expression in the embryo (Figure S1, B–D), was weak in second-early third-instar larva, and showed no expression in late third-instar larva and adults (Figure S3, A–K), but the relative number of plasmatocytes was similar in srpHemo-3xmCherry and hml-DsRed in third-instar larvae (Figure 3, C and E), and we detected ≥10 times more fluorescent-positive plasmatocytes in srpHemo-3xmCherry than hml-DsRed adults (Figure 3, D and E), consistent with microscopic examination (Figure S2, F–G), indicating very weak expression of hml-DsRed at this time. Using srpHemo-3xmCherry, we identified 0.25 and 0.6% of total cells as plasmatocytes in larvae and adults, respectively (Figure 3E and Figure S4, B and C). Thus, these srpHemo-derived constructs permit in vivo visualization and efficient FACS sorting, and analysis of plasmatocytes from the embryo to the adult independent of GAL4-based expression, unlike any other extant direct fusion line.

**QF2 lines allowing genetic manipulation of plasmatocytes from the embryo to the adult**

To permit genetic manipulation of plasmatocytes along with separate modulation of other tissues, we have taken advantage of the Q system (Potter et al. 2010; Potter and Luo 2011) and a nontoxic variant of the relevant transcription factor called QF2 (Riabinina et al. 2015). Our srpHemo-QF2 driver integrated at the attP16a landing site on the second chromosome can control the expression of QF2 constructs such as QF2::GFP in plasmatocytes (Figure 4A), starting at embryonic stage 10. We additionally observed lower-level expression from srpHemo-QF2 either in the amnioserosa, mesoderm, and/or in punctate cells in the germband ectoderm in 11% of embryos (Figure S5, A and B). As QF2 does not bind to UAS sites, it can be combined with the known large repertoire of GAL4 drivers, which can then independently drive UAS constructs in other tissues. We illustrate this capability by combining srpHemo-QF2::QF2::GFP with repo-GAL4 UAS-moc::mCherry to simultaneously label plasmatocytes and the embryonic nervous system (Figure 4B and File S6). In the larval stage, we see srpHemo-QF2-dependent expression detectable with a stereomicroscope again in the circulating and resident plasmatocyte population at the body wall during all larval stages (Figure 4, C and D), and in the third-instar larval lymph gland as well as pericardial cells (data not shown). Extraneous expression is seen in a subset of the fat body (arrowhead in Figure 4C). Plasmatocyte expression continues into the adult, which can be detected with a stereomicroscope (Figure 4E). Thus, the srpHemo-QF2 line permits the independent visualization or genetic modification of plasmatocytes and surrounding tissues.

**GAL80 line blocking GAL4 action in plasmatocytes from the embryo to the adult**

Finally, we wished to be able to genetically alter Drosophila using broadly expressed GAL4 drivers while not affecting plasmatocytes themselves. To this end, we utilized GAL80, which blocks the activity of GAL4 (Lee and Luo 1999), and created srpHemo-GAL80 lines. This construct was integrated on the second and third chromosome at the split white attP landing sites at ZH-51D and ZH-86Fb, which contain 3xP3-RFP and can be recognized in larvae by the remaining landing site red fluorescence in the brain (Figure S6A), hindgut (Figure S6B), and intersegmental nerves (asterisk in Figure S6C), and in the top of the head (Figure S6, C and C’) (Bischof et al. 2007) in the adult, aiding detection of the chromosome in crosses. Should this extraneous RFP be deleterious for planned experiments, it can be eliminated from the line by expressing cre recombinase. To demonstrate the use of this construct,
we visualized plasmatocytes using the above-described *srpHemo-H2A::3xmCherry* and utilized the ubiquitous driver *tub-GAL4* to express UAS-CD8::GFP in the entire embryo (Figure 5, A–A”). The addition of *srpHemo-GAL80* was able to block *tub-GAL4*-based expression of CD8::GFP in plasmatocytes (Figure 5, B and B”), but did not affect any of the surrounding cells (Figure 5, B and B”). To visualize this effect in larvae and adults, we shifted to GAL4-based expression of GFP just in plasmatocytes using *srp-GAL4 UAS-2xGFP*. We observed the same capacity of the *srpHemo-GAL80* to suppress the effect of GAL4 in plasmatocytes, resulting in no GFP expression in first- to third-instar larvae (compare Figure 5, C–C” to Figure 5, D–D” and Figure S6, D–D” to Figure S6, E–E”). Adults (compare Figure 5, E–E” to Figure 5, F–F”). We also noted that *srp-GAL4* at these stages labeled only a subset of the plasmatocytes visualized with *srpHemo-3xmCherry* (Figure S6, D and E). Thus, *srpHemo-GAL80* can insulate plasmatocytes from the effects of broadly expressed GAL4 drivers in the embryo, larva, and adult.

**DISCUSSION**

In recent years, plasmatocytes have been shown to be able to detect multiple physiological conditions, and produce adaptive and sometimes deleterious responses to them. Much of this work has focused on the signals sent from plasmatocytes to the surrounding tissues and the resulting effects (Ayyaz et al. 2015). To investigate the reverse aspect, namely how tissues signal to plasmatocytes and influence immune cell number or behavior, tools permitting the visualization or isolation of plasmatocytes in conditions where only surrounding tissues have been genetically altered are required. We have created three extremely bright lines that allow the easy detection of the plasmatocyte nucleus, cytoplasm, or actin cytoskeleton live or upon fixation from embryonic stage 8 until the adult in homozygotes and heterozygotes. The cytoplasmic linea is particularly effective for FACS purification at all stages, facilitating quantitative assessment of the numbers of plasmatocytes and the levels of proteins expressed in them. This will also support next-generation sequencing analysis of the plasmatocyte transcriptome at many stages and eventually at the single-cell level. Our additional creation of *srpHemo-QF2* and *srpHemo-GAL80* facilitate targeted genetic manipulations in combination with other GAL4 drivers. Thus, we have produced a comprehensive set of tools permitting the analysis and genetic screening of plasmatocyte behaviors at all stages of the *Drosophila* life cycle.

Several of these new tools will permit experiments on plasmatocyte migration that were not feasible until now. Plasmatocytes are born from the mesoderm and start to migrate at embryonic stage 8, three stages and
3 hr before stage 11 when the previous visualization techniques using GAL4 and fluorescent reporters allowed their detection. Thus, the mechanisms that trigger the initiation of their movement, their coordination while they are in closer contact, or their choices to split into different paths (all of which occur prior to stage 10) have not been investigated. The extant direct fusion srp-moc:GFP line is weakly expressed in the embryo, absent in late larvae and adults, and utilizes a fluorophore whose activation and emission spectra is shared by many autofluorescent molecules in the fly. Thus, our srpHemo direct fusion lines that start expression at stage 8 will serve as the foundation for studies to address these migratory questions, with the nuclear line facilitating tracking and the actin labeling line aiding examination of the cytoskeletal underpinnings of this developmental movement. These lines will also aid investigations into the migration posited to underlie the final homing of plasmatocytes to their positions on the larval body wall, where they proliferate (Makhijani et al. 2011; Van De Bor et al. 2015), and to the dorsal clusters in the adult (Ghosh et al. 2015), which could shed light on resident macrophage homing in vertebrates.

The movement of plasmatocytes allows them to reach tissues where they are known to play important roles, responding to wounds (Stramer et al. 2005; Wood et al. 2006), engulfing dead cells (Tepass et al. 1994; Franc et al. 1996; Weavers et al. 2016a), promoting or killing tumors (Cordero et al. 2010; Parisi et al. 2014), regulating stem cell proliferation (Ayyaz et al. 2015; Van De Bor et al. 2015), and monitoring metabolism (Woodcock et al. 2015). The nature, though not the identity, of the cues that guide them to these tissues is somewhat understood for wounds (Razzell et al. 2013; Weavers et al. 2016b) and tumors (Pastor-Pareja et al. 2008), and remains completely unknown for the rest. Screens utilizing GAL4 expression of RNAi constructs in these tissues and monitoring plasmatocyte responses will be greatly aided by all three of our direct fusion lines, which are visible as heterozygotes. Such screens seeking to quantitatively examine effects on plasmatocyte proliferation throughout the organism should utilize FACS analysis and our srpHemo-3xmCherry line, which is effective from stage 8 to the adult. FACS analysis will detect changes in proliferation in both the lymph gland and the tissue-resident populations, as we see expression in plasmatocytes in both regions. If the chosen driver expresses broadly, our srpHemo-GAL80 can be used to block the activity of GAL4 in plasmatocytes from stage 9 in the embryo to the adult and allow the RNAi screen to only affect surrounding tissues. How tissues and plasmatocytes signal back and forth to one another can be investigated using our srpHemo-QF2 line in addition to extant GAL4 drivers to modulate the genetic behavior on both sides. If the process is only being investigated in L3 larvae and beyond, then the extant hml-QF2 (Lin and Potter 2016) can be used (Table 3). Thus, these lines should allow the identification of new mechanisms underlying plasmatocyte migration, and regulatory interactions between plasmatocytes and surrounding tissues at all stages of the *Drosophila melanogaster* life cycle.

We hope that these reagents will also spur on new types of studies in the adult. The previously created hml-DsRed is visible in third-instar larvae, yet in adults hml-DsRed is hard to detect; our srpHemo-3xmCherry line (Figure 2, C–G and Figure 3D) thus enables experiments that were previously difficult. While plasmatocytes have been shown to regulate metabolism and affect aging (Woodcock et al. 2015), further investigations of how aging tissues signal to stimulate adaptive or deleterious plasmatocyte responses require direct visualization and FACS analysis of plasmatocytes in the adult. The role of other tissues in potentially influencing plasmatocyte responses to infection (Buchon et al. 2014) is another area that these lines could beneficially impact, by enabling screens as described above.

Given the wide range of processes *Drosophila* plasmatocytes have been shown to participate in, this set of tools will immediately prove useful to a broad number of scientists studying *Drosophila* development, aging, cancer, stem cells, wounds, immunity, and metabolism. Since plasmatocytes interact with tissues throughout the organism at all stages, these tools will also facilitate the discovery and investigation of many as yet unidentified regulatory processes. The genetic conservation observed between *Drosophila* and vertebrates strongly suggests that this future work will also prove beneficial for studies in higher organisms.

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**Table 3 QF2 and GAL80 lines: previously published and described in this paper**

| Promoter Source-Txion Factor | Tissue Expression | Time and Level of Effect in Plasmatocytes | Reference for Creation and Expression |
|------------------------------|-------------------|------------------------------------------|--------------------------------------|
| hemolectin-QF2               | P, CC             | +++L2–L3                                  | Expression based on hml-GAL4, same promoter (see Table 1) Lin and Potter (2016) |
| srpHemo-QF2                  | P, CC until embryonic stage 15, LG, PC in third-instar larvae and adult, small patch in larval FB | +++ | This paper |
| srpHemo-GAL80                | P, CC until embryonic stage 15, LG, PC in larvae and adult, SGS from embryonic stage 16 till LL3 | +++ | This paper |

P, Plasmatocytes; CC, Crystal Cells; LG, lymph gland; FB, Fat Body; SGS, Stomatogastric nervous system; PC, Pericardial Cells.
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