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Methods for studying planar cell polarity

Jessica Olofsson and **Jeffrey D. Axelrod**
Department of Pathology, Stanford University School of Medicine, 300 Pasteur Drive, L235, Stanford, CA 94305, USA, Tel: 650-498-7543

Jeffrey D. Axelrod: jaxelrod@stanford.edu

Introduction

Planar cell polarity (PCP) is the polarity of epithelial cells in the plane orthogonal to the apical-basal axis, and is controlled by a partially defined signaling system (McNeill, 2010; Singh & Mlodzik, 2012). PCP related signaling also plays roles in cell migration, tissue re-organization and stem cell differentiation during embryonic development, and later, in regeneration and repair (Dworkin et al, 2011; Munoz-Soriano et al, 2012). Aberrant signaling has been linked to a broad range of pathophysiologies including cancer, developmental defects, and neurological disorders (Dworkin et al, 2011; Munoz-Soriano et al, 2012; Tissir & Goffinet, 2013). The deepest mechanistic insights have come from studies of PCP in *Drosophila* (Maung & Jenny, 2011; Singh & Mlodzik, 2012). In this chapter we review tools and methods to study PCP signaling in *Drosophila* epithelia, where it was found to involve asymmetric protein localization that is coordinated between adjacent cells. Such signaling has been most extensively studied in wing, eye, and abdomen, but also in other tissues such as leg and notum (Adler, 2012; Lawrence & Casal, 2013). In the adult fly, PCP is manifested in the coordinated direction of hairs and bristles, as well as the organization of ommatidia in the eye. The polarity of these structures is preceded by asymmetric localization of PCP signaling proteins at the apical junctions of epithelial cells. Based on genetic and molecular criteria, the proteins that govern PCP can be divided into distinct modules, including the core module, the Fat/Dachsous/Four-jointed (Fat/Ds/Fj) module (often referred to as the ‘global’ module) as well as tissue specific effector modules (Goodrich & Strutt, 2011; McNeill, 2010; Vladar et al, 2009). Different tissues and tissue regions differ in their sensitivity to disturbances in the various modules of the PCP signaling system, leading to controversies about the interactions among the modules, and emphasizing the value of studying PCP in multiple contexts (Lawrence & Casal, 2013; Peng & Axelrod, 2012). Here, we review methods including those generally applicable, as well as some that are selectively useful for analyses of PCP in eye (including eye discs), wing (including wing discs), pupal and adult abdomen, and the cuticle of larvae and embryos.

Correspondence to: Jeffrey D. Axelrod, jaxelrod@stanford.edu.

Mosaic techniques

While a broad range of genetic manipulations are useful in the study of PCP, only methods used to create mosaic tissue are discussed here. PCP relies on intercellular communication and coordination. By studying how a genetically altered group of cells affects its surroundings and vice versa, it is possible to identify which proteins are important for sending information *vs.* receiving information, to observe instances in which loss- or gain-of-function mutations are rescued by surrounding cells (non-autonomy) or perturb the polarity of surrounding cells (domineering non-autonomy), or to make inferences about the direction of signal transmission across the tissue. These features have been highly informative in working out the logic of PCP signaling. Another important use of mosaics in PCP is to assign the asymmetric localization of PCP proteins to a specific cell edge, as light microscopy cannot resolve whether a given protein resides on, for example, the proximal edge of one cell or the distal edge of the adjacent neighboring cell (or both). By expressing a tagged version of a protein in a clone and observing the edges of such clones it is possible to distinguish to which cell that protein belongs. In addition, mosaic techniques can be used when ubiquitous expression or mutation would lead to poor viability.

Targeted gene expression can be achieved using the GAL4/UAS binary system (Duffy, 2002). GAL4 encodes a yeast transcription activator protein that binds to a UAS enhancer element that activates an adjacent gene upon binding. By driving the expressing of GAL4 in tissue or cell specific patterns, the activity of targeted genes can be spatially limited as both the GAL4 protein and the UAS element is necessary for activation (Brand & Perrimon, 1993). A large variety of GAL4 and UAS fly stocks is available. Other less commonly used systems based on the same principles are available, and can be used in combination with the GAL4/UAS system (del Valle Rodriguez et al, 2012; Ting et al, 2011).

Clones of genetically different cells can be created using the FRT/FLP recombination system where FLP is used to induce recombination between FRT sites (Blair, 2003; Xu & Rubin, 1993; Xu & Rubin, 2012). A terminator cassette flanked by FRTs and inserted between a promoter/enhancer and a gene of interest is excised and the gene coupled to the promoter/enhancer is expressed. FLP can be expressed ubiquitously or locally using tissue specific promoters, or regulated in time using heat-shock or drug induction of FLP expression (del Valle Rodriguez et al, 2012). Heat-shock induction is often used to control the frequency of clone induction (Blair, 2003). This method can be coupled with the GAL4/UAS system (Duffy et al, 1998) to express GAL4. Induced GAL4 expression can be used to drive multiple genes each independently coupled to UAS, including fluorescent clone markers, and is effective in cells that do not divide (Blair, 2003). Alternatively, the enhancer chosen for the Flp-out construct can be the UAS element, thereby directing clones to specific locations depending on the choice of GAL4 line. In a variant of the Flp-out strategy, a coding sequence (instead of a stop sequence) is removed, thereby eliminating expression in the clone (Blair, 2003; del Valle Rodriguez et al, 2012).

In a second approach to producing mutant clones, effective in dividing cells, FLP induces site-specific mitotic recombination between FRT sites inserted on two homologous chromosome arms. Beginning with a heterozygous allele of interest, recombination creates

one clone that is homozygous for the allele, and a sister clone that does not inherit the allele. Introduction of a cell marker on the chromosome arm opposite the allele marks all cells outside the clone (Xu & Rubin, 1993; Xu & Rubin, 2012). While this makes it possible to identify the clone, it might be practically difficult to find infrequent unlabeled cells. Positive clone labeling is accomplished by the Mosaic Analysis with a Repressible Cell Marker (MARCM) technique (Lee & Luo, 1999; Wu & Luo, 2006). In MARCM, the GAL4 inhibitor GAL80 is eliminated from the clone, allowing expression of a GAL4-dependent clone marker. Variations on these techniques have been used to produce adjacent 'twin' clones of defined genetic composition that have proven useful for dissecting the communication between cells (see for examples (Chen et al, 2008; Strutt & Strutt, 2007)). Additional useful information and protocols may be found in the following references (Bankers & Hooper, 2007; Blair, 2007b; Dourlen et al, 2013; Theodosiou & Xu, 1998).

Mounting of adult, larval and embryonic tissue to study ommatidia, hair and denticle polarity without staining

Multiple structures in the developing as well as adult fly exhibit polarity that can be read out without the use of staining or tagged proteins. Those structures include the adult hair and bristles and the larval and embryonic denticles. Several different media for mounting those tissues are available, and not all are treated here. Likewise a number of approaches to dissecting and preparing the samples can be used, and the choice is often based on personal preference. Here, variants of the involved steps are described; complete protocols can be found in the references. The *Drosophila* eye consists of close to 800 planar polarized ommatidia and is an important model system for studying of PCP. While the polarity of ommatidia in adult eye can be studied without staining, the techniques differ substantially from the other tissues, and are therefore treated separately at the end of this section.

Mounting media

Hoyer's medium is suitable for embryo, larval, and adult cuticle and is commonly chosen as it dissolves soft tissue which might otherwise interfere with visualization of the cuticle (Stern & Sucena, 2012b). A related drawback is that the structures of interest are slowly digested over time. Tissue can be transferred directly from water, which can also be used to dilute the medium to facilitate spreading between coverslips. Hoyer's medium can also be mixed with lactic acid which increases contrast, but creates crystals over time (Stern & Sucena, 2012b). A recipe for Hoyer's medium can be found in (Stern & Sucena, 2012b). For mounting of wings and other tissue that do not benefit from soft-tissue digestion, alternative mounting media are preferentially used. Samples mounted in the medium Canada balsam can be stored for over a century, but specimens must be dehydrated and samples demand more experience to prepare. Canada balsam can be diluted in xylene to facilitate mounting, but care should be taken to avoid addition of too much xylene, as the volume of the medium might shrink so much during hardening that the sample is disturbed (Stern & Sucena, 2012a). DPX is a medium which hardens and provides long term storage. It can be used as a replacement for xylene-based media such as diluted Canada balsam (Arbouzova & McNeill, 2008). Euparal is another commonly selected mounting medium that hardens to provide long-term preservation of samples. Euparal can be diluted with ethanol to slow hardening

and to lower viscosity. Commonly the sample is dehydrated through exposure to ethanol before mounting or, in the case of water based media, rehydrated (Arbouzova & McNeill, 2008; Stern & Sucena, 2012a; Stern & Sucena, 2012b). This list of media is certainly not exhaustive.

Tissue is typically mounted between a microscope slide and a coverslip by placing a drop of mounting media on the slide, dragging the tissue into the drop using forceps and placing the coverslip on top, taking care not to trap air. Although not necessary, we often place lead weights (fishing sinkers are convenient) on top of the coverslip to ensure appropriate flattening of the sample. Care must be taken to avoid shearing the sample when adding weights, and more importantly, to avoid distorting the polarity pattern of hairs. Many mounting media, including Hoyer's medium, Canada balsam, Euparal and DPX, can be hardened before viewing the sample through baking on a hot plate or in a hot chamber. Clear nail polish can be used as a mounting medium, but is more commonly used to seal the edges of coverslips mounted in other media. If the mounting medium is not solidified, it is advised to first add nail polish dots at the corners of the coverslip, and to wait before sealing the edges until this polish has dried. When using Hoyer's medium, complete hardening of the mounting medium might not be optimal, as the tissue of interest might then be degraded in the process (Stern & Sucena, 2011b).

Dissection and preparation of adult tissue

Adult cuticle samples can be mounted fresh, or be stored in 70% ethanol for extended periods of time before mounting, which might also facilitate dissection (Stern & Sucena, 2012a). Storing in a mix of 30% glycerol and 70% absolute ethanol has been suggested to be ideal, as glycerol does not evaporate and protects the tissue (Stern & Sucena, 2012a). This mixture has also been suggested as a dehydration solution before mounting in DPX and Canada balsam (Arbouzova & McNeill, 2008).

Adult tissue can be dissected in air without any pretreatment, but can also be dissected in 70% ethanol (Stern & Sucena, 2012a; Stern & Sucena, 2012b). Dissection is typically done under a stereomicroscope. Wings can be pulled off the body by grabbing high up in the hinge region with fine forceps and directly introduced into a drop of mounting medium on a slide. Ideally it is noted whether the dorsal or ventral side of the wing faces up in the final mount (or more conveniently, if it is a right- or left-side wing), as the hair polarity can differ between the dorsal and ventral sides of a wing.

Stern and Sucena use soft tissue digestion by heating the entire fly in 10% KOH before dissection of body parts and mounting in Canada balsam (Stern & Sucena, 2012a). Dissection is then performed in 70% ethanol and head, abdomen and thorax are separated by pulling with forceps. While the abdomen is not further dissected they advise separating the thorax from appendages and dividing it in half using a dissecting scissor. For mounting in Hoyer's medium they dissect in the same way, except without KOH treatment (Stern & Sucena, 2012b).

We dissect adult structures in air and mount in Euparal. A razor blade or a dissection scissor is used to separate the thorax from the head, and the abdomen from the thorax. Abdomens

can either be cut in halves, or be mounted with only rough emptying of interior contents using tweezers. Further emptying is accomplished by pressing flat with a coverslip during mounting. This method is quick and easy, but may yield samples with more debris as compared to more extended protocols. Some improvement may be gained by using Hoyer's medium to digest soft tissue.

For studying abdomen and thorax, an en face view of a flattened cuticle is typically chosen. If it is important to visualize a certain area that is situated in a curved region of the thorax or abdomen, it can be beneficial to cut out a small square of the region of interest to obtain a flatter mount (Struhl et al, 1997). Microscopes with exceptional depth of field are also useful in these situations. At usual magnification, it can sometimes be difficult to see, and even more difficult to photograph, in which direction a hair points. For this reason we find it helpful to study also side views of the hairs and cuticle. Suitable samples can be obtained by allowing the tissue to fold during mounting, which tends to happen spontaneously if the tissue is not stretched. The polarity of hairs in a line corresponding to the location of the fold is then evident.

Note that a significant part of the posterior abdominal segments and the most anterior part of each anterior segment is folded under the rest of the adult cuticle unless it is stretched out, which can be done using forceps or by pinning (Krzemien et al, 2012). Alternatively, if not emptying the abdomen of its contents, the cuticle is stretched out as the body is pressed flat during mounting. Well stretched cuticles can also be obtained by fixing entire flies in 1% glutaraldehyde overnight, which bloats the contents and expands the cuticle (Struhl et al, 1997).

Dissection and preparation of larval and embryonic tissue

Larval and embryoic cuticles are commonly studied by mounting in Hoyer's medium. There are several techniques to mount larvae and their suitabilities vary with the instar of the larvae. It can be advantageous to first incubate larvae at 4°C for 15 min which causes them to stretch out (Stern & Sucena, 2011a). In the pricking method, which can be used for all instars, the larva is placed in 70% glycerol and pricked with a fine tungsten needle. Subsequently the larva is fixed and mounted in Hoyer's medium (Stern & Sucena, 2011a). In the pressure method, a larva is fixed and after rinsing in water directly mounted in Hoyer's medium. By using the right amount of media the cover slip is pulled down by the liquid (due to adhesive and cohesive forces) such that the larva bursts, typically in its rear, and the interior is squeezed out (Stern & Sucena, 2011a). The pressure method works only for first and second instar larvae. Hirano et al mounted first instar larvae directly in Hoyer's medium without pretreatment (Hirano et al, 2009). As an alternative to pricking, third instar larvae can be prepared by incubating in 10% KOH at 70 °C before mounting in Hoyer's medium (Stern & Sucena, 2011a), or heated, fixed and dissected as described in (Repiso et al, 2010) before the mounting.

Preparation of cuticles from unhatched first instar larvae can be advantageous to prepare, especially if the larvae are unable to hatch. The embryos are allowed to develop until cuticle is formed (approximately 18 h at 25 °C), at which time they are dechorionated using bleach (Stern & Sucena, 2011b). If desired, the embryos can then be allowed to develop in water

for another 18–24h before devitellinization, followed by mounting in Hoyer's medium (Stern & Sucena, 2011b).

Taking images of mounted tissue

Even for tissue mounted flat, the high magnification microscopes available in most laboratories often do not allow for taking perfect in focus images due to a too shallow depth of field. In those cases, software such as Auto-Montage (Syncroscopy) and Helicon Focus (Heliconsoft) that automatically create focus stacks can be used to produce sharp images (Krzemien et al, 2012; Lawrence et al, 2004). Images taken at different focal planes can also be stacked manually using more commonly available software, for example Adobe Photoshop. To create wide-field high resolution images we have stitched images using Fiji and available stitching plugins (Preibisch et al, 2009).

Wing hairs can be nicely visualized using bright field microscopy. Larger larval structures such as the ventral denticles are often studied using dark field illumination. Finer structures such as the dorsal denticles of first instar larvae and the small hairs covering most of the adult body are usually best studied using phase contrast microscopy, and in some cases dark-field microscopy (Stern & Sucena, 2012b). For observation with dark-field optics it is especially important to clean microscopy slides and coverslips, which can be done with lens paper moistened with 70% ethanol. If dark field microscopy is not available, a similar effect can be obtained by using a low magnification objective in combination with a high-magnification-objective phase-contrast setting for the condenser (Stern & Sucena, 2011b). For cases when it is hard to judge in which direction a hair points, it can be helpful to study the specimen in varying focal planes, and to compare with a sample with known polarity. For studying wing ridge polarity, as described by Collier and colleagues (Doyle et al, 2008; Hogan et al, 2011), cuticle refraction microscopy is useful (Neff et al, 2012).

Mounting and imaging of adult eye sections

The orientation of ommatidia is often studied in thin sections of eye, as described in detail in (Arbouzova & McNeill, 2008; Gaengel & Mlodzik, 2008; Jenny, 2011; Mishra & Knust, 2013). Each ommatidium comprises eight photoreceptors whose arrangement can then be judged without staining based on the position and appearance of their light-harvesting organelles, the rhabdomeres (Gaengel & Mlodzik, 2008). Fly heads with one eye cut away to allow fixative to penetrate into the head are fixed in steps including osmium solution. The heads are then embedded in plastic, and sectioned using a microtome. The sections can be stained with toluidine blue for improved contrast (Arbouzova & McNeill, 2008; Gaengel & Mlodzik, 2008; Jenny, 2011). The sections can be mounted between a microscope slide and a coverslip in medium such as DPX. Well stained samples can be studied using high magnification bright field microscopy, while unstained sections are best visualized using phase contrast or dark field optics (Gaengel & Mlodzik, 2008; Jenny, 2011).

Orientation of ommatidia and polarity defects can be registered in intact heads or even living flies using the Deep Pseudopupil Analysis (DPP) and optical neutralization techniques, which can be useful as an initial assessment in genetic screens (Mishra & Knust, 2013). Those techniques are further mentioned in the Live imaging section below.

Live imaging

Live imaging allows for studying the dynamics of developmental processes. In addition it can be advantageous for being less labor intensive and for better preserving tissue compared to other strategies. For live imaging, genetically encoded fluorescent probes, such as GFP, fused to a protein of interest, are typically used for visualization. For example, the evolution of asymmetrically localized PCP proteins has been tracked in live tissues (Aigouy et al, 2010; Cooper & Bray, 1999). In other examples, intracellular apical transport of PCP core proteins such as Vang, Fz and Dsh, tagged with fluorescent proteins has been investigated in this way (Shimada et al, 2006). Live imaging and expression of fluorescently tagged proteins has also been used in combination with FRAP to study PCP complex stability and protein turnover or to study the direction of predominant microtubule growth using EB1::GFP comets (Harumoto et al, 2010; Strutt et al, 2011).

A number of different protocols have been developed for live imaging of *Drosophila*. Important considerations for choice of protocol include the tissue of interest, and whether the microscope is inverted or upright. Live imaging is typically performed using confocal microscopy and objectives with a short working distance. It is then critical to mount the specimen so that the area of study is sufficiently close to the cover slip facing the objective. For some tissues this is challenging, especially when using high magnification and upright microscopes, and the tissue might need support to stay in position. Typically the specimen is immersed, or the area of interest covered, in a medium such as water or gas permeable halocarbon oil (for example Voltalef 10S and Halocarbon oil 700). This is done to prevent dehydration and to provide improved matching of refractive indices along the path of the light during imaging. Furthermore, because live tissue needs sufficient oxygenation, particularly for extended periods of imaging, inaccurate experimental results due to hypoxia must be avoided. In addition to using gas permeable mounting media, gas permeable membranes are therefore used as an alternative to glass microscopy slides or glass coverslips in some situations. Additional concerns to take into account during live imaging include phototoxic effects and heating of the tissue (Parton et al, 2010).

Pupal tissue

The outer case of the *Drosophila* pupa is typically removed before imaging as it is not transparent and exhibits autofluorescence. Either a small window is opened in the pupal case over the area of interest, or the entire case can be peeled off to obtain a naked pupa (Classen et al, 2008; Peng et al, 2012). Limited peeling is preferable if the pupa is to be recovered and allowed to develop after imaging, and also provides the advantage of continued mechanical protection during handling. To facilitate dissection of the pupa, it can be attached to a hard surface such as a microscopy slide using double-sided tape. Only dry pupae attach well; therefore pupae may be air dried for a few minutes before attachment. This also renders the pupal case more crisp, which considerably facilitates dissection. To detach the pupa from the tape, liquid such as water or the imaging medium can be used. Useful tools for dissection are dissection-scissors, fine tungsten and hypodermic needles and forceps with fine tips. Forceps with the tips slightly bent inwards are often found easy to work with, as this both facilitates tearing the pupal case and makes accidently punctuating the cuticle less likely. It is helpful

to learn where there is space between the pupal case and the cuticle, and to start dissections in those regions. A common entry point for dissection is the anterior dorsal side of the pupa at the operculum. The dissection is typically done under a stereomicroscope.

When using inverted microscopes, peeled pupae can simply be placed in a glass-bottom dish with the area of interest abutting the glass, and imaged from below. A drop of grease (Dow Corning Toray), wax or pieces of wetted filter paper can be used to support the pupae (Harumoto et al, 2010; Shimada et al, 2006). If a pupa is placed in a drop of halocarbon oil with the right size, this alone can stabilize the pupa close to the abutting glass and can keep it in place even if the dish is inverted for imaging using an upright microscope (Ninov & Martin-Blanco, 2007). Working distance is, of course, limited by the thickness of the glass. Peng et al. placed completely peeled pupae in a dish with a hydrophobic gas-permeable bottom moistened by a drop of water with the ventral side of the pupae abutting the glass. Using this configuration, the pupae stayed in place when the dish was turned upside down for use on an upright microscope (Peng et al, 2012). Classen et al. dissected pupae attached to a coverslip with a piece of double-sided tape. After the dissection the tape and the pupa were lifted up together, inverted, and taped to the bottom of a glass-bottomed Petri dish. This was done such that the wing to be studied abutted the glass and the tape covered the pupae to keep it stably in place (Classen et al, 2008). Only the pupal case over the wing was peeled off and addition of halocarbon oil was limited to this region. This was noted to be critical to ensure healthy pupae during longer imaging periods. For studying wing or abdomen on an upright microscope we mount pupae between a microscope slide or a dish with gas permeable membrane as bottom, and a coverslip. The distance between the slide and the coverslip is managed using stacks of coverslips glued together, or kept together by immersion media such as halocarbon oil. While the pupae can easily be positioned stably without support for imaging of the abdomen, we often place a strip of wet filter paper on each side of the pupa to position it properly for visualization of the wing. The strips of filter paper can also be used to carefully rotate the pupa into proper position. Note however that wings can easily be twisted in this process if much of the wing has been exposed. A similar approach has been used for live imaging of notum (Bellaiche et al, 2001; Bosveld et al, 2012) and can be used for other tissue such as pupal eye.

Imaginal discs and larvae

Methods for live imaging of imaginal discs, either isolated or attached to a piece of the larval body, using confocal microscopy were developed by Aldaz et al. (Aldaz et al, 2010). The methods involve the use of mounting media with sufficient viscosity (through addition of 2.5% methyl cellulose), and a simple imaging chamber created from adhesive tape, a coverslip, a semipermeable membrane and a metal slide with a cut-out panel. The viscous medium minimizes movement of the discs during the measurement. Live imaging of ommatidial rotation of prepupal discs was reported by Escudero et al. (Escudero et al, 2007). In this study, an imaging chamber was created using a microscope slide, a coverslip, Parafilm “M” as spacer and petroleum jelly for sealing 3 sides of the arrangement. The forth side was “sealed” using gas permeable halocarbon oil. The prepupa was paralyzed using levamisole. Similar methods can be used for larvae that need to be paralyzed or

anesthetized, and are therefore studied in closed mounting arrangement with such agents added (Liu et al, 2012; Zhang et al, 2010).

Adult eye

DPP (deep pseudopupil analysis) and further refinements of this method using optical neutralization can be used to score defects in ommatidial organization in living flies (Mishra & Knust, 2013). The perfect arrangement of rhabdomeres in combination with their light absorbing properties can under illumination produce an image of a pseudopupil that is disturbed when defects in ommatidial organization are present. In optical neutralization, the eye is covered by a medium with a similar refractive index as chitin (for example water, nail polish or immersion oil) which makes it possible to study the retina and the positioning of rhabdomeres through the corneal lenslets (Franceschini et al, 1981). Using optical neutralization, planar polarity defects can be scored both using the rhabdomeres auto-fluorescent properties, or using expression of fluorescent proteins (Franceschini et al, 1981; Pichaud & Desplan, 2001). Dourlen et al. combined the imaging of fluorescent proteins through cornea neutralization with FLP induced mitotic recombination to study mosaic adult photoreceptor cells in living adult flies with confocal microscopy. In vivo imaging was enabled by immobilizing the flies in agarose solution (Dourlen et al, 2013).

Embryo

Drosophila embryos are surrounded by an eggshell called the chorion. When the embryo is submerged in halocarbon oil, the chorion becomes transparent, and imaging of embryonic structure is possible without dechorination. Nevertheless, removing the chorion improves the imaging quality and is therefore often done (David et al., 2012). The chorion can be removed using bleach, as described in (David et al., 2012) or manually (Reed et al, 2009). Live imaging of embryos has been used to study planar polarity and cytoskeletal dynamics during the growth of denticles (Price et al., 2006). Prince et al. mounted embryos in halocarbon oil between a gas permeable membrane and a coverslip, as described in detail in (David et al., 2012). In other studies embryos have been mounted between coverslips and using 70% glycerol (Sanson et al, 1999). In both cases the embryos will be slightly compressed which increases the area that can be viewed in the same focal plane. Embryos can also be glued to a coverslip which allows for imaging hanging embryos and embryos in otherwise unstable positions (David et al, 2012; Figard & Sokac, 2011). The glue is made by dissolving the adhesive from clear tape in heptane. This mounting method is more labor intensive but has the advantage of being milder in terms of mechanical forces. In an alternative method embryos are imaged in a hanging drop of halocarbon oil (Reed et al, 2009).

Dissection, fixations and stainings

The choice of fixation buffer depends on what structures are to be preserved and studied. While the common fixation buffer, 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS), is often used to study PCP proteins, Classen et al. suggest instead using 8% PFA with Cacodylate added as this speeds up fixation and therefore might better preserve the pattern of polarized cortical proteins (Arbouzova & McNeill, 2008; Axelrod, 2001;

Classen et al, 2008). Alternative fixatives include 100% methanol, as well as PLP (Periodate-Lysine-Paraformaldehyde) fixative and PFA in PEM buffer, for which recipes can be found in (Arbouzova & McNeill, 2008). Methanol as fixative is usually avoided if F-actin is studied (the main component of prehairs) as it can destabilize the filaments. Tissue is more fragile before fixation and therefore often not fully dissected until after fixation, or dissected in fixative with pausing to allow for stepwise fixation during the procedure. For imaging, the fixed and stained tissue is placed in a drop of mounting medium on a microscope slide and covered with a cover slip. Common mounting media include Aqua-Polymount, Vectashield, Fluoromount-G, ProLong Gold, Slowfade Gold, and glycerol based media (Swedlow, 2011). Media that harden have the advantage of preventing mechanical disturbance to the tissue during imaging. Alternatively, nail polish can be used to seal and immobilize the coverslip as described above.

Phalloidin staining

Phalloidin, a toxin isolated from the mushroom *Amanita phalloides*, binds F-actin with high affinity. Phalloidin conjugated with fluorescent molecules is commonly used to study actin structures such as prehairs. Protocols for phalloidin staining are similar to those for antibody staining. When only staining with phalloidin, this can be done in washing buffer without blocking, and if co-staining with antibodies is desired the phalloidin can be added to the mix of secondary antibodies (Arbouzova & McNeill, 2008). As noted above, F-actin can be destabilized by methanol, which should therefore be avoided when staining with phalloidin. Phalloidin stains the cortical actin that outlines the cell perimeter and therefore both the positioning and direction of prehairs can be judged. Another advantage of this approach compared to studying adult hairs is that the shorter prehairs are less sensitive to the introduction of spurious polarity defects during tissue handling. For the same reason, prehairs are preferentially studied as early as possible. Wing prehairs appear around 32h after pupa formation (APF) in pupae grown at 25 °C. We see dorsal abdominal prehairs for similar conditions starting around 42h APF, with a spread in timing of several hours. Note that times for prehair initiation can vary between labs and that some genotypes are known to show delayed growth of prehairs (Strutt & Strutt, 2002; Wong & Adler, 1993). To keep track of the age of pupae they can be picked as white prepupae (a stage lasting approximately 30 minutes, and defined as 0 h APF, during which they stop moving, take on the shape of pupae, but still have the same color as larvae) and placed in a Petri dish with a lid and a wetted tissue to maintain humidity.

Wing

To prepare pupal wings for fixation and staining we decapitate the pupae in PBS, first puncturing the head with a sharp instrument to relieve pressure, and then severing the head with scissors or blunt dissection (Axelrod, 2001). Cut pupae are placed in fixative for between an hour and over night. After fixation the head-less fly can easily be pulled out from the pupal case after which wings can be pulled from the body, and the cuticle removed from the wings (or in opposite order). Removing the cuticle allows antibodies to more easily access the wing and avoids interference from autoflorescence during imaging.

Alternatively, Classen et al. fixed during the dissection by working in a drop of fixative. Working on several pupae simultaneously ensured enough time for proper fixation between each dissection step (Classen et al, 2008). Arbouzova et al. first remove the pupal case and then fix the entire naked pupa for 30 min to >1 h. Subsequently, the wings are removed and fixed for another 20 min before continuing with the staining procedure (Arbouzova & McNeill, 2008).

Typically, only low levels of detergent are needed to permeabilize pupal wing tissue for immunolabeling. Using low detergent levels better preserves membrane structures such as intracellular vesicles (Classen et al, 2008). Fixed wings can be transferred between different solutions using a 20 μl pipettor to move small amounts of solution containing the wings.

Classen et al describe a separate protocol for wings 1–7h APF (Classen et al, 2008). The pupa is placed in 50 μl of PBS, divided into a posterior and an anterior half, and the developing wings accessed from the inside of the anterior part. At this stage the wings are attached to one tracheal branch each, and can be pulled out by the trachea so that the fragile wings do not have to be touched. Fixative is added to the PBS drop and gently mixed. Staining then proceeds as for more mature wings. Pupal wings <1hAPF are dissected by the same protocol as for imaginal discs (see below) (Classen et al, 2008).

Imaginal discs

Wing and eye PCP is established in the respective imaginal discs. Polarity in wing discs can be assessed by staining for the same set of proteins as studied in the later pupal wings. Polarity of ommatidia in eye discs can be determined by staining for specific photoreceptor cell fate markers by standard methods (see for example (Cooper & Bray, 1999). Imaginal discs are relatively easy to access and manipulate, and disturbances in their development do not threaten survival of the larva. By grabbing the head of a third instar larva at the mouth hooks and the spiracles, or slightly more posteriorly, the head can be pulled off such that all imaginal discs except the genital discs follow. The discs of interest can then be separated out and cleaned by careful removal of other tissue, either before or after fixation and staining (Phalle Bde, 2004; Purves & Brachmann, 2007). Pulling at only the mouth hooks facilitates removal of just the eye-antenna disc complex (Arbouzova & McNeill, 2008; Wolff, 2007). Using an alternative strategy, the pupa can be severed around mid section with a sharp blade, and the anterior half inverted (Blair, 2007a). Discs can be separated out and detached from the inverted body wall either before or after proceeding with fixation and staining, or even as late as during final mounting (Klein, 2008; Phalle Bde, 2004). Using this method third instar larvae can easily be dissected, while younger larvae demand more experience (Klein, 2008). The location of different imaginal discs and differences in their appearance can be found in (Klein, 2008) and (Phalle Bde, 2004). Details on dissection and subsequent treatment such as fixation and staining can be found in (Arbouzova & McNeill, 2008; Blair, 2007c; Klein, 2008; Phalle Bde, 2004; Purves & Brachmann, 2007; Wolff, 2007).

Pupal abdomen

Pupae can be cut in halves longitudinally using dissection scalpels and the internal abdominal structures gently cleaned away before fixation, for example by flushing with a

pipettor. The pupal case can be left as a stabilizing structure until final mounting. Wang and Yoder used deoxycholic acid as detergent in the initial fixation step, and omitted permeabilizing agents to minimize cell loss (Wang & Yoder, 2011). In addition, rocking during incubation was minimized. Alternative fixation protocols used for abdomen can be found in (Fabre et al, 2008) and (Krzemien et al, 2012). The dissected abdomen halves are curved and therefore hard to mount between flat surfaces. We find the mounting technique suggested by Wang and Yoder very efficient (Wang & Yoder, 2011). In short, several abdomens are positioned in a drop of mounting medium in a depression slide. Using the right amount of mounting medium, the abdomens automatically move into good and stable imaging position around the edges of the well when a coverslip is slowly placed on top (Wang & Yoder, 2011).

Larval and adult epidermis, and adult and pupal eye

Immunohistochemistry of larval epidermis has not been commonly reported in studies of PCP, but is possible. For example, Repiso et al. fixed pieces of third instar larval cuticle with attached epidermis and stained with anti-Fasciclin 3 antibodies (Repiso et al, 2010). Galko et al. and Lesch et al. dissected larvae and performed whole mounts of the tissue after fixation and subsequent β-galactosidase histochemistry (Galko & Krasnow, 2004; Lesch et al, 2010). β-galactosidase histochemistry has also been used on adult epidermis to study expression patterns of PCP related proteins (Casal et al, 2002). The protocol is described in (Struhl et al, 1997), with a correction presented in (Lawrence et al, 1999).

Likewise, immunohistochemmistry of adult and pupal eye are not commonly used to study PCP, but protocols can be found in (Mishra & Knust, 2013; Ramos et al, 2010; Walther & Pichaud, 2006).

Embryo

The chorion surrounding the embryo can be removed by incubation in solution containing 50% bleach. Inside the chorion, the water impermeable vitelline membrane protects the embryo. Therefore, for water based fixative solutions, embryos are exposed simultaneously to fixative and heptane through agitation. The vitelline envelope is permeabilized by the heptane, which allows the fixative to work. Before incubation in antibodies, the vitelline membrane is removed, either by methods involving osmotic shock or manually using a tungsten needle (Celis et al, 2005; Muller, 2008; Swedlow, 2011). Methanol treatment, either for removing the vitelline membrane or for fixation, can affect antigens and the structural integrity of the embryo, and can destabilize F-actin and should therefore not be used in combination with phalloidin-staining (Muller, 2008). Discussion of fixation protocols for embryos can be found in (Celis et al, 2005; Harris & Peifer, 2007; Marcinkevicius & Zallen, 2013; Miller et al, 1989; Muller, 2008; Swedlow, 2011; Tamada et al, 2012).

Measuring polarization

Although trained eyes are quite good at detecting polarity, there is a need to quantify polarity in various forms. Furthermore, occasionally eyes fail to detect polarity that can be

measured using more rigorous tools (Casal et al, 2006; Donoughe & DiNardo, 2011). Therefore, several ways to quantitively characterize and compare polarization have been developed. Intrinsic to the PCP signaling mechanism, certain proteins become asymmetrically localized. In the wing for example, proteins including Dishevelled and Diego accumulate at distal cell edges, while other proteins including Vang and Prickle accumulate at proximal cell edges. As part of this process cell edges orthogonal to the polarization axis become depleted of these proteins, resulting in the appearance of zigzaging stripes of protein accumulation at proximal-distal cell contacts. Such asymmetric protein localization can be characterized by quantifying cell perimeter variations in fluorescence intensity from proteins that have been tagged with, for example, GFP or stained using fluorescently labeled antibodies. The vector direction of the polarity can be assessed through comparing fluorescence intensity, representing protein localization, at proximal *vs.* distal (or anterior *vs.* posterior, depending on the polarization axis) edges of cells. However, because optical resolution normally does not distinguish to what side of an intercellular junction the signal is localized, clones of cells expressing a tagged version of the protein may be introduced to this end, as described above. Alternatively, comparing the protein abundance between anterior/posterior *vs* proximal/distal cell boundaries can be used as a proxy to assess the axis of polarity without specifying its vector direction, and to quantify the extent of polarization. Cell perimeter segments for comparing protein abundance can be defined manually by drawing lines or marking areas in images and fluorescence intensity measured using software such as ImageJ (or it's newer variant Fiji), and Adobe Photoshop (Brittle et al, 2012; Simoes Sde et al, 2010; Tree et al, 2002). This procedure is painstaking and prone to error or bias.

A number of automated tools have been developed for this purpose. Aigouy et al. created a freely available multifunctional software named "packing analyzer" that automatically decides the axis and strength of polarization (Aigouy et al, 2010; Sagner et al, 2012). A watershed algorithm is used to segment cell edges in images of tissue containing fluorescently labeled proteins. After segmentation, the polarization is estimated based on an integration of the fluorescence intensity along the perimeter of the cells. (Aigouy et al, 2010; Sagner et al, 2012). Using this method the vector direction of the polarization is not decided, but the axis and level of polarization are quantified. Inspired by denotations for order in fields such as material sciences, those properties are together called the "PCP nematic".

Polarity can also be decided by comparing the localization of two different structures, such as the cell-center and prehairs. This can be done by drawing vectors between points representing the structures and analyzing the length and/or angular distribution of the vectors. A commonly used program for such analyses is ImageJ (or it's newer variant Fiji), which contain functions for doing this manually as well as for implementing it in more automated ways (Lawlor et al, 2013). A similar approach can be used to characterize in what direction extended structures such as hairs and denticles point. Donoughe and DiNardo used ImageJ and the plugin "Particles8" to assign approximate angles to denticles in pictures that first had been thresholded (Donoughe & DiNardo, 2011). A freely available suite of MATLAB scripts described by Wu and coworkers, based on similar principles, analyzes the orientation of adult wing hairs (Wu et al, 2013). Sagner et al. used pixel intensity

correlations to automatically characterize the axis of hair orientation (i.e. the hair nematics) from bright field images of adult wings (Sagner et al, 2012).

One method to assess polarity in relative localization between structures measures the fluorescence intensity along a line in two fluorescence images taken of the same tissue, but using different channels to visualize distinct structures (such as for example prehairs marked with GFP and cell edges marked with RFP). By comparing the localization of fluorescence intensity peaks in the two different images, possible polarization can be detected and quantified. A method for doing this automatically across an entire image was developed by Matis et al. (Matis et al, 2012). In this method, correlative fluorescent comparison is used to assess the apparent co-localization between the two structures as the two images taken with different channels are shifted relative to each other. By iteratively applying this algorithm in all possible directions, one can automatically discover and quantify polarization in any orientation. Besides being automatic, advantages of this method are that the artifacts resulting from erroneous cell segmentation are avoided, and that specific structures do not have to be identified (Matis et al, 2012). The method uses freely available plugins for measuring cross-correlation in ImageJ, in combination with a freely available MATLAB script.

Mathematical Modeling

PCP is governed by a multiprotein system with a complexity level making the interpretation of experiments based on intuition difficult. To infer the molecular interactions and system characteristics underlying experimental results, mathematical modeling is therefore a useful tool. For a review of this topic see (Axelrod & Tomlin, 2011).

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