Modelling Podocyte Biology Using Drosophila Nephrocytes

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Abstract

Vertebrate podocytes are kidney glomerular cells critically required for normal renal filtration. To fulfil their role, podocyte's form molecular sieves known as slit diaphragms that contribute to the glomerular filtration barrier. The disruption of podocyte biology or slit diaphragm formation in humans is a precursor to albuminuria, renal failure and cardiovascular morbidity. Due to genetic and functional similarities, the nephrocytes of *Drosophila* are increasingly used to model the genetic and metabolic basis of human podocyte biology. They have the advantage that they are a much quicker system to study compared to other murine transgenic models. In this chapter we present methods to modulate and study *Drosophila* nephrocyte function and diaphragm formation.

1. Introduction.

The nephrocytes of insects are genetically similar to podocytes {Zhuang, 2009 #180}{Weavers, 2009 #186}. They are large filtration cells that contribute to cardio-renal homeostasis by clearing from circulation unwanted circulating proteins {Hartley, 2016 #341}.

Due to the genetic tractability of *Drosophila melanogaster,* the insect nephrocyte is increasingly used to model podocyte genetics and slit diaphragm biology (reviewed in {Hermle, 2017 #507}).

Drosophila genetics allows for cell and temporally restricted gene modulation in a range of contexts including studies of development, metabolism and ageing. Flies are particularly amenable to long term studies of ageing due to their relatively short lifespan of 70-90 days under standard laboratory conditions.

Gene expression in nephrocytes is achieved using a nephrocyte-restricted driver based on the *dKlf15* enhancer region {Ivy, 2015 #448}. The *dKlf15* gene's expression is restricted to and required for the development of mature nephrocytes. The enhancer sequence of *dKlf15* has been engineered upstream of the yeast *Gal4* gene, allowing use in the widely employed bipartite *Gal4-UAS* system.

2. Materials

Food for routine propagation of *Drosophila*.
Although diets vary between labs they typically consist of agar to make the food solid, a carbohydrate source (sucrose), protein (yeast or yeast extract), food preservatives (methylparaben / nipagin and propionic acid).

Transgenic *Drosophila* lines.

1. *dKlf15-Gal4* (Hartley lab, described in {Ivy, 2015 #448})
2. *Dot-Gal4* (available from Bloomington Drosophila Stock Center; stock number
3. *Tubulin-Gal80ts* .

Dissection:

1. Triethylamine solution (anaesthetic); made up at the following ratio 50% trethylamine: 25% ethanol: 25% water). A 7mL bijou containing 5mL will last for several weeks depending on usage. A small volume is preferable because of the solution's pungent odour.
2. Empty *Drosophila* vial, with cotton stopper
3. 30mm diameter polycarbonate petri dishes
4. Vacuum grease or petroleum jelly
5. Hanks Balanced Salt Solution
6. 1mM stock of Calcium Chloride
7. 1mM stock of Magnesium Chloride
8. Two pairs of No. 5 Dumont forceps
9. 3mm curved vannas scissors
10. Benchtop aspirator
11. Paper towels

Vital staining / endocytosis

1. Dextran (10kDa).
2. Albumin
3. Lysotracker
4. Wheat Germ Agglutinin
5. pHrodo

Slit diaphragm imaging

1. Formaldehyde (stock 38% solution)
2. Phosphate buffered saline (PBS)
3. Goat anti-sticks and stones (sns) primary antisera
4. Rabbit anti-dumbfoudned (duf) primary antisera
5. Wheat Germ Agglutinin
6. Secondary anti-goat antisera
7. Secondary anti-rabbit antisera
8. Triton X-100

Solid Food Ingestion Assay (SOFI).

1. 7mL plastic bijou tubes with caps
2. Agar (1% made with distilled water)
3. Molten *Drosophila* food (made with sucrose to either 5% or 25% w/v, for low and high sugar diets, respectively).

4. 25 gauge needles
5. Benchtop heat block at 60°C
6. Microbalance capable of measuring in the sub-milligram range
7. Bijou tube racks (or polystyrene 15mL centrifuge tube packaging moulds).

3. Methods.

Drosophila propagation and ageing.

Maybe add a sentence about where you keep them and the temperature CO2 etc that is needed.

Gene modulation in pericardial nephrocytes.

Dissecting adult flies to reveal pericardial nephrocytes.

Prepare a 30mm diameter petri dish by smearing a thin coat of vacuum grease or petroleum jelly onto the surface. This should be deep enough to lightly embed an anesthetised fly but not too thick to impede dissection. These dishes allow for 20-30 flies to be dissected and stained at the same time. With practise, dissection takes a few seconds and 30 flies can be neatly dissected within 30 minutes. One petri dish accommodates 3mL of any solution; this completely submerges dissected abdomen preparations. All dissections are done using a standard dissecting microscope, using two pairs of No.5 Dumont forceps, as well as 3mm curved Vannas scissors. All dissections are done at ambient temperature, with age-matched flies and Hanks Balanced Saline solution supplemented with 2mM $CaCl_2$ and 4mM $MgCl_2$ (HBSS). Normally, females are used because they are bigger and easier to dissect than males. Males can also be examined and, despite size differences, results from them are similar to those found in females.

1. Flies are transferred from their food vial to an empty 95mm long vial and a 20µL aliquot of trethylamine solution, is added to the innermost surface of the vial's cotton stopper. Flies are fully anaesthetised within 60-120 seconds and remain so for up to 20 minutes. (note - although anaesthesia can be achieved either with CO_2 , this has a major effect on heart function and can limit analyses should quantification of cardiac function been needed. Typically, we use trethylamine as the agent of choice.)
2. Flies are then gently placed on their backs into the vacuum grease, with wings outstretched so that the abdomen is in direct contact with the grease (otherwise preparations can float off).
3. Typically a 'four cut' preparation is performed in order to expose pericardial nephrocytes (**figure 1A**). The first two cuts are done 'dry', the subsequent cuts are done with flies under HBSS. Firstly, with the curvature of the vannas scissors facing upwards the anterior abdomen is cut to remove most of the thorax, head and legs. The second cut is best done with the vannas scissors facing downwards and is done to remove the final abdominal section. This allows scissors to make the cuts either side of the abdomen, allowing access to the abdominal cavity. The intestinal and reproductive tissues are then removed by pulling them away with forceps. This will reveal a beating dorsal vessel and adjacent pericardial nephrocytes (a stained heart, showing wild type architecture, is presented in Figure 1B).
4. The dish is rinsed with HBSS to remove residual tissues floating in the buffer and the dissections 'tidied' with forceps to remove any extraneous tissues accidentally embedded in grease.
5. Due to the natural curvature of the abdomen, the preparation is gently flattened into the grease so that solutions have free access to the heart and nephrocytes.
6. Finally, it is sometimes necessary to remove excess fat-body and trachea that would otherwise obscure the view of the nephrocytes. This is best done by aspiration with a 10mL syringe attached to a small bore tube and pulled glass pipette with a narrow aperture. Gentle negative pressure from the syringe can be applied with one hand whilst the other guides the tip with the other, when looking down the dissecting scope.
7. It is imperative that aspiration is done so as to not damage the heart or remove pericardial nephrocytes (the former is more easily done than the latter).

8. Note – aspiration is the step that most likely disrupts the overall architecture of the heart and nephrocytes. Novices can avoid this step in order to retain architecture but remaining tissues may obscure some nephrocytes. This issue is a problem when counting but not when assessing individual nephrocyte morphology or staining.

Analysis of pericardial nephrocyte endocytic function.

1. The endocytic function of pericardial nephrocytes can be imaged and quantified using fluorescently tagged cargoes such as dextran and albumin linked to Alexa dyes (**Figure 2**). For routine analysis, we use a 3mL solution of 50 µg / mL 10kDa dextran-Alexa^{488} (made with HBSS), which is added to dissected abdomens to cover the entire preparation in the dissecting dish.
2. This is then incubated at 25°C for 0–30 minutes, depending on the experiment. Under these conditions the signal for Alexa-conjugated 10kDa dextran does not increase significantly after about 20 minutes. (note – as the dextran is trafficked into more acidic compartment the fluorescence is quenched. Dyes that fluoresce in lower pH environment such as the pHrodo conjugated dextrans can be also be ustilised to assess endocytosis).
3. Endocytosis is stopped by placing the dishes onto ice, aspirating the dextran solution (*note – this can be reused several times as long as results are not compared between assays*) and briefly rinsing dissections three times with ice-cold HBSS. The nephrocytes can then be fixed with cold 2% formaldehyde in HBSS for 10 minutes and then rinsed three rinses with HBSS.
4. Nephrocytes are counterstained with a cell surface marker to confirm internalisation of the endocytic cargo. The final HBSS rinse is replaced with 3mL of 5 µg/mL wheat-germ agglutinin (WGA) conjugated to the Alexa^{594} fluorochrome. A thirty minute incubation of WGA at ambient temperature is sufficient to obtain excellent signal-to-noise ratio. (note: We find that the signal for fixable endocytic cargoes such as dextran wanes over longer periods of incubation (e.g. overnight), so nephrocytes are imaged on the day of the assay).
5. (note: Wheat germ agglutinin binds N-acetylglucosamine and N-acetylneuraminic acid (sialic acid) residues on cell surface proteins, it preferentially binds to pericardial nephrocytes but will also bind the surfaces of other cell types, making it a useful counterstain for immunofluorescent imaging. WGA is available conjugated to a wide range of fluorescent molecules with different excitation and emission spectra (see thermofisher / molecular probes for more information).
6. Fixed abdomens are then transferred to a glass chamber slide. These are prepared beforehand by adhering two glass coverslips (No.1 size) to a standard microscopy slide so that the distance between the coverslips is approximately 1cm. The coverslips are adhered to the slide using clear nail polish.
7. A thin layer of vacuum grease is smeared onto the glass slide in order to facilitate placement of the dissected abdomens. Applying abdomens to bear glass will cause problems later because coverslipping will displace them.
8. The fixed abdomen is cut from the thorax and the cuticle trimmed on either side of the heart tube using curved vannas scissors.
9. The trimmed abdomen is then taken from the dish using forceps that hold the corner of the tissue. It is then placed onto the glass slide and gently flattened out. (note – as the abdomen comes out of the liquid in the dissecting dish it will curl up and this can be fiddly but by gently pressing the tissue into the grease on the glass slide, the tissue should come off the forceps and remain in place on the slide).

Analysis of pericardial nephrocyte morphology and enumeration after counterstaining with WGA.

1. Flies should be dissected and counterstained with WGA as described above.
2. Morphometric analysis is best done using a confocal microscope so that the z-plane midpoint of nephrocytes can be established.

3. Although pericardial nephrocytes show a range of folds and indentations, their overall morphology approximates to an elipse. For this reason we typically measurement both the longest and shortest axes at the cell's midpoint. These numbers are combined to provide a measure of nephrocyte size at the cell's midpoint (μm^2).
4. We aim to quantify at least 5 nephrocytes from five to six flies per experimental cohort. This provides a minimum of 25 measurements.
5. Enumeration of pericardial nephrocyte abundance can be done using wide-field fluorescence optics, usually with a 10x objective (assuming the use of a 10x eyepiece).
6. It is sometimes not possible to see all nephrocytes due to overlapping fat body or because the dissection has disrupted heart morphology; quantification is avoided when this problem is encountered.
7. In adult flies, pericardial nephrocyte abundance ranges between twenty to thirty nephrocytes per fly, with the mode approximating twenty-six per fly.

Staining for the nephrocyte marker Amnionless and the slit diaphragm proteins Duf and Sns.

Assessment of food intake and modulation of dietary sugar.

One of the issues with modulating diets is quantifying food ingestion. Numerous assays have been developed, one of the most useful being the CAFÉ assay. However this relies on measurements of liquid food intake, rather than intake of the solid food that flies are routinely propagated on. We have developed a simple solid food intake assay (SOFI) that works to quantify food intake by flies on a daily basis. It is limited to the use of male flies because females will lay eggs on the food and only groups of flies are used to reduce variability. Nonetheless it provides a reliable, quantitative measure of food ingestion for a group of flies over several days. It is simple to set up and easily monitored.

1. To a 7mL plastic bijou tube 2mL of 1% agar is added in order to maintain hydration of the flies.
2. Five holes are then punched into one half of the bijou tube's cap using a 25G needle.
3. A small bolus of molten *Drosophila* food is placed into the cap so that none is obstructing the air holes and all the food is within the bevel demarking the thread of the cap's screw. (note – this is easily achieved if the food is molten and pipette tips are pre-wamred to approximately 60°C in a benchtop heat block).
4. Typically, ten male flies are added to a tube (note – add flies that have been anesthetised with CO_2 and place them in the tube when its horizontal then flies can't get stuck on the agar, which can sometimes happen whilst they recover from anaesthetic). (note – males are used because females will lay eggs on the food and this affects the weighing)
5. To control for evaporation from food, each experiment should had an equal number of tubes that do not contain flies but whose caps do contain food.
6. Vials should be placed horizontally and so that the air holes in the cap are uppermost and food is closest to the horizontal plane.
7. Maintain flies and control vials at 25°C on a 12hr:12hr light dark cycle.
8. To quantify solid food ingestion the cap of a tube is removed and weighed on a microbalance. The first measurement is regarded as 'day zero'. (note removing the cap can be troublesome as flies will want to escape; with a little practise and dexterity it is possible with the leading hand to remove the cap and place it on the balance whilst capping the tube with the thumb of the other hand; the leading hand is then free to right down the measurement).
9. Replace the weighed cap without trapping flies in the thread.
10. The caps are re-weighed each day for several days. The amount of food eaten is calculated by establishing the difference in weight between each day and subtracting the amount that has also evaporated in the control tubes. This figure can then be divided by the number of flies in the vial to obtain the milligrams of food eaten per day per fly. **The mean (±SEM) of 8-12 SoFI assays is presented**.

11. Using this assay it is possible to ascertain that flies adjust their food intake according to the amount of sucrose in the diet (e.g. they reduce overall intake when the concentration rises from from 5% to 25% sucrose). Whilst this satiety feedback is tightly regulated, there appears to be a limit as to how much flies can compensate. We find that, despite reducing food intake, flies will over-ingest sucrose when at 25% in the diet (**FIGURE 2**).

***Ex vivo* incubation of nephrocytes**

This method does not necessarily require specialist cell culture incubators nor aseptic technique, although it does require antibiotics to reduce the risk of infection in cultures. Dissected abdominal preparations can be maintained for short periods of time (typically overnight but sometimes up to 48 hours), which is sufficient to assess the impact of genotypes or pharmacological agents or both, on nephrocyte function, slit diaphragm homeostasis nephrocyte cell viability. Schneider's medium is aliquoted and frozen until required (usually as 13mL aliquots in 15mL centrifuge tubes; this is sufficient for three independent cultures using 4mL per dish). Antibiotics are added to the culture medium (1:00 of a 5000 units /mL stock of penicillin/ streptomycin), as too is foetal calf serum (to a final concentration of 10%).

1. Flies should be dissected as described above. Different genotypes can be grouped within the same culture dish so that any pharmacological treatments are applied equally.
2. Rinse the dissected abdomens with HBSS.
3. Add 4mL of culture medium to the dish(es), place lids on them and transfer to a 150mm petri dish (also containing wetted paper, if the incubator is not humidified). The use of a larger petri dish is simply for ease of carriage.
4. Make sure the surface you are placing the culture dish on is level, if not medium will be unequally distributed in the culture dish.
5. Cultures can be maintained at 25°C for 24-48 hours; after 24 hours the heart is still beating and nephrocytes are still endocytically active and slit diaphragms (as evidenced by anti-Duf staining) are largely intact / expressed at the cell surface as regular linear arrays.
6. Nephrocytes can be stained, fixed and imaged as described.

Notes

Although diets vary between labs they typically consist of agar to make the food solid, a carbohydrate source (sucrose), protein (yeast or yeast extract), food preservatives (methylparaben / nipagin and propionic acid).

This driver has the advantage over other systems, such as *prospero* and *dorothy* based drivers, because it does not require additional controls due to expression in neurons (*prospero*) and the haematopoietic system (*dorothy*, {Kimbrell, 2002 #269}).

Dissection is greatly aided by removal of air bubbles that attach to the fly. A thin smear of vacuum grease added to the 30mm diameter petri dish is used to fix the fly on its back during dissection.

Figure 1.

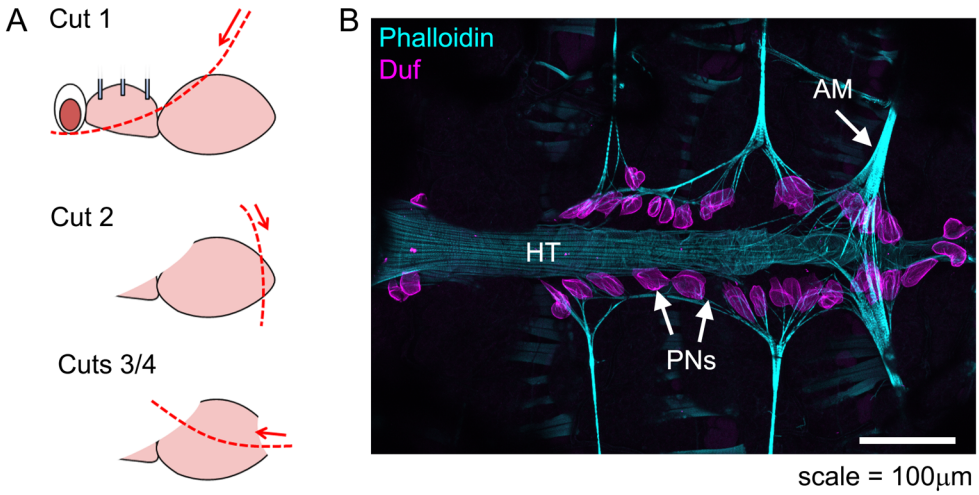

Figure 2.

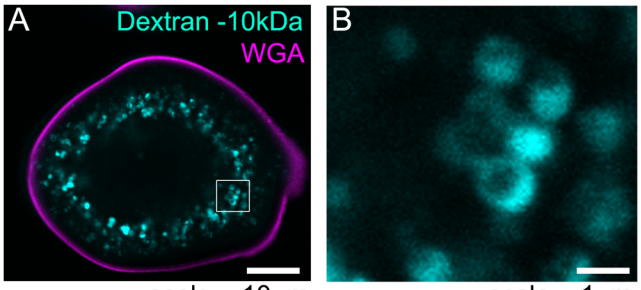

Figure 3.