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1 **Modelling Podocyte Biology Using *Drosophila* Nephrocytes**

2

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4

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9

10

11 **Abstract**

12

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

21

22

23

24 **1. Introduction.**

25

26 The nephrocytes of insects are genetically similar to podocytes {Zhuang, 2009 #180}{Weavers, 2009
27 #186}. They are large filtration cells that contribute to cardio-renal homeostasis by clearing from
28 circulation unwanted circulating proteins {Hartley, 2016 #341}.

29

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

32

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

37

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

42

43

44

45

46 **2. Materials**

47

48 **Food for routine propagation of *Drosophila*.**

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

52

53 **Transgenic *Drosophila* lines.**

54

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

58

59 **Dissection:**

- 60 1. Triethylamine solution (anaesthetic); made up at the following ratio 50% triethylamine: 25%
61 ethanol: 25% water). A 7mL bijou containing 5mL will last for several weeks depending on
62 usage. A small volume is preferable because of the solution's pungent odour.
- 63 2. Empty *Drosophila* vial, with cotton stopper
- 64 3. 30mm diameter polycarbonate petri dishes
- 65 4. Vacuum grease or petroleum jelly
- 66 5. Hanks Balanced Salt Solution
- 67 6. 1mM stock of Calcium Chloride
- 68 7. 1mM stock of Magnesium Chloride
- 69 8. Two pairs of No. 5 Dumont forceps
- 70 9. 3mm curved vannas scissors
- 71 10. Benchtop aspirator
- 72 11. Paper towels

73

74 **Vital staining / endocytosis**

- 75 1. Dextran (10kDa).
- 76 2. Albumin
- 77 3. LysoTracker
- 78 4. Wheat Germ Agglutinin
- 79 5. pHrodo

80

81

82 **Slit diaphragm imaging**

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

91

92 **Solid Food Ingestion Assay (SOFI).**

- 93 1. 7mL plastic bijou tubes with caps
- 94 2. Agar (1% made with distilled water)
- 95 3. Molten *Drosophila* food (made with sucrose to either 5% or 25% w/v, for low and high sugar
96 diets, respectively).

- 97 4. 25 gauge needles
- 98 5. Benchtop heat block at 60°C
- 99 6. Microbalance capable of measuring in the sub-milligram range
- 100 7. Bijou tube racks (or polystyrene 15mL centrifuge tube packaging moulds).
- 101
- 102

103 **3. Methods.**

104

105

106 **Drosophila propagation and ageing.**

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

108

109 **Gene modulation in pericardial nephrocytes.**

110

111 **Dissecting adult flies to reveal pericardial nephrocytes.**

112

113 Prepare a 30mm diameter petri dish by smearing a thin coat of vacuum grease or petroleum jelly
114 onto the surface. This should be deep enough to lightly embed an anaesthetised fly but not too thick
115 to impede dissection. These dishes allow for 20-30 flies to be dissected and stained at the same
116 time. With practise, dissection takes a few seconds and 30 flies can be neatly dissected within 30
117 minutes. One petri dish accommodates 3mL of any solution; this completely submerges dissected
118 abdomen preparations. All dissections are done using a standard dissecting microscope, using two
119 pairs of No.5 Dumont forceps, as well as 3mm curved Vannas scissors. All dissections are done at
120 ambient temperature, with age-matched flies and Hanks Balanced Saline solution supplemented
121 with 2mM CaCl₂ and 4mM MgCl₂ (HBSS). Normally, females are used because they are bigger and
122 easier to dissect than males. Males can also be examined and, despite size differences, results from
123 them are similar to those found in females.

124

- 125 1. Flies are transferred from their food vial to an empty 95mm long vial and a 20µL aliquot of
126 trethylamine solution, is added to the innermost surface of the vial's cotton stopper. Flies are
127 fully anaesthetised within 60-120 seconds and remain so for up to 20 minutes. (note - although
128 anaesthesia can be achieved either with CO₂, this has a major effect on heart function and can
129 limit analyses should quantification of cardiac function been needed. Typically, we use
130 trethylamine as the agent of choice.)
- 131 2. Flies are then gently placed on their backs into the vacuum grease, with wings outstretched so
132 that the abdomen is in direct contact with the grease (otherwise preparations can float off).
- 133 3. Typically a 'four cut' preparation is performed in order to expose pericardial nephrocytes (**figure**
134 **1A**). The first two cuts are done 'dry', the subsequent cuts are done with flies under HBSS. Firstly,
135 with the curvature of the vannas scissors facing upwards the anterior abdomen is cut to remove
136 most of the thorax, head and legs. The second cut is best done with the vannas scissors facing
137 downwards and is done to remove the final abdominal section. This allows scissors to make the
138 cuts either side of the abdomen, allowing access to the abdominal cavity. The intestinal and
139 reproductive tissues are then removed by pulling them away with forceps. This will reveal a
140 beating dorsal vessel and adjacent pericardial nephrocytes (**a stained heart, showing wild type**
141 **architecture, is presented in Figure 1B**).
- 142 4. The dish is rinsed with HBSS to remove residual tissues floating in the buffer and the dissections
143 'tidied' with forceps to remove any extraneous tissues accidentally embedded in grease.
- 144 5. Due to the natural curvature of the abdomen, the preparation is gently flattened into the grease
145 so that solutions have free access to the heart and nephrocytes.
- 146 6. Finally, it is sometimes necessary to remove excess fat-body and trachea that would otherwise
147 obscure the view of the nephrocytes. This is best done by aspiration with a 10mL syringe
148 attached to a small bore tube and pulled glass pipette with a narrow aperture. Gentle negative
149 pressure from the syringe can be applied with one hand whilst the other guides the tip with the
150 other, when looking down the dissecting scope.
- 151 7. It is imperative that aspiration is done so as to not damage the heart or remove pericardial
152 nephrocytes (the former is more easily done than the latter).

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

157

158 **Analysis of pericardial nephrocyte endocytic function.**

159

- 160 1. The endocytic function of pericardial nephrocytes can be imaged and quantified using
161 fluorescently tagged cargoes such as dextran and albumin linked to Alexa dyes (**Figure 2**). For
162 routine analysis, we use a 3mL solution of 50 µg / mL 10kDa dextran-Alexa⁴⁸⁸ (made with HBSS),
163 which is added to dissected abdomens to cover the entire preparation in the dissecting dish.
- 164 2. This is then incubated at 25°C for 0–30 minutes, depending on the experiment. Under these
165 conditions the signal for Alexa-conjugated 10kDa dextran does not increase significantly after
166 about 20 minutes. (note – as the dextran is trafficked into more acidic compartment the
167 fluorescence is quenched. Dyes that fluoresce in lower pH environment such as the pHrodo
168 conjugated dextrans can be also be utilised to assess endocytosis).
- 169 3. Endocytosis is stopped by placing the dishes onto ice, aspirating the dextran solution (note – this
170 can be reused several times as long as results are not compared between assays) and briefly
171 rinsing dissections three times with ice-cold HBSS. The nephrocytes can then be fixed with cold
172 2% formaldehyde in HBSS for 10 minutes and then rinsed three rinses with HBSS.
- 173 4. Nephrocytes are counterstained with a cell surface marker to confirm internalisation of the
174 endocytic cargo. The final HBSS rinse is replaced with 3mL of 5 µg/mL wheat-germ agglutinin
175 (WGA) conjugated to the Alexa⁵⁹⁴ fluorochrome. A thirty minute incubation of WGA at ambient
176 temperature is sufficient to obtain excellent signal-to-noise ratio. (note: We find that the signal
177 for fixable endocytic cargoes such as dextran wanes over longer periods of incubation (e.g.
178 overnight), so nephrocytes are imaged on the day of the assay).
- 179 5. (note: Wheat germ agglutinin binds N-acetylglucosamine and N-acetylneuraminic acid (sialic
180 acid) residues on cell surface proteins, it preferentially binds to pericardial nephrocytes but will
181 also bind the surfaces of other cell types, making it a useful counterstain for immunofluorescent
182 imaging. WGA is available conjugated to a wide range of fluorescent molecules with different
183 excitation and emission spectra (see thermofisher / molecular probes for more information).
- 184 6. Fixed abdomens are then transferred to a glass chamber slide. These are prepared beforehand
185 by adhering two glass coverslips (No.1 size) to a standard microscopy slide so that the distance
186 between the coverslips is approximately 1cm. The coverslips are adhered to the slide using clear
187 nail polish.
- 188 7. A thin layer of vacuum grease is smeared onto the glass slide in order to facilitate placement of
189 the dissected abdomens. Applying abdomens to bear glass will cause problems later because
190 coverslipping will displace them.
- 191 8. The fixed abdomen is cut from the thorax and the cuticle trimmed on either side of the heart
192 tube using curved vannas scissors.
- 193 9. The trimmed abdomen is then taken from the dish using forceps that hold the corner of the
194 tissue. It is then placed onto the glass slide and gently flattened out. (note – as the abdomen
195 comes out of the liquid in the dissecting dish it will curl up and this can be fiddly but by gently
196 pressing the tissue into the grease on the glass slide, the tissue should come off the forceps and
197 remain in place on the slide).

198

199

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

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

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

217

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

219

220 **Assessment of food intake and modulation of dietary sugar.**

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

228

- 229 1. To a 7mL plastic bijou tube 2mL of 1% agar is added in order to maintain hydration of the flies.
- 230 2. Five holes are then punched into one half of the bijou tube's cap using a 25G needle.
- 231 3. A small bolus of molten *Drosophila* food is placed into the cap so that none is obstructing the air
232 holes and all the food is within the bevel demarking the thread of the cap's screw. (note – this is
233 easily achieved if the food is molten and pipette tips are pre-warmed to approximately 60°C in a
234 benchtop heat block).
- 235 4. Typically, ten male flies are added to a tube (note – add flies that have been anaesthetised with
236 CO₂ and place them in the tube when its horizontal then flies can't get stuck on the agar, which
237 can sometimes happen whilst they recover from anaesthetic). (note – males are used because
238 females will lay eggs on the food and this affects the weighing)
- 239 5. To control for evaporation from food, each experiment should had an equal number of tubes
240 that do not contain flies but whose caps do contain food.
- 241 6. Vials should be placed horizontally and so that the air holes in the cap are uppermost and food is
242 closest to the horizontal plane.
- 243 7. Maintain flies and control vials at 25°C on a 12hr:12hr light dark cycle.
- 244 8. To quantify solid food ingestion the cap of a tube is removed and weighed on a microbalance.
245 The first measurement is regarded as 'day zero'. (note removing the cap can be troublesome as
246 flies will want to escape; with a little practise and dexterity it is possible with the leading hand to
247 remove the cap and place it on the balance whilst capping the tube with the thumb of the other
248 hand; the leading hand is then free to right down the measurement).
- 249 9. Replace the weighed cap without trapping flies in the thread.
- 250 10. The caps are re-weighed each day for several days. The amount of food eaten is calculated by
251 establishing the difference in weight between each day and subtracting the amount that has also
252 evaporated in the control tubes. This figure can then be divided by the number of flies in the vial
253 to obtain the milligrams of food eaten per day per fly. **The mean (\pm SEM) of 8-12 SoFI assays is
254 presented.**

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

260

261 ***Ex vivo* incubation of nephrocytes**

262 This method does not necessarily require specialist cell culture incubators nor aseptic technique,
263 although it does require antibiotics to reduce the risk of infection in cultures. Dissected abdominal
264 preparations can be maintained for short periods of time (typically overnight but sometimes up to
265 48 hours), which is sufficient to assess the impact of genotypes or pharmacological agents or both,
266 on nephrocyte function, slit diaphragm homeostasis nephrocyte cell viability. Schneider's medium is
267 aliquoted and frozen until required (usually as 13mL aliquots in 15mL centrifuge tubes; this is
268 sufficient for three independent cultures using 4mL per dish). Antibiotics are added to the culture
269 medium (1:100 of a 5000 units /mL stock of penicillin/ streptomycin), as too is foetal calf serum (to a
270 final concentration of 10%).

271

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

284

285 Notes

286

287

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

291

292

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

296

297

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

300

301

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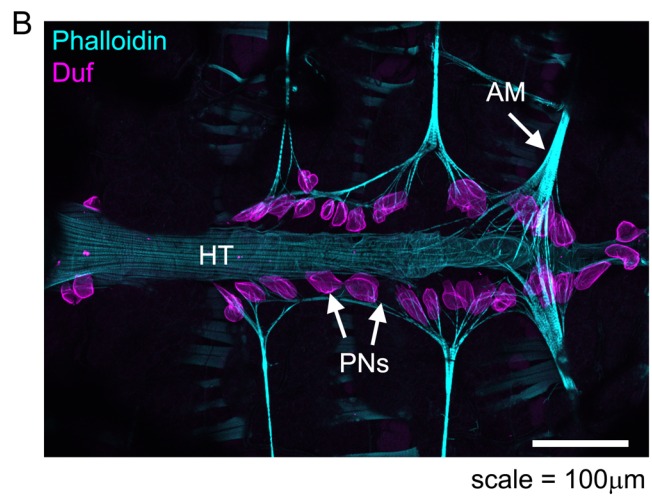
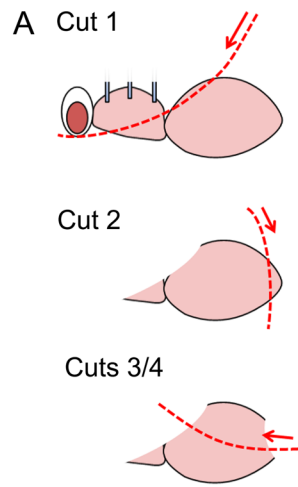
303 Figure 1.

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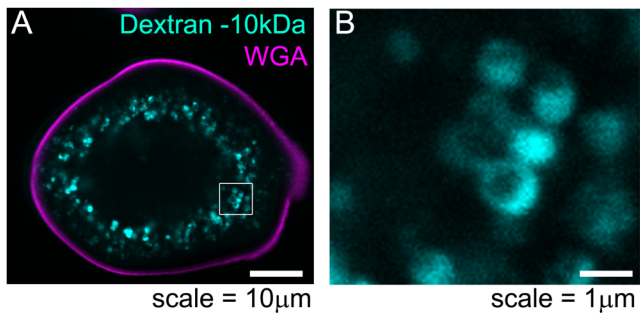
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311 Figure 2.

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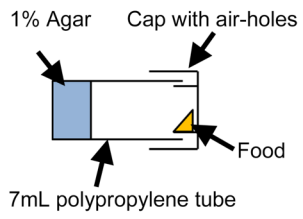
318 Figure 3.

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325