Video Article

Use of the Ramsay Assay to Measure Fluid Secretion and Ion Flux Rates in the *Drosophila melanogaster* Malpighian Tubule

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Abstract

Modulation of renal epithelial ion transport allows organisms to maintain ionic and osmotic homeostasis in the face of varying external conditions. The *Drosophila melanogaster* Malpighian (renal) tubule offers an unparalleled opportunity to study the molecular mechanisms of epithelial ion transport, due to the powerful genetics of this organism and the accessibility of its renal tubules to physiological study. Here, we describe the use of the Ramsay assay to measure fluid secretion rates from isolated fly renal tubules, with the use of ion-specific electrodes to measure sodium and potassium concentrations in the secreted fluid. This assay allows study of transepithelial fluid and ion fluxes of ~20 tubules at a time, without the need to transfer the secreted fluid to a separate apparatus to measure ion concentrations. Genetically distinct tubules can be analyzed to assess the role of specific genes in transport processes. Additionally, the bathing saline can be modified to examine the effects of its chemical characteristics, or drugs or hormones added. In summary, this technique allows the molecular characterization of basic mechanisms of epithelial ion transport in the *Drosophila* tubule, as well as regulation of these transport mechanisms.

Video Link

The video component of this article can be found at <http://www.jove.com/video/53144/>

Introduction

Renal epithelial ion transport underlies organismal iono- and osmoregulation. The *Drosophila melanogaster* Malpighian (renal) tubule offers an unparalleled opportunity to study the molecular mechanisms of epithelial ion transport. This is due to the combination of the powerful genetics of *Drosophila*, paired with the accessibility of its renal tubules to physiological study. The Ramsay assay, named after the investigator who pioneered the technique¹, measures fluid secretion rates from isolated Malpighian tubules, and was established in *Drosophila* in 1994 by Dow and colleagues². This paved the way for further studies using *Drosophila* genetic tools, such as the GAL4-UAS system^{3,4}, to define cell-specific signaling pathways regulating fluid secretion. An example includes calcium signaling in response to a peptide hormone⁵, amongst many others^{6,7}.

A combination of genetic techniques and classical physiological study has shown that urine generation in the fly occurs through the secretion of a potassium chloride-rich fluid from the main segment of the tubule. This occurs through the parallel transepithelial secretion of cations, primarily K⁺ but also Na⁺, through the principal cell, and Cl⁻ secretion through the stellate cell⁸⁻¹². The ability to separately measure transepithelial K⁺ and Na⁺ fluxes allows a more detailed characterization of transport mechanisms than the measurement of fluid secretion alone. For example, in unstimulated *Drosophila* tubules, the Na⁺/K⁺-ATPase inhibitor ouabain has no effect on fluid secretion², even when its uptake into principal cells is inhibited by the organic anion transporter inhibitor taurocholate¹³. However, Linton and O'Donnell showed that ouabain depolarizes the basolateral membrane potential, and increases Na⁺ flux⁹. As shown in the Representative Results, we replicated these findings, and showed that K⁺ flux is concomitantly decreased¹⁴; the increased Na⁺ flux and decreased K⁺ flux have opposing effects on fluid secretion, resulting in no net change in secretion. Thus, there are two resolutions to the "ouabain paradox," i.e., the initial observation that ouabain has no effect on fluid secretion in the *Drosophila* tubule: first, in stimulated tubules, the effect of ouabain on fluid secretion is not apparent due to its uptake by the organic anion transporter¹³; and second, in unstimulated tubules, ouabain has opposing effects on transepithelial Na⁺ and K⁺ flux, resulting in no net change in fluid secretion (see Representative Results and ref. 9). Therefore, the primary role of the Na⁺/K⁺-ATPase in unstimulated tubules is to lower intracellular Na⁺ concentration to generate a favorable concentration gradient for Na⁺-coupled transport processes across the basolateral membrane. Indeed, by separately measuring Na⁺ and K⁺ fluxes, we demonstrated that tubules lacking the fly sodium-potassium-2-chloride cotransporter (NKCC) have decreased transepithelial K⁺ flux, with no further decrease after ouabain addition, and no change in transepithelial Na⁺ flux¹⁴. These findings supported our conclusion that Na⁺ entering the cell through the NKCC is recycled through the Na⁺/K⁺-ATPase. In another example, Iwanowski *et al.* observed that lowering bath K⁺ concentration from 10 mM to 6 mM decreased transepithelial K⁺ flux and increased transepithelial Na⁺ flux in tubules from *Rhodnius prolixus*, with no net change in fluid secretion¹⁵. Differential effects on Na⁺ flux and K⁺ flux across larval tubules have also been observed in *Drosophila* tubules in response to varying salt diets¹⁶ and in two mosquito species in response to rearing salinity¹⁷.

The greatest challenge in the measurement of transepithelial ion flux in the Ramsay assay preparation is the determination of ion concentrations within the secreted fluid. This challenge has been met with varying solutions, including flame photometry¹⁸, use of radioactive ions¹⁹, and electron probe wavelength dispersive spectroscopy²⁰. These techniques require transfer of the secreted fluid drop to an instrument for measurement of ion concentrations. Since the volume of fluid secreted by the unstimulated *Drosophila* tubule is small, typically ~0.5 nL/min, this poses a technical challenge and also introduces error if some of the secreted fluid is lost upon transfer. In contrast, the use of ion-specific electrodes allows the measurement of ion activity (from which ion concentration can be calculated) *in situ*. The current protocol was adapted from that used by Maddrell and colleagues to measure transepithelial K⁺ flux across the *Rhodnius* tubule using valinomycin as the K⁺ ionophore²¹, and also describes the use of a 4-*tert*-butylcalix[4]arene-tetraacetic acid tetraethyl ester-based Na⁺-specific ion-specific electrode characterized by Messerli *et al.*²². Ion-specific electrodes have also been used to measure ion concentrations in fluid secreted by Malpighian tubules in the Ramsay assay in adult^{9,23} and larval¹⁶ *Drosophila melanogaster*, the New Zealand Alpine Weta (*Hemideina maori*)²⁴ and in mosquitoes¹⁷.

Here, we describe in detail the use of the Ramsay assay to measure fluid secretion rates in Malpighian tubules from *Drosophila melanogaster*, as well as the use of ion-specific electrodes to determine the concentrations of K⁺ and Na⁺ within the secreted fluid and thus the calculation of transepithelial ion fluxes. An overview of the assay is provided in **Figure 1**.

Figure 1. Schematic of the Malpighian Tubules and the Ramsay Assay with Use of Ion-specific Electrodes to Measure Ion Concentrations.

This figure illustrates the setup for the Ramsay assay. **(A)** Each fly has four tubules, a pair of anterior tubules and a pair of posterior tubules, that float in the abdominal cavity surrounded by hemolymph. In each pair, the two tubules join at the ureter, which then empties the urine at the junction of the midgut and hindgut. The tubules are blind-ended. Urine is generated by the fluid-secreting main segment (shown in red), and flows toward the ureter and out into the gut. After dissection, the tubule pair is dissociated from the gut by transecting the ureter. **(B)** The pair of tubules is then transferred into a droplet of bathing saline within a well of the assay dish. One of the two tubules, referred to here as the “anchor tubule,” is wrapped around a metal pin and is inert. The other tubule is the secreting tubule. The initial segment (which does not secrete fluid) and main segment of the secreting tubule remain within the droplet of bathing saline. Ions and water move from the bathing saline and into the tubule lumen of the main segment, and then move toward the ureter, as would occur *in vivo*. The lower segment (blue) is outside the bathing saline and therefore inert. Since the ureter is cut, the secreted fluid emerges as a droplet from the cut end of the ureter. The secreted fluid droplet enlarges over time as secretion continues, and its diameter is measured using an ocular micrometer. A layer of mineral oil prevents evaporation of the secreted fluid. The reference and ion specific electrodes measure the ion concentration of the secreted fluid. [Please click here to view a larger version of this figure.](#)

Protocol

1. Preparing the Dissection, Calibration and Assay Dishes

Note: In this step, three plastic petri dishes lined with silicone elastomer are prepared: one for dissection, one for performing the Ramsay assay (“assay dish”), and one for performing calibration. These dishes are re-used from experiment to experiment, and thus this step only needs to be repeated if a dish breaks. A picture of the assay dish is shown in **Figure 2**.

Figure 2. The Assay Dish. The dish used for the Ramsay assay is shown here. It is a 10 cm petri dish that is lined with silicone elastomer. Between 20 and 25 wells are carved out of the elastomer. A Minutien metal pin, cut in half, is placed to the right of each well (or to the left, if the experimenter is left-handed). [Please click here to view a larger version of this figure.](#)

1. Using gloves, pour ~80 g silicone elastomer base into a glass beaker. Add 1/10 weight (8 g) silicone elastomer cure. Stir with a metal stirrer. Place on an orbital shaker with a flat top at a gentle speed, for example 100 rpm, for several hours until all bubbles have been removed.
2. Pour the silicone elastomer into clean plastic petri dishes: 100 mm x 15 mm dishes for the dissection and assay dishes, and 35 x 10 mm for the calibration dish. The thickness of the elastomer layer should be ~6 - 7 mm for the 100 mm dishes, and ~5 mm for the 35 mm dish.
3. Place the dishes on the bench at room temperature (RT) to cure (harden), ~24 - 48 hr.
4. Once the assay dish has cured, prepare a smaller batch of silicone elastomer as in step 1.1, e.g., 10 g silicone elastomer base with 1 g cure. Shake on orbital shaker as in step 1.1 until air bubbles are no longer present. This elastomer will be used in step 1.5.4 and should not be allowed to harden prior to that step.
5. Using a surgical scalpel and standard sharps precautions, make wells in one of the 100 mm silicone elastomer-coated petri dishes. This will be the assay dish.
 1. Make wells ~1 cm apart and with a diameter of ~3 - 4 mm. 25 wells can easily fit in a 100 mm assay dish. Wells should be at least 6 mm removed from the walls of the dish. **Figure 2** illustrates the spacing of the wells. Each well will contain one fluid-secreting tubule during the experiment. Thus, a dish containing 25 wells will allow 25 tubules to be analyzed.
 2. Use a permanent marker to mark the position of the wells if needed.
 3. Make the walls of the well as smooth as possible by placing the scalpel in the elastomer, and then using the opposite hand to rotate the dish 360°.
 4. Then, using standard sharps precautions, dip a 30 G needle into the non-hardened elastomer from step 1.4 and place a small drop at the bottom of each well. This smoothens out the bottom of the well. Allow to cure (harden) x 24 - 48 hr.
6. Prepare the Minutien pins.
 1. Place 0.15 mm black anodized Minutien pins in a row on a piece of 1 inch standard lab labeling tape. The pins' long axis should be orthogonal to the tape's long axis. Cut the tape along its length in order to sever each pin into two approximately equal halves (**Figure 3**). Use one half-pin for each well.

Figure 3. Cutting the Minutien Pins. The pins are lined up on a piece of labeling tape in parallel. Then, scissors are used to cut the pins in half. [Please click here to view a larger version of this figure.](#)

2. Insert each half-pin into the silicone elastomer approximately 1 mm to the right of each well (if right-handed; if left-handed, insert the pin to the left of each well). This is most easily done when visualizing the wells at low power under a dissecting stereomicroscope and is aided by use of a blunt forceps. **Figure 2** illustrates the positioning of the pins.

2. Preparing Fine Glass Rods

Note: In this step, a glass rod is prepared that will be used to transfer the tubules from the dissecting dish into the bathing drop. The glass rod is re-used from experiment to experiment, so this step is performed only once unless the rod breaks and a new one is needed.

1. Obtain sheets of 3 mm (1/8 inch) thick stained black glass from a hobby store and appropriate glass-cutting equipment, such as a glass cutter and pliers. Use appropriate safety equipment (thick gloves, goggles).
2. Cut the glass into strips ~ 6 mm wide x 10 cm long
3. Hold a glass strip in each hand. Soften the short end of each strip over the flame of a Bunsen burner, using appropriate safety precautions. Then, push the ends of the two strips together and pull apart in a smooth movement to create a fine glass rod with a handle.

3. Physiology Setup

Note: In this step, the microscope, electrometer and electrical circuit is set up. Other than periodic re-chloriding (step 3.2) of the silver wires and re-calibration of the electrometer (step 3.8), this step is performed only once. **Figure 4** illustrates the setup.

Figure 4. Physiology Setup. The physiology setup is pictured here. **(A)** Overview of the setup. The stereomicroscope is placed inside the Faraday cage with the micromanipulators on either side. A fiber optic light is threaded through a hole in the side of the Faraday cage. The electrometer is placed outside the Faraday cage. **(B)** To chloride coat the silver wires, the wire is immersed into bleach. **(C)** Close-up of the setup. The straight microelectrode holder, shown in this picture on the left, is threaded onto the probe of the electrometer. The ion-specific electrode will then be threaded over the silver wire into the electrode holder. On the right, the reference electrode is threaded over the silver wire of the 45° microelectrode holder. The circuit must then be appropriately grounded. The assay dish is shown as it will be positioned when performing measurements. [Please click here to view a larger version of this figure.](#)

1. Place the stereomicroscope with ocular micrometer inside the Faraday cage. Ground to the interior of the Faraday cage, which is then grounded to the chassis ground of the electrometer. Place the micromanipulators on either side of the microscope (**Figure 4A**).
2. Chloride two silver wires by immersing in bleach for at least 1 hr. Extend overnight (O/N) if necessary (**Figure 4B**). Repeat this step whenever the silver wires need to be re-chlorided, for example if they are grey in appearance rather than black.
3. Thread one chlorided silver wire into each of the microelectrode holders.
4. Establish the electrical circuit with appropriate grounding. For example, place the vented, straight microelectrode holder, which will hold the ion-specific electrode (ISE), on the electrometer probe, which is secured onto the micromanipulator (**Figure 4C**).
5. Secure the vented, 45° microelectrode holder, which will hold the reference electrode, onto the other micromanipulator (**Figure 4C**). Then ground to the circuit ground on the electrometer.
6. Ground the "A-B out" output BNC of the electrometer to the chassis ground of the electrometer.
7. Place the fiber optic light source outside of the Faraday cage, with the gooseneck pipe threaded through a hole into the Faraday cage (**Figure 4A**).
8. Set up and calibrate the electrometer according to the manufacturer's instructions. Re-calibrate the electrometer on a regular basis (every 1 - 2 weeks). Once complete, and between measurements, leave the electrometer in "standby" mode with the position toggle set to "IN," meter input set to "A" and range set to "200 mV."

4. Prepare the Dissecting and Bath Solutions

1. Prepare *Drosophila* saline as detailed in **Table 1**. For use in experiments, pour ~40 ml into a 50 ml conical tube and keep at RT. Discard if there is evidence of bacterial or fungal growth.
2. To prepare standard bathing medium (SBM), mix *Drosophila* saline 1:1 with Schneider's medium, and pass through a 0.22 μ m syringe filter. Prepare in small aliquots (~10 - 15 ml), store at 4 °C and discard if there is evidence of bacterial or fungal growth. The components of Schneider's medium are listed in **Table 2**.

5. Making the Ion-specific Electrode: Silanizing Pipets

Note: In this step, dichlorodimethylsilane is used to lightly "silanize" the ion-specific electrode. This adds a hydrophobic coat to the inside of the electrode that allows it to retain the hydrophobic ionophore. Excessive silanization is avoided to prevent uptake of mineral oil when making measurements in drops under oil. Silanized electrodes are good for several weeks. Therefore, this step is performed every few weeks.

1. Flame-polish the ends of 5 - 6 unfilamented borosilicate glass capillary tubes (outer diameter 1.2 mm, inner diameter 0.69 mm, length 10 cm) over a low flame, using appropriate safety precautions.
2. Place the capillary tubes in the bottom of a 1 L glass beaker.
3. In a hood and using appropriate personal protective equipment, pour 70% nitric acid (CAUTION: flammable and corrosive, see Material Safety Data Sheet for safe storage and handling instructions) over the capillary tubes and soak for 5 min.
4. Pour the nitric acid back into a glass bottle. Re-use for subsequent nitric acid washing.
5. Add ~200 ml of deionized H₂O to the beaker. Empty waste into a dedicated glass bottle for nitric acid waste. Repeat with an additional 200 ml of deionized H₂O. Follow institutional guidelines for the safe disposal of acid waste.
6. Do three additional washes with large volumes of deionized water. Empty into sink.
7. In the hood, place capillary tubes onto a hot plate with ceramic top set to 200 °C and dry for a minimum of 20 min, optimally 1 hr. It can also be left for longer periods of time.
8. On a pipet puller (**Figure 5A**), pull pipets to a tip diameter of ~1 - 2 μ m.
9. Place pulled pipets back onto hotplate, taking care not to break tips, for at least 10 min, optimally 30 min but can be left for longer.
10. Add 20 μ l of dichlorodimethylsilane (CAUTION: Flammable, corrosive, acute toxicity, see Material Safety Data Sheet for safe storage and handling instructions) into a 15 cm glass petri dish and invert dish over the pipets on the hot plate (**Figure 5B**). Leave in place for at least 20 min, optimally 2 hr. The same petri dish can be re-used in subsequent experiments.
 1. Determine the amount of dichlorodimethylsilane added by trial and error. After ionophore is added (step 8.4), ensure that the interface between the ionophore and the backfill solution is flat (**Figure 5C**). If the interface is concave, this indicates over-silanization, and less silane should be used. If the interface is convex, this indicates under-silanization, and more silane should be used.

Note: The dichlorodimethylsilane tends to go "off" over time, *i.e.*, less effective silanization is achieved with the same volume of silane. At this point, either new silane can be ordered, or the amount adjusted to achieve equivalent silanization.
11. Turn off hot plate and allow to cool. Remove glass petri dish and transfer pipets to storage jar containing silica gel, which maintains desiccation. Handle pipets carefully (forceps helpful) to prevent breaking tip.

A

B

C

Figure 5. Silanizing Pipets. (A) Example of pipet puller. (B) Picture of the pulled pipets on the hot plate. The glass dish containing a drop of dichlorodimethylsilane has been inverted over the pulled pipets. (C) Schematic illustrating the interface between the ionophore cocktail and the backfill solution. A flat interface indicates optimal silanization. [Please click here to view a larger version of this figure.](#)

6. Preparing the Negative Suction Device

Note: In this step, a simple negative suction device is prepared (**Figure 6**) that will be used to fill the ion-specific electrode. This step is performed only once.

1. Attach a 3 ml syringe to a 3 way stopcock with luer lock. At the opposite end of the stopcock, containing the rotating collar and guard, screw in a female locking luer connector with barbed end. Then, attach silicone tubing, 1/16 inch inner diameter with 1/8 inch outer diameter, to the barbed end of the connector. Then, insert plastic tubing with 1/32 inch inner diameter and 3/32 inch outer diameter

Figure 6. The Negative Suction Device. Picture of the components of the negative suction device (3 ml syringe with luer lock, 3-way stopcock with rotating collar and guard, female luer locking connector with barbed end, silicone tubing, plastic tubing) and the final product. [Please click here to view a larger version of this figure.](#)

7. Collect Flies for Dissection

1. Use standard fly husbandry techniques²⁵ to set up fly crosses and modify as needed. For example, use increased temperature (such as 28 °C) in experiments where increased GAL4 activity is desired.
Note: It is important that the flies are not reared in overly crowded conditions; the numbers of male and female parents should be decreased in this case. If different genotypes are used, the numbers of male and female parents should be adjusted to obtain approximately similar number of progeny.
2. Collect flies for dissection (step 11) using standard fly husbandry techniques²⁵ within 1 - 2 days of eclosion. Tubules from female flies are more easily dissected, but tubules from male flies can also be used if necessary or desired. Place flies into a vial containing fly food. Place vials at the desired temperature for 3 - 5 days prior to dissection.

8. Filling the Ion-specific Electrode (ISE)

Note: In this step, the ISE is backfilled with a salt solution and then ionophore is introduced into the tip. The ISE can be re-used from day to day as long as it is working well. Therefore, this step is performed every few days as needed.

1. To make a K^+ -specific electrode, backfill a silanized pipet with 0.5 M KCl using a 1 ml syringe and microfilament (re-use microfilaments from experiment to experiment). Ensure that the backfill solution fills to the very tip of the pipet – no air in tip. If uncertain, visualize under a compound microscope. Dislodge air bubbles by gently flicking the pipet.
 1. For a Na^+ -specific electrode, backfill with 150 mM NaCl.
2. Insert the back end of the ISE into the plastic tubing of the negative suction device prepared in step 6. In the hood, place the ISE onto an inverted plastic 3.5 cm petri dish with a piece of modeling clay to secure in place.
3. Generate negative pressure using the suction device. Draw back on the syringe with the “off” of the stopcock handle pointing toward the side port. The amount of drawing back will vary but is usually in the range of 0.6 – 0.7 ml. Then turn the stopcock handle so the “off” is pointing toward the tubing.
4. In the hood and using appropriate personal protective equipment, dip a 1 – 10 μ l pipet tip into the ionophore solution (CAUTION: toxic. See Material Data Safety Sheet for safe storage and handling instructions). Expel a small drop by placing a gloved finger over the larger opening of the pipet tip. Then, touch the drop of ionophore to the tip of the ISE, without touching the ISE tip with the pipet tip to avoid breaking the ISE tip.

1. Use the potassium ionophore listed in the Materials Table "as is." To prepare the sodium ionophore, make a solution of (in % w/w) 10% 4-*tert*-butylcalix[4]arene-tetraacetic acid tetraethylester, 89.75% nitrophenyl octyl ether, and 0.25% sodium tetraphenyl borate (CAUTION: toxic. See Material Data Safety Sheet for safe storage and handling instructions). Store in a glass vial wrapped in foil to shield from light.
5. Examine the ISE under 40X magnification using a compound microscope to determine whether the ionophore was "taken up" into the ISE tip and whether the ionophore/backfill solution interface is flat (see step 5.10.1 and **Figure 5C**).
 1. If no ionophore was taken up, increase the amount of negative pressure generated by the suction device. If this is not successful, the electrode may have been inadequately silanized. Repeat step 5 using a larger amount of dichlorodimethylsilane.
6. Place the ISE, tip down, onto the wall of a beaker partially filled with 150 mM KCl. Secure the ISE by placing modeling clay onto the side of the beaker. The tip lies within the 150 mM KCl. Continue to use the ISE over multiple days as long as it is working well (see step 10.6).
 1. For a Na⁺ ISE, store the electrode in 150 mM NaCl.

9. Prepare the Reference Electrode

Note: Steps 9.1 - 9.3 can be performed in advance. Steps 9.4 - 9.6 are performed each experimental day.

1. Flame-polish the ends of 10 filamented borosilicate glass capillary tubes (outer diameter 1.2 mm, inner diameter 0.69 mm, length 10 cm) over a low flame, using safety precautions.
2. On a pipet puller, pull pipets to a tip diameter of ~1 - 2 μ m.
3. Store pipets in a pipet storage jar until use. Pipets can be stored indefinitely.
4. On the day of the experiment, using a microfilament and syringe, fill the tip and shank of the pipet with 1 M sodium acetate. Ensure there are no air bubbles and that solution goes to the tip. Gently flick the pipet if air bubbles are present.
5. Using a second microfilament and syringe, backfill the pipet with 3 M KCl. Again, ensure air bubbles are not present.
6. Store the reference electrode in a beaker containing 150 mM KCl (see step 8.6).

10. Calibration of ISE

Note: This step is performed three times on the experiment day: early in the day to make sure the ISE is working, and then before and after measurements of the 20 - 25 secreted fluid drops (**Table 3**).

1. For calibration of the potassium ISE, place two 0.6 μ l drops each of the following four concentrations of KCl onto the silicone elastomer-coated 3.5 cm petri dish (prepared in step 1): 15 mM, 75 mM, 150 mM and 200 mM. Carefully layer 2 ml of mineral oil over the drops.
 1. For sodium ISE, use calibration drops of 15 mM and 150 mM NaCl.
2. Place the calibration dish onto the stage of the stereomicroscope in the Faraday cage and illuminate.
3. Thread the ISE and reference electrode over the silver wires and fasten into microelectrode holders.
4. Using the micromanipulators, advance the ISE and reference electrodes into the 15 mM KCl drop.
5. Switch the electrometer to "operate" mode. Allow reading to settle.
6. Record reading in notebook. Repeat with the 75 mM, 150 mM and 200 mM drops. Calculate slope to determine whether ISE is working well (see step 13.1 and **Table 3**). If not, prepare a new ISE.

Note: Signs that an ISE is not working well: failure to get a reading; slow to equilibrate (several seconds or longer); unstable reading; slope <49 mV/decade change in K⁺ or Na⁺ concentration.
7. Between the first calibration of the day and the measurements performed in step 12, ie during the performance of step 11 (tubule dissections), store the ISE and reference electrodes in 150 mM KCl (as described in step 8.6).

11. Tubule Dissection

Note: This step is performed on the experiment day.

1. Aliquot out a small amount (~500 - 600 μ l) of standard bathing medium (SBM), prepared in step 4.2, for use on the day of experiment and allow to warm to RT. This should be done at least 30 min prior to dissections but can also be done earlier. Also, have at least 20 ml of RT *Drosophila* saline (step 4.1) available prior to starting dissections.
2. Immediately prior to starting dissections: view the assay dish at 10x magnification under a stereomicroscope and add enough SBM to almost fill each well in the assay dish, typically between 10 and 30 μ l. If drugs or peptide are going to be added to SBM mid-experiment, make a note of the exact volume of SBM added to each well. Avoid over-filling the well as this can lead to the tubule floating away during the experiment.
3. Carefully layer ~12 - 13 ml of mineral oil on top so that the wells are covered. This will prevent evaporation of the secreted fluid drops during the experiment.
4. Place flies to be dissected on CO₂ pad.
5. Pick up a fly via its leg or wing with a forceps and place on its back (ventral side up) on the silicone elastomer-coated dissecting dish prepared in step 1. Impale the thorax with a Minutien pin to secure the fly in place.
6. Add a drop of RT *Drosophila* saline (step 4.1) to immerse the fly in saline.
7. Optional: clip off wings and legs. In practice, this is not usually necessary.
8. Use the non-dominant hand forceps to "hold" the fly's abdomen at the thoraco-abdominal junction. Use the dominant hand forceps to peel the abdominal cuticle away, starting at the thoraco-abdominal junction and moving towards the tail end of the fly. The gut, with attached Malpighian tubules, should be exposed with this maneuver.

9. Without touching the tubules, dissect free the midgut/hindgut and attached tubules. Hold the gut in the non-dominant hand forceps and use a 30 G needle to sever the ureter from the gut, detaching the tubules from the gut and free from the fly. It is essential that no tears or rents be introduced into the tubule, other than at the ureter.
Note: The anterior pair of tubules is most easily dissected, however the posterior tubules may be used as well.
10. Using the fine glass rod (step 2), pick up the tubule pair and transfer into a well of the assay dish.
11. Immediately after the tubule pair has been transferred into the well, pick up the end of one of the tubules with the glass rod, withdraw from the bathing drop until the cut end of the ureter is halfway between the pin and the bathing drop, and wrap the end of the tubule around the pin using the glass rod. At the end of this maneuver, one tubule remains in the bathing saline drop and will secrete fluid from the cut end of the ureter, as illustrated in **Figure 1**. The other tubule, labeled the "anchor tubule" in **Figure 1**, is wrapped around the pin. It anchors the secreting tubule in place, is surrounded by oil, and does not secrete fluid.
12. Immediately after step 11.11, write down the well (e.g., A, B, C), tubule identifying information (e.g., genotype or condition), and the time (this is the start time when fluid will begin to be secreted by the tubule in the droplet of bathing saline).
13. Proceed with the next dissection. Once the experimenter is skilled in this technique, it typically takes 3 - 4 min to dissect a pair of tubules, transfer them to the bathing saline, and wrap the anchor tubule around the pin. Therefore, 20 - 25 tubules can be set up in the Ramsay assay within 1.5 hr. The start time of each tubule will therefore be about 3 - 4 min after the start time of the previous tubule.

12. Making Measurements

Note: This step is performed on the experiment day.

1. Calibrate the ISE (step 10) approximately 20 min before the first measurement. This allows time to make a new ISE if needed.
Note: At the desired time, for example after 2 hr of secretion, the secreted fluid drop of each tubule is ready for measurement.
2. Record the time of measurement. Measure the diameter of the secreted fluid drop using the ocular micrometer and record. Note the magnification, e.g., 50X.
3. Advance the ISE and reference electrode into the fluid drop. Switch the electrometer to "operate." Allow the reading to stabilize. Record the value.
4. Repeat for the next drop.
5. At the end of the experiment, repeat the calibration measurements (step 10).

13. Calculations

Note: This step can be performed either at the end of the experiment day, or at a later time.

1. Calculate the mean slope/decade change in potassium concentration. See **Table 3** for an example.
Note: For sodium, the values will be for the difference between the 15 mM and 150 mM NaCl measurements.
2. Determine the mean value of the two measurements (before and after) of 200 mM KCl (or 150 mM NaCl).
3. Calculate the volume of each droplet. $V = \pi d^3/6$, where d is the diameter of the droplet measured with the ocular micrometer in step 12.2.
4. Calculate the secretion rate = V/time (nL/min/tubule), where V is the volume of the droplet determined in step 13.3, and time is the length of time the tubule secreted fluid (= time of measurement - time of pulling ureter out of bathing droplet).
5. Calculate the ion concentration using the formula $[K] = 10e(\Delta v/S)*200$ or $[Na] = 10e(\Delta v/S)*150$, where Δv = the difference (in mV) between the potential measured of the secreted fluid drop, and the potential of the 200 mM calibration drop (for potassium; 150 mM drop for sodium). S = the slope determined in step 13.1.
6. Calculate ion flux = $[\text{ion}] \times \text{fluid secretion rate}$. For *Drosophila* tubules, this will be pmol/min/tubule.

14. Cleaning Up

Note: This step is performed at the end of the experiment day.

1. Thorough cleaning of the wells is essential to ensure that residual salt crystals do not remain in the wells, altering the ion concentration and osmolarity in future experiments.
2. Allow the mineral oil to drain off.
3. Rinse the wells of the assay dish. A 200 μ l pipet tip can be used to gently scrape off residual salt crystal. Using plastic tubing attached to the faucet, squeeze the tubing to create a high-pressure jet of hot water to thoroughly rinse out each well.
4. Allow to dry O/N. Alternatively, a blow dryer can be used to dry out the wells.
5. Rinse off forceps with deionized H₂O and soak in ethanol for 15 min to several hours.
6. Drain oil off of calibration dish and wash with hot water. Use soap as well as long as it is thoroughly rinsed off. Perform the final rinse with distilled H₂O.
7. Flush microfilaments and syringes with distilled H₂O.

Representative Results

Figures 7 and 8 demonstrate that use of the Ramsay assay with ion-specific electrodes to measure K⁺ and Na⁺ concentrations can distinguish genetically and pharmacologically distinct K⁺ and Na⁺ fluxes, information that is not captured by measuring fluid secretion rates alone. **Figure 7** shows that decreased fluid secretion in tubules from flies carrying a homozygous null mutation in the NKCC is driven by a decrease in K⁺ flux in the mutants (**Figure 7A**), while Na⁺ flux is not changed (**Figure 7B**). **Figure 8** shows that the Na⁺/K⁺-ATPase inhibitor, ouabain, has distinct effects on each of the three parameters measured: in wild-type tubules, fluid secretion rate is unchanged (**Figure 8A and 8B**), Na⁺ flux is increased (**Figure 8A**), and K⁺ flux is decreased (**Figure 8B**). Furthermore, ouabain has no effect on transepithelial K⁺ flux in the *NKCC^{null}* mutant tubules (**Figure 8B**). Together, these data indicate that the Na⁺/K⁺-ATPase provides the driving force for K⁺ uptake through the NKCC,

while the Na^+ is recycled through the Na^+/K^+ -ATPase¹⁴. Between 13 and 18 tubules were analyzed in each genotype/condition; differences observed were statistically significant with a $p < 0.01$ (or less) by unpaired, two-tailed Student's t-test. Since ~15-20 tubules can easily be analyzed in a single day's experiment, the results shown here represent ~10 days of experimentation.

Figure 7. Fluid Secretion Rate and Transepithelial K^+ Flux are Decreased in Tubules Carrying a Homozygous Null Mutation in *NKCC*, While Na^+ Flux is Unchanged. (A) Fluid secretion rate (nl/min/tubule) and K^+ flux (pmol/min/tubule) were measured in tubules from control flies (*wBerlin*) or from flies carrying a homozygous null mutation in the fly *NKCC* (*w; Ncc69²/Ncc69²*). $n = 18$ tubules/genotype. (B) Fluid secretion rate (nl/min/tubule) and Na^+ flux (pmol/min/tubule) were measured from control flies (*wBerlin*, $n = 13$ tubules) or from flies carrying a homozygous null mutation in the fly *NKCC* (*w; Ncc69²/Ncc69²*, $n = 14$ tubules). Values shown are mean $\pm$ standard error of the mean (SEM). NS, not significant; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. This figure was adapted from Rodan *et. al.*¹⁴ [Please click here to view a larger version of this figure.](#)

Figure 8. The Na⁺/K⁺-ATPase Recycles Na⁺ Entering through the NKCC. (A) Fluid secretion rate (nl/min/tubule) and Na⁺ flux (pmol/min/tubule) were measured in tubules from control flies (*wBerlin*) in the absence (n = 13 tubules) or presence (n = 15 tubules) of 100 μM ouabain, an inhibitor of the Na⁺/K⁺-ATPase. (B) Fluid secretion rate (nl/min/tubule) and K⁺ flux (pmol/min/tubule) were measured in tubules dissected from control flies (*wBerlin*) in the absence (n = 17 tubules) or presence (n = 17 tubules) of 100 μM ouabain. K⁺ flux was also measured in *NKCC^{null}* tubules in the absence or presence of ouabain (*w; Ncc69²/Ncc69²*, n = 16 - 17 tubules/condition). Values shown are mean ± SEM. NS, not significant; **, p < 0.01; ***, p < 0.001. This figure was adapted from Rodan *et al.*¹⁴ [Please click here to view a larger version of this figure.](#)

Drosophila saline			
For 1L:			
Weigh out 3.6 g glucose and place into a beaker; final concentration = 20 mM			
Then add:			
Component	Volume (ml)	Final concentration (mM)	
H ₂ O	~800		
2.5 M NaCl	47	117.5	
1 M KCl	20	20	
1 M CaCl ₂	2	2	
1 M MgCl ₂	8.5	8.5	
1 M NaHCO ₃	10.2	10.2	
1 M HEPES	15	15	
1 M NaH ₂ PO ₄	4.3	4.3	
Stir, adjust pH to 7.0			
Add H ₂ O to final volume of 1L			
Filter sterilize; avoid autoclaving (glucose will caramelize)			
Store at 4 °C			
Discard if evidence of bacterial or fungal growth			

Table 1. Making *Drosophila* Saline.

Schneider's medium	
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Component	Concentration (mM)
glycine	3.33
L-arginine	2.3
L-aspartic acid	3.01
L-cysteine	0.496
L-cystine	0.417
L-glutamic acid	5.44
L-glutamine	12.33
L-histidine	2.58
L-isoleucine	1.15
L-leucine	1.15
L-lysine hydrochloride	9.02
L-methionine	5.37
L-phenylalanine	0.909
L-proline	14.78
L-serine	2.38
L-threonine	2.94
L-tryptophan	0.49
L-tyrosine	2.76
L-valine	2.56
β -alanine	5.62
CaCl_2	5.41
MgSO_4	15.06
KCl	21.33
KH_2PO_4	3.31
NaCl	36.21
NaHCO_3	4.76
Na_2HPO_4	4.94
α -ketoglutaric acid	1.37
D-glucose	11.11
fumaric acid	0.862
malic acid	0.746
succinic acid	0.847
trehalose	5.85
yeastolate	2,000 mg/L

Table 2. Composition of Schneider's Medium.

Pre		Post		Interval	Slope/decile
mM	mV	mM	mV	15 - 150 (pre)	52.8
15	-28.4	15	-29.4	15 - 150 (post)	54.4
75	9.1	75	9.4	75 - 150 (pre)	51
150	24.4	150	25	75 - 150 (post)	52
200	30.6	200	31.9	150 - 200 (pre)	49.6
				150 - 200 (post)	55.2
				Mean	52.5

Table 3. Calibration of K⁺ ISE. The K⁺ ISE is calibrated in solutions containing 15, 75, 150 and 200 mM KCl before and after the measurement of experimental secreted fluid; an example of actual measurements is shown in this table. Calculation of the slope/decile change in K⁺ concentration is illustrated. For the 15-150 mV interval, slope/decile = mV for the 150 mM calibration drop – mV for the 15 mM calibration drop. For the 75 - 150 interval, the difference is divided by 0.3 [log (150/75)]. For the 150 - 200 interval, the difference is divided by 0.125 [log (200/150)]. The six slopes (three pre-experiment, three post-experiment) are averaged together to get the mean slope/decile. This table was adapted from Rodan *et al.*¹⁴

Discussion

The use of the Ramsay assay, together with ion-specific electrodes, allows the measurement of fluid secretion rates and ion fluxes in isolated insect Malpighian (renal) tubules. Twenty or more tubules can be assayed at a time, allowing higher throughput compared to the assay of individual *in vitro* microperfused tubules. In addition, ion-specific electrodes allow the determination of ion concentrations within the secreted fluid *in situ*, limiting errors that may be introduced in the transfer of the small volumes of fluid to a second apparatus. Critical steps within the protocol include ensuring that tubules are dissected without rents or tears (which will cause fluid to be secreted from the tear, rather than the cut end of the ureter), and ensuring that the ISE is working well by determining its slope prior to starting the experiment. Even with careful dissections, it is not unusual for occasional tubules to fail to secrete (up to ~10% of tubules dissected), but if a high proportion of tubules do not secrete this may be for biological reasons (*i.e.*, very low secretion rates under that particular condition). Some pitfalls that may lead to an ISE not working well include: ionophore leaking out (which may be due to the electrode tip breaking or inadequate silanization), or introduction of an air bubble into the tip. These problems can be ascertained by visualization of the electrode under a compound microscope and usually require filling a new ISE with ionophore before proceeding. Additionally, an ISE may work well for several days, or even weeks, but will eventually fail, again mandating construction of a new ISE. It is also critical that the assay dish is thoroughly washed at the end of the experiment, because residual salts will alter the ionic and osmotic composition of the bathing saline in subsequent experiments.

In our experience, the Na⁺ ISE described in this protocol is less stable than the K⁺ ISE, often exhibiting deterioration of its slope over the course of the experiment. Indeed, Jayakannan *et al.* described an improved Na⁺ ISE based on the same ionophore with better selectivity and stability. Their cocktail included 3.5% 4-*tert*-butylcalix[4]arene-tetraacetic acid tetraethylester, 95.9% 2-nitrophenyl octyl ether, and 0.6% potassium tetrakis (4-chlorophenyl) borate²⁶.

Once the experimenter is comfortable performing this assay, many permutations can be introduced to probe the underlying biology of epithelial ion transport. For example, tubules of differing genotypes may be examined to determine the role of a specific transporter or ion channel on transepithelial fluid and ion transport¹⁴, or of extracellular or intracellular signaling pathways regulating these transporters or channels^{14,27}. The bathing droplet composition can be modified to look at the role of its specific components, such as ions, glucose or amino acids⁹, or osmolarity^{27,28}. Or, hemolymph itself obtained from animals reared under different conditions may be used in place of the bathing saline¹⁶. Drugs, hormones (or their second messengers) or peptides can be added to ascertain their effects^{2,9,14,23,29}. In the current protocol, fluid and ion fluxes in the main segment are examined, since the initial segment does not secrete fluid², and the lower segment is outside the bathing drop. However, the protocol can be modified to examine activity of the initial or lower segments³⁰. Transgenic reporters, such as the calcium-sensing aequorin transgene⁵, or the pH- and chloride-sensing ChloHensor transgene¹², can allow correlation of intracellular ion concentrations with transepithelial flux. Similarly, Efetova *et al.* demonstrated cell-specific manipulation of cAMP levels using optogenetic control of a photoactive adenylyl cyclase, demonstrating differential effects of cAMP on stellate and principal cells³¹.

While the current protocol is focused on the Malpighian tubules of adult *Drosophila melanogaster*, the techniques used have been applied in other insects, including *Rhodnius prolixus*²¹, *Hemideina maori*²⁴, and multiple mosquito species¹⁷, as well as on larval *Drosophila* tubules¹⁶. Similarly, other ions besides Na⁺ and K⁺, such as H⁺, Ca²⁺ or tetraethylammonium may be assayed^{30,32}, as well as toxins such as salicylate³³. Finally, the ion-specific electrode technique can also be used to measure ion concentrations in microperfused mammalian tubules, obviating the need for radiolabeled ions^{34,35}.

In summary, use of the Ramsay assay with ion-specific electrodes allows measurement of transepithelial fluid and ion fluxes from isolated *Drosophila melanogaster* renal tubules. Together with the powerful *Drosophila* genetic tools available, molecular mechanisms of epithelial ion transport can be elucidated.

Disclosures

The authors have nothing to disclose.

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