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METHODS & TECHNIQUES

Determining rates of epithelial solute transport by optical measurement of fluorochrome concentration gradients in the unstirred layer

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SUMMARY

We describe a method for calculating rates of fluorochrome transport from unstirred layer (USL) concentration gradients measured using confocal microscopy. Isolated Malpighian tubules or guts of *Drosophila melanogaster* were secured to depression slides and bathed in saline containing a fluorescent compound. By measuring the concentration gradient of fluorescent organic anions (fluorescein, Texas Red) or the P-glycoprotein substrate daunorubicin in the USL adjacent to the epithelium we were able to calculate the transepithelial flux of the fluorochrome using Fick's equation. Dose–response curves for fluorescein and Texas Red based on USL concentration gradients near the surface of the Malpighian tubule were comparable to those based on collection and analysis of secreted fluid droplets. Rates of Texas Red and daunorubicin secretion were also calculated for the gut of second instar *D. melanogaster* larvae, a tissue that is too small for measurement of transport rates by other *in vitro* techniques such as cannulation and perfusion. Our results suggest that measurement of USL concentration gradients by confocal microscopy may be applicable to any fluorescent indicator of rapidly transported compounds.

Key words: confocal microscopy, epithelial transport, fluorochrome, organic ion transport, Malpighian tubule, gut transport.

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INTRODUCTION

Transport of solutes into or out of cells produces measurable gradients in solute concentration in the unstirred layer (USL) near the surface of many cells or epithelia. In the scanning ion electrode technique, a self-referencing ion-selective microelectrode is used to measure ion concentration gradients, typically within 30–50 μm of the cell surface. The flux of ions into or out of cells can be calculated using Fick's equation:

$$J = D\Delta C/\Delta X, \quad (1)$$

where J is the flux (mol cm^{-2} s^{-1}), D is the diffusion coefficient (cm^2 s^{-1}) and ΔC is the ion concentration gradient (mol cm^{-3}) measured between two sites within the USL separated by the distance ΔX (cm). Potentiometric electrodes are available for common physiological ions (Na^+, K^+, Cl^-, H^+, Ca^{2+} and NH_4^+) and for organic anions such as salicylate and organic cations such as tetraethylammonium. In addition, amperometric electrodes and/or optrodes have been developed for molecules such as oxygen, glucose and nitric oxide (Smith et al., 2007; McLamore and Porterfield, 2011).

The limitations of self-referencing microelectrodes and optrodes are the requirement for specialized hardware to precisely control sequential movement of the measurement device within the USL and the inability to scan rapidly or at a constant distance for many morphologically complex biological preparations. For potentiometric and amperometric electrodes there is the additional problem of electromagnetic interference. By contrast, there is a wide range of fluorescent probes that can be used for optical measurement of solute transport.

Fluorescent indicators anchored in the plasma membrane have been used in the past to measure surface pH (Miesenböck et al., 1998; Stüwe et al., 2007; Urra et al., 2008). In addition, a pH-sensitive fluorescent dye conjugated to 10 kDa dextran has been used to measure extracellular pH near the surface of growing plant root tips. However, the proton fluxes across the root tips were not calculated from Fick's equation, but from accompanying measurements with self-referencing pH microelectrodes (Monshausen et al., 2007).

In this paper we describe a simple method for the measurement of fluorochrome concentration gradients near the surface of rapidly transporting cells using a single confocal micrograph. The technique is potentially suitable for any transported fluorochrome and permits the spatial patterns of transport to be resolved both qualitatively and quantitatively. Fluxes can be calculated using Fick's equation and both transport kinetics and the effects of transport inhibitors can be measured. Given the widespread availability of confocal microscopes, the technique may be applicable to many rapidly transporting epithelia or cells.

MATERIALS AND METHODS

Drosophila stocks

Flies of the Oregon R strain of *Drosophila melanogaster* Meigen 1830 were reared at 22°C on standard yeast medium (Roberts and Stander, 1998). Malpighian tubules or whole guts were dissected from second instar larvae in saline. Experiments were performed at room temperature (17–22°C). Saline contained (in mmol l^{-1}): NaCl (117.5), KCl (20), $CaCl_2$ (2), $MgCl_2$ (8.5), $NaHCO_3$ (10.2), NaH_2PO_4 (4.3), Hepes (8.6), L-glutamine (10) and glucose (20). Saline was titrated to pH 7 with NaOH.

Confocal image acquisition

Dissected Malpighian tubules or whole guts were gently straightened and adhered to depression slides coated with a thin layer of

125 ng µl^{-1} of poly-L-lysine. The tissue was bathed in saline containing one of the organic ions (fluorescein, Texas Red or daunorubicin) and covered with a cover slip. Images were collected on a Leica TCS SP5 confocal microscope (Leica Microsystems, Concord, ON, Canada). The samples were placed under a HC X PLAPO CS 10.0×0.40 dry UV objective (Leica Microsystems). Digital zooms of 3.37 and 1.37 were used for Malpighian tubules and whole gut, respectively. The lasers used in our experiments are preinstalled in the Leica TCS SP5 confocal and manipulated through preinstalled computer software that also came as part of the confocal package. Switching lasers and changing the exciting and emitting wavelengths could be completed in less than 30 s. For fluorescein, the argon laser was set to 40% of maximum, and the 488 nm laser line was used at 15% power. A triple dichroic beam splitter, TD 488/543/633 (Leica Microsystems), was employed and the photomultiplier tube (PMT) detectors were set to detect 500–535 nm wavelengths of light. For Texas Red, the HeNe 594 laser was set at 33% of maximum. A dual dichroic beam splitter, DD 488/594 (Leica Microsystems), was used and the PMT detection range was 605–700 nm. To detect daunorubicin, the argon laser was set to 40% of maximum with the 514 nm laser line set to 61% power and the PMT detectors were tuned to detect wavelengths of light between 599 and 780 nm. The pinhole was adjusted to 1 airy unit for fluorescein and Texas Red and 4.03 airy units for daunorubicin. The pinhole is located before the image plane and acts to eliminate unfocused light. The image thickness was 4.12, 6.24 and 21.97 µm for fluorescein, Texas Red and daunorubicin, respectively. Using the over-glow/under-glow mode, the Smart Offset was adjusted so that all the pixels in the bathing solution were at the minimum detectable level. In the normal colour mode, the Smart Gain was increased until the lumen of the Malpighian tubules or the whole gut was saturated. A resolution of 12 bits was the minimum used. If the bathing saline contained low concentrations of the fluorescent organic ion, 16 bit resolution was used. An image size of 512×512 pixels and scan speed of 200 Hz was used to collect brighter images. For samples containing lower levels of fluorescence, an image size of 1024×1024 pixels and a scan speed of 100 Hz was used. For daunorubicin, the fluorescence was quite low and an image size of 2048×2048 pixels and scan speed of 10 Hz was used with 16 bit resolution.

Images were opened in the public domain Java image processing program ImageJ (version 1.43u) obtained from the National Institutes of Health (http://rsbweb.nih.gov/ij/) and the scale was set to the scale embedded in the image file. The protocol for measurement of fluorochrome concentration gradients within the USL is described in the Results.

Statistical analysis

All statistical analysis was completed using SigmaPlot 2000 (Systat Software, Chicago, IL, USA). All data are expressed as means ± s.e.m. For comparison between two means, a two-tailed *t*-test was performed. A *P*-value of less than 0.05 was used as the level of statistical significance.

RESULTS

Fig. 1 shows a confocal image of the main (secretory) segment of a *D. melanogaster* Malpighian tubule bathed in saline containing 5 µmol l^{-1} Texas Red, an organic anion known to be secreted by Na^+-independent mechanisms in the tubule (Leader and O'Donnell, 2005). The darker regions near the surface of the tubule (Fig. 1A) indicate a reduction in Texas Red concentration below the bulk concentration. Plots of fluorescence intensity (FI) within the two

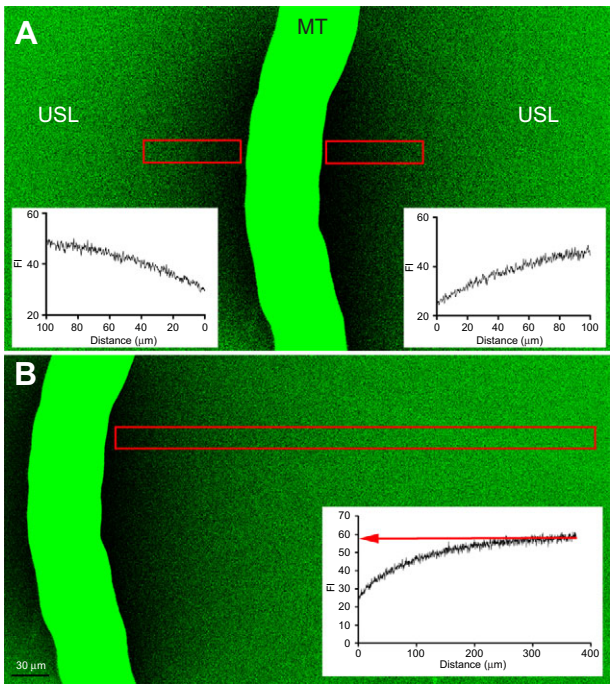

Fig. 1. Confocal image of the main secretory segment of a Malpighian tubule from a second instar larval *Drosophila melanogaster*. The tubule was bathed in saline containing 5 µmol l^{-1} Texas Red. (A) The intensity of Texas Red fluorescence in the unstirred layer (USL) can be seen to increase with increasing distance from the tubule. The graphs plot the profile of average fluorescent intensity (FI) measured from the red 20×100 µm rectangles. The profile function in ImageJ averages single rows of vertical pixels and plots the average against distance. Only gradients that displayed symmetry on both sides of the tubule, as shown, were used to measure flux. MT designates the Malpighian tubule and the USL is indicated. (B) To obtain the FI corresponding to 5 µmol l^{-1} Texas Red, the tubule was moved to the edge of the image and a 20 µm tall box was extended from the edge of the tubule to the edge of the image. The point at which the FI plateaus (red arrow) corresponds to a concentration of 5 µmol l^{-1}.

20×100 µm rectangular areas are shown in the bottom corners of Fig. 1A. The *x*-axis in each plot indicates the horizontal distance from the tubule surface and the *y*-axis shows the vertically averaged pixel intensity at each *x*-coordinate within the rectangle. Depending on the magnification and bit resolution of the images, FI was measured at 200–400 *x*-coordinates over a distance of 100 µm. FI gradients were symmetrical along both sides of the tubule, consistent with transport towards the lumen reducing the concentration of Texas Red in the USL near the basolateral surface of the tubule. Images were discarded if the gradients were asymmetrical, which occurred, for example, if the field of view contained cellular debris or if there was leakage of the lumenal contents into the field of view from the opening created when the ureter was transected during isolation of the tubules. Images were also discarded if the tubule was twisted or looped, resulting in asymmetric gradients. Care was thus taken to position the tubule along a straight line prior to microsocopy. At distances greater than 300 µm from the tubule, FI plateaus at a value corresponding to the concentration of 5 µmol l^{-1} Texas Red (Fig. 1B).

Fig. 2 shows how solute flux was estimated from plots of FI for the same preparation shown in Fig. 1. The tubule was bathed in saline containing 5 µmol l^{-1} Texas Red. Plots of FI as a function of distance from the tubule surface (Fig. 2A) are shown for the six 20×100 µm

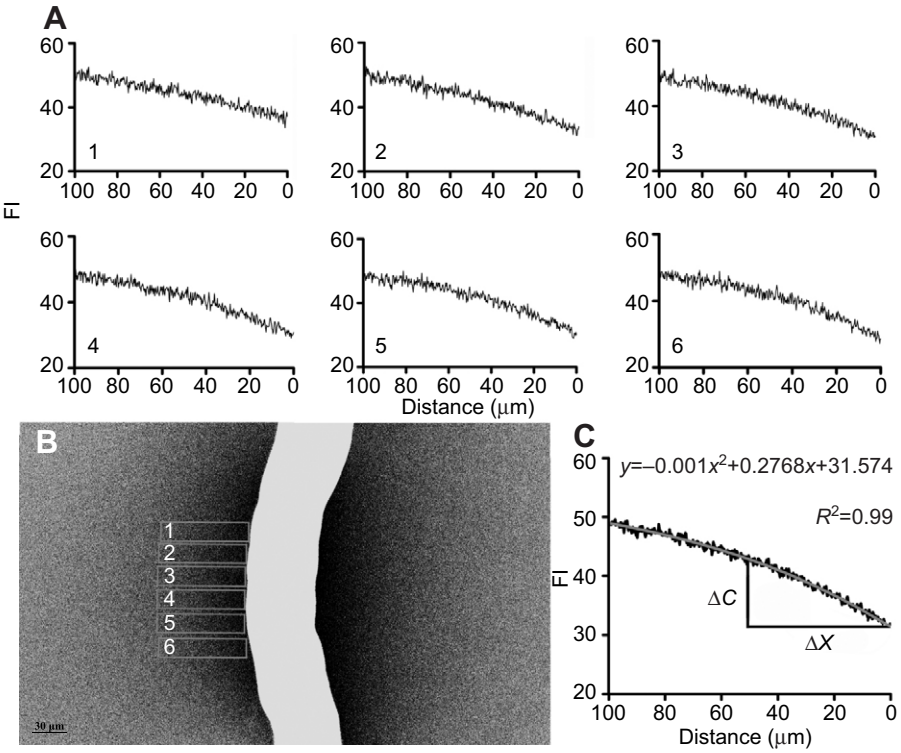

Fig. 2. Estimation of solute flux from plots of FI in the USL near a second instar *Drosophila melanogaster* Malpighian tubule bathed in 5 μmol l^{-1} Texas Red. (A) Plots of FI in the six 20×100 μm rectangles. (B) Confocal image of a *D. melanogaster* second instar Malpighian tubule bathed in 5 μmol l^{-1} Texas Red. The six rectangles used for plots of FI are indicated. (C) Plot of the average concentration gradient for the six plots shown in A. The average plot was fitted to a second-order polynomial equation (grey line). The equation was used to calculate flux using Fick's law, as described in the text.

rectangles indicated in Fig. 2B. A second-order polynomial curve was then fitted by nonlinear regression to the average FI at each distance for the six plots (Fig. 2C). The number of rectangular selections used to calculate the average FI at distances from the tubule surface was always ≥5 so that the r^2-value of the fitted curve was ≥0.95. The bulk concentration of 5 μmol l^{-1} Texas Red in the bath ([Texas Red]$_{bath}$) corresponded to a FI of 57.8 units (Fig. 1B), measured as the average FI on the plateau (FI$_{plateau}$) of the plot at 300–375 μm from the tubule surface (Fig. 1B). The equation for the second-order polynomial curve fit (Fig. 2C) was then used to solve for the values of FI at 0 μm (FI$_{0\,\mu m}$) and 50 μm (FI$_{50\,\mu m}$) from the tubule surface. The concentration difference (ΔC) between these two points was then estimated as:

$$\Delta C = [(FI_{0\,\mu m} - FI_{50\,\mu m})/FI_{plateau}]\,[\text{Texas Red}]_{bath}. \quad (2)$$

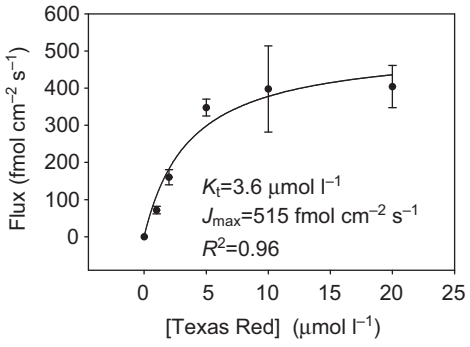

Fig. 3. Flux of Texas Red as a function of bathing concentration of Texas Red in the saline. Each point shows the mean ± s.e.m. for N=5–8 tubules. The data were fitted to the Michaelis–Menten equation by nonlinear regression. K_t, the concentration at which the rate of transport was half-maximal; J_{max}, maximum rate of transport.

The corresponding flux (fmol cm^{-2} s^{-1}) was then calculated from the Fick equation (Eqn 1), where D is the diffusion coefficient for Texas Red (2×10^{-6} cm^2 s^{-1}) (Saylor, 1997) and ΔX is the distance (0.005 cm) over which the gradient was measured.

Transport of Texas Red by the larval tubule was saturable (Fig. 3) and rates of transport as a function of the concentration of the fluorochrome in bathing saline were fitted by the Michaelis–Menten equation. The repeatability of the technique can be estimated from the error bars; the standard error for measurements on five or more tubules was 6–14% of the mean at each concentration, with the exception of the point at 10 μmol l^{-1} (29%). The concentration at which the rate of transport was half-maximal (K_t) was 3.6 μmol l^{-1}, comparable to the value of 7.1 μmol l^{-1} in a previous study in which transport was calculated as the product of fluid secretion rate and the concentration of Texas Red in droplets secreted by isolated tubules of adults set up in a Ramsay assay (Leader and O'Donnell, 2005). The maximum rate of Texas Red secretion of adult tubules in the Ramsay assay is 118 fmol min^{-1} tubule^{-1} (Leader and

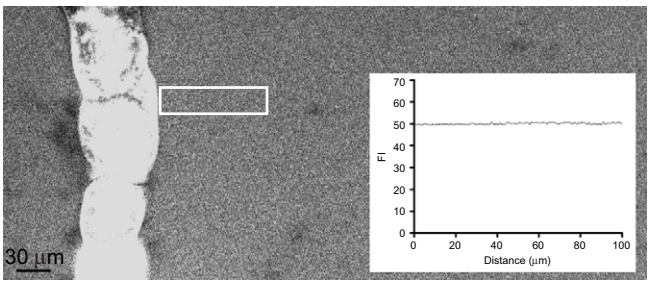

Fig. 4. Confocal image of a Malpighian tubule from *Drosophila melanogaster* bathed in fluorescein with 1 mmol l^{-1} of cyanide. There was no gradient in FI in the USL near the tubule surface, as indicated in the profile plot of average FI for the rectangle indicated in white.

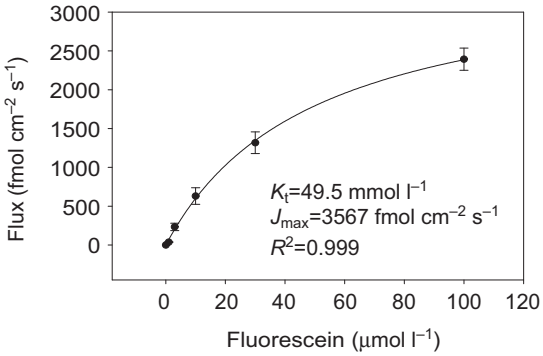

Fig. 5. Flux of fluorescein as a function of the concentration of fluorescein in the bathing saline. Each point shows the mean ± s.e.m. for *N*=6–10 tubules. The data were fitted to the Michaelis–Menten equation by nonlinear regression.

O'Donnell, 2005). This value can be converted to 617 fmol cm^{-2} s^{-1} by dividing by 60 and by an estimate of the surface area of the tubule (3.2×10^{-3} cm^2) (Rheault and O'Donnell, 2004). The maximum rate of Texas Red transport by isolated tubules in a Ramsay assay is thus in reasonable agreement with the value of 515 fmol cm^{-2} s^{-1} (Fig. 3) determined by measurement of USL concentration gradients for the tubules of second instar larvae. The USL gradients were also reduced by probenecid, an inhibitor of organic anion transport (e.g. Leader and O'Donnell, 2005). The flux of Texas Red by tubules bathed in saline containing the fluorochrome at 5 μmol l^{-1} was reduced by 66% to 78±11 fmol cm^{-2} s^{-1} (*N*=8) within 10 min of the addition of 1 mmol l^{-1} probenecid in 1% dimethylsulphoxide (DMSO), relative to the value for tubules bathed in saline containing 1% DMSO alone (231±19 fmol cm^{-2} s^{-1}, *N*=5).

We also measured USL concentration gradients for the organic anion fluorescein, which is transported *via* Na^+-dependent processes by insect Malpighian tubules. Gradients of fluorescein concentration in the USL were not detectable when metabolism was inhibited in a second instar tubule by addition of 1 mmol l^{-1} cyanide to the bathing saline 2 min before imaging (Fig. 4). Gradients were also undetectable when the tubule was frozen and thawed prior to analysis (data not shown), indicating that gradients are not due simply to the interaction of fluorescein with charged sites in the basement membrane. These findings, plus the saturable nature of transport (Fig. 5), indicated that transport was active and metabolically dependent. The concentration at which the rate of fluorescein transport was half-maximal (K_t) was 49.5 μmol l^{-1}, comparable to the value of 31.9 μmol l^{-1} in a previous study in which transport was calculated as the product of fluid secretion rate and the concentration of fluorescein in droplets secreted by isolated tubules set up in a Ramsay assay (Leader and O'Donnell, 2005). Although the fluorescence spectra of sulphorhodamines such as Texas Red are pH insensitive (e.g. Ji et al., 2000), the spectra of fluorescein and its derivatives are known to be pH sensitive. Because pH gradients are present in the USL of active cells such as Malpighian tubules (Collier and O'Donnell, 1997), we tested whether fluxes calculated from USL concentration gradients were altered by changes in bathing saline pH. The flux was 233±47 fmol cm^{-2} s^{-1} in saline containing 3 μmol l^{-1} fluorescein and adjusted to pH 7 (*N*=10 tubules). This value did not differ significantly from the value of 220±23 fmol cm^{-2} s^{-1} at pH 8 (*N*=5 tubules), suggesting that USL gradients near the tubule surface are not an artefact of pH-dependent changes in absorption or emission spectra.

Qualitative and quantitative estimates of fluorochrome transport derived from USL concentration gradients were also obtained for the isolated gut of second instar *D. melanogaster* larvae (Fig. 6A). Concentration gradients for Texas Red were particularly apparent in the posterior midgut (Fig. 6B). Transport of Texas Red by the posterior midgut was saturable (Fig. 6C) and the Michaelis–Menten parameters J_{max} (maximum rate of transport) and K_t were of magnitudes similar to those determined for the Malpighian tubules. Gradients were also detectable in posterior midguts bathed in saline containing 20 μmol l^{-1} of the organic cation daunorubicin, a P-glycoprotein substrate. The flux calculated from the USL concentration gradients was 238±21.90 fmol cm^{-2} s^{-1} (*N*=7).

DISCUSSION

Measurement of concentration gradients of fluorochromes within the USL of actively transporting epithelia such as the Malpighian tubules and gut provides a convenient means of assessing transport sites and mechanisms. Transport can be assessed qualitatively from confocal images of regionalized structures such as the insect gut. In essence, the technique relies upon adjustment of laser intensity to values well above those normally used to study subcellular

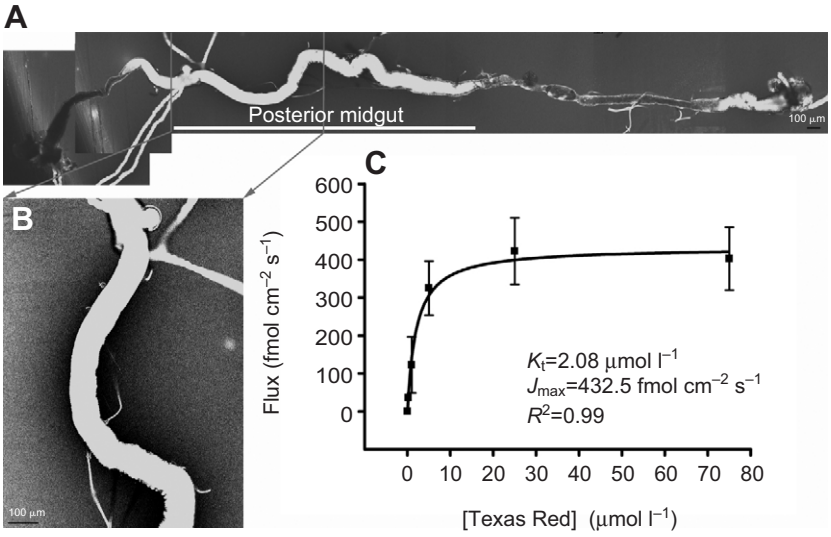

Fig. 6. Concentration gradients were measured on the posterior midgut from second instar *Drosophila melanogaster* larvae. (A) Confocal image of a whole gut from second instar *D. melanogaster* larvae bathed in 5 μmol l^{-1} Texas Red. The posterior midgut is indicated on the image. (B) The posterior midgut clearly showed a gradient in FI in the USL. Fluorochrome concentration increased with increasing distance from the gut. For this preparation, gradients were measured in the middle third of the image. The region of the gut in the bottom 25% of the image was curved and the FI gradients were asymmetric; this region was therefore not used for measurements. (C) Flux of Texas Red as a function of concentration of Texas Red in the bathing saline. Each point shows the mean ± s.e.m. for *N*=5–8 guts. The data were fitted to the Michaelis–Menten equation by nonlinear regression.

structure. Although the fluorescence intensity is saturated within the cells of the Malpighian tubules and the gut, the increase in gain allows the gradients of fluorescence intensity in the USL and bathing saline to be visualized. In addition, quantitative estimates of the rates of transport can be determined from plots of FI within the USL. In conjunction with Michaelis–Menten kinetic analysis and the effects of transport inhibitors, such information provides useful information on the mechanisms of fluorochrome transport.

Fluorescence correlation spectroscopy (FCS) has previously been used to measure extracellular morphogen gradients in zebrafish embryos (Yu et al., 2009) and osmotic water flow (Erokhova et al., 2011). FCS measures changes in FI that are due to the Brownian motion of a small number (typically 1–100) particles in a small volume (~1 fl) of fluid and is most appropriate for concentrations in the nanomolar to picomolar range, well below those associated with saturable transport of organic ions. FCS also requires multiple measurements at discrete locations in order to reconstruct the concentration gradient over distances of ~100 μm. By contrast, our method is suitable for higher concentrations (micromolar to millimolar) up to and above the K_t value for transport of organic anions by isolated epithelia. Furthermore, our approach allows concentration gradients to be measured from a single confocal image and it is particularly well suited for differentiating transport over distances of several hundred micrometers in functionally regionalized structures such as the gut.

We first applied the technique to analysis of the Malpighian tubules because previous studies based on collection of secreted fluid provided a means of independently confirming the rates and characteristics of fluorochrome transport. However, the technique may be of much greater value for preparations that are too small (e.g. the gut of second instar *D. melanogaster* larvae) to study using other *in vitro* techniques such as cannulation and collection and analysis of lumenal perfusate. Texas Red is a substrate of transporters such as multidrug-resistance associated protein 2 (MRP2), and its transport by the posterior midgut is consistent with our previous evidence for expression of multiple organic anion transporters in the midgut (Chahine and O'Donnell, 2009). It is worth noting that the *Drosophila* gut has recently been shown to be a valuable model for studies of intestinal disease (Cognigni et al., 2011), and techniques such as those described in the present study may be of use in characterizing transport by the gut of flies in which molecular genetic tools have been used to alter specific transport mechanisms.

Future studies will determine whether epithelial transport of nutrients such as sugars and amino acids can be measured by analysis of USL concentration gradients of fluorescent analogs.

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