Long-term live imaging of the *Drosophila* adult midgut reveals real-time dynamics of division, differentiation, and loss

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ABSTRACT

Organ renewal is governed by the dynamics of cell division, differentiation, and loss. To study these dynamics in real time, we present a platform for extended live imaging of the adult *Drosophila* midgut, a premier genetic model for stem cell-based organs. A window cut into a living animal allows the midgut to be imaged while intact and physiologically functioning. This approach prolongs imaging sessions to 12-16 hours and yields movies that document cell and tissue dynamics at vivid spatiotemporal resolution. Applying a pipeline for movie processing and analysis, we uncover new, intriguing cell behaviors: that mitotic stem cells dynamically re-orient, that daughter cells use slow kinetics of Notch activation to reach a fate-specifying threshold, and that enterocytes extrude via ratcheted constriction of a junctional ring. By enabling real-time study of midgut phenomena that were previously inaccessible, our platform opens a new realm for dynamic understanding of adult organ renewal.

INTRODUCTION

Stem cell-based organs rely upon the coordinated control of cell division, differentiation, and loss to maintain tissue homeostasis. Studies of the *Drosophila* adult midgut (Fig. 1A) have elucidated conserved processes and pathways that control these events during healthy turnover and cause their dysfunction during aging and in cancer. These contributions, which include mechanisms of multipotency and asymmetric-symmetric fates, endocrine and immune regulation, and injury and stress responses, span the range of adult stem cell biology (Biteau et al., 2008; Buchon et al., 2009; Deng et al., 2015; Guo and Ohlstein, 2015; Hudry et al., 2016; Jiang et al., 2009; O’Brien et al., 2011; Ohlstein and Spradling, 2007; Siudeja et al., 2015).

However, investigation of midgut cell dynamics has been constrained by the lack of a viable platform for extended live imaging. At present, fixed midguts provide static snapshots of cells and tissues but do not allow dynamic behaviors to be observed over time. Meanwhile, cultured midguts have been imaged *ex vivo* for 60-90 minutes—a time window long enough for faster events such as calcium oscillations, cell divisions, and acute toxicity responses (Antonello et al., 2015; Deng et al., 2015; Lee et al., 2016; Montagne and Gonzalez-Gaitan, 2014; Scopelliti et al., 2014) but too short for slower events such as differentiation and apoptosis. Indeed, the power of extended live imaging is demonstrated by studies of numerous other stem cell-based organs, including *Drosophila* ovary
testis (Fichelson et al., 2009; Lenhart and DiNardo, 2015; Morris and Spradling, 2011) and mouse epidermis, testis, muscle, and intestine (Bruens et al., 2017; Gurevich et al., 2016; Hara et al., 2014; Ritsma et al., 2014; Rompolas et al., 2012; 2016; Webster et al., 2016). For the midgut, long-term live imaging would synergize with the organ’s existing genetic tractability and well-characterized cell lineages to open exciting investigative possibilities.

To enable such studies, here we present a simple platform that substantially extends imaging times by keeping the midgut within a living animal. The live animal is secured in a petri dish, and the midgut is visualized through a window cut into the dorsal cuticle. The organ’s structural integrity stays largely intact, which allows routine acquisition of movies ~12-16 hours long. Furthermore, digestive function is preserved; the animals ingest food, undergo peristalsis, and defecate even while being imaged. As a result, these long-term movies vividly capture midgut cell dynamics in a near-native physiological context.

To mine the data in these movies, we also present a systematic approach for image processing and segmentation and for spatiotemporal analysis of single cells and whole populations. These proof-of-principle analyses both corroborate prior fixed-gut observations and reveal intriguing, dynamic behaviors that relate to cell division, differentiation, and loss. (1) For division, we find that mitotic stem cells frequently re-orient—sometimes repeatedly—but can be ‘anchored’ in place by two immature enteroblast cells. (2) For differentiation, we analyze kinetics of Notch activation that reveal the transition from a stem-like to a terminal cell state, and we find that, contrary to expectation, real-time activation does not correlate with contact between Notch- and Delta-expressing siblings. (3) For cell loss, we perform morphometric analysis of enterocyte cell extrusion over time and find that extrusion occurs via ratcheted constriction of a basal junctional ring. These analyses demonstrate the power of examining midgut cell dynamics in a near-native context over multi-hour timescales. By allowing real-time observation of cellular events that were previously inaccessible, our platform holds promise to advance understanding of the fundamental cell behaviors that underlie organ renewal.
RESULTS and DISCUSSION

An apparatus for midgut imaging within live *Drosophila* adults

We designed a ‘fly mount’ for imaging the midgut in live *Drosophila* adults (Fig. 1B). Our mount, similar to an apparatus for imaging adult *Drosophila* brains (Seelig et al., 2010), is assembled from inexpensive, common materials and can be configured for upright, inverted, or light sheet microscopes (Fig. 1-fig. supplement 1A-D). In the mount, a live animal is stabilized by affixing its abdomen in a cutout within a petri dish (upright or inverted microscopes) or a syringe barrel (light sheet microscopes) (Fig. 1-fig. supplement 1A-D, Fig. 1-fig. supplement 2, Video 1). The midgut’s R4a-b (P1-2) region (Buchon et al., 2013a; Marinas and Spradling, 2013) is exposed through a window cut in the dorsal cuticle (Fig. 1B-C, Video 1). This arrangement leaves midgut-associated trachea and neurons largely intact (Video 2). Steps to assemble the fly mount and prepare the midgut are illustrated in a detailed tutorial (Video 1).

Three design features prolong animal viability. First, the animal is provided liquid nutrition through a feeder tube and allowed to ‘breathe’ through unoccluded spiracles (Fig. 1B, Fig. 1-fig. supplement 1A). Second, the exposed organ is stabilized by an agarose bed and bathed in media (Fig. 1C). Third, the animal is kept hydrated in a humidity box (Fig. 1-fig. supplement 1B). Throughout imaging, animals continue to ingest food, undergo peristalsis, and defecate, which suggests that midguts remain in a state that approaches native physiology.

A crucial element is the use of a 20x, high-NA dipping objective, which captures z-stacks that are both wide field (100-300 cells) and high resolution (~1 μm) (Video 3). Time intervals between z-stacks ranged from 5-15 min. At room temperature, 72% of animals were alive and responsive after 12-16 hours of continuous imaging (N=18 animals; median imaging duration, 14.6 h) (Fig. 1-source data 1, Video 4). Nearly all cells remained viable, as revealed by the cell death marker Sytox Green (93-98% viability; Fig. 1-fig. supplement 3, Video 5). However at elevated temperatures (≥29 °C), the midgut was prone to rupture, so temperature-controlled gene expression by GAL80° or heat-shock induction proved impracticable. Progesterone-induced GeneSwitch drivers (Mathur et al., 2010) could be a feasible alternative.

To minimize interference with native digestion, we used no anesthetics. Hence, ~90% of raw movies were blurred due to involuntary midgut contractions and voluntary animal movements (Fig.
A systematic approach for comprehensive spatiotemporal tracking of single cells

Study of dynamic cellular events requires that individual cells be identified, tracked, and analyzed in space and over time. To facilitate these analyses, we generated a ‘fate sensor’ line with fluorescent, nuclear-localized markers for live identification of the midgut’s four major cell types (egGAL4, UAShis::CFP, GBE-Su(H)-GFP:nls, ubi-his::RFP) (Fig. 2A-B; Video 7): (1) Stem cells, which are marked by CFP and RFP. Stem cells are responsible for virtually all cell divisions. (2) Enteroblasts, which are marked by CFP, GFP and RFP. Enteroblasts are Notch-activated stem cell progeny that will mature into enterocytes. (3) Enterocytes, which are marked by RFP and have polyploid nuclei. Enterocytes are terminally differentiated cells that absorb nutrients and that form the bulk of the epithelium. (4) Enteroendocrine cells, which are marked by RFP and have small, diploid nuclei. Enteroendocrine cells are terminally differentiated cells that secrete enteric hormones.

To analyze these multichannel, volumetric movies, we developed a semi-automated workflow. ImageJ and Bitplane Imaris are used to digitally separate marked populations, identify all cells in each population, and track these cells for the duration of the movie (Fig. 2C-D). Comprehensive, single-cell tracking enables features such as fluorescence intensity, spatial position, and nuclear size to be measured for each individual cell. Multiplying the 100-300 cells in a movie over the hundreds of time points in a 12-16 hour imaging session, we collect tens of thousands of real-time measurements. Unlike prior approaches, which relied on manual identification and tracking of a few cells, our approach generates single-cell and population-level data in an unbiased manner.

To demonstrate the utility of this imaging platform and workflow, we performed proof-of-principle analyses for three core behaviors of midgut renewal: enterocyte extrusion and loss (Fig. 3A-F), stem cell division (Figs. 3G-H, 4), and enteroblast differentiation (Figs. 5-6).

Enterocyte extrusion: Spatiotemporal dynamics of ring closure, ring travel, and nuclear travel

Enterocytes in the Drosophila midgut, like enterocytes in the mammalian intestine, are lost through apical extrusion (Buchon et al., 2010; Eisenhofer et al., 2012; Harding and Morris, 1977; Madara, 1990; O’Brien et al., 2011). During extrusion, a cell is ejected out of the epithelium and
into the lumen by the concerted contractions of its neighbors (Eisenhoffer and Rosenblatt, 2012).
Because this process is seamless, extrusion eliminates apoptotic cells while preserving the epithelial
barrier (Gudipaty and Rosenblatt, 2017). Since apoptotic enterocytes secrete stem cell-activating mi-
togens (Liang et al., 2017), understanding when and how apoptotic enterocytes are extruded is im-
portant for understanding midgut turnover.

In fixed tissues, extrusion has been challenging to study because extruded cells leave no trace
in the epithelium. Although fixed sections can catch extruding cells ‘in the act’, they do not reveal
the dynamics of these transient events.

Our imaging platform enabled us to study extrusions live. Most extrusions were enterocytes,
which exited the epithelium either as single cells (18 of 34 total extrusions in 6 independent movies;
Figs. 3A, F, Fig. 3-fig. supplement 1; Videos 8, 9) or as clusters of 2-5 cells (16 of 34 total extru-
sions). We also observed one extrusion of an enteroendocrine cell (Video 10). Extrusions were dis-
tributed comparably across the first and second halves of individual movies. All extrusions were apic-
al.

To gain insight into extrusion dynamics, we performed fine-grained morphometric analysis
on 3 single-enterocyte extrusions (Figs 3A-E; Fig. 3-fig. supplement 1). Enterocytes exited the epi-
thelium through constriction of a basal junctional ‘ring’. Junctional rings were six-sided and marked
by E-cadherin::YFP and myosin::GFP (myosin light chain kinase, or sqh::GFP) (Fig. 3A, Video 8).
As extrusions progressed, rings closed to a point and eventually vanished (Fig. 3B, Video 8). Mean-
while, neighbor enterocytes drew into rosettes with the extruding cells at their center. Ring closure
required ~4-6 hours to complete (Fig. 3-fig. supplement 1D).

Unexpectedly, we found that ring closure was pulsatile and ratcheted. Ratcheted processes
are characterized by pulses of constriction that alternate with pulses of stabilization or even relaxation
(Coravos et al., 2017). All three extrusions exhibited 6-12 of such alternating pulses (Figs. 3C, Fig.
3-fig. supplement 1A-C). Cumulative times of constriction were similar to cumulative times of stabi-
lization/relaxation. By contrast, rates of constriction generally exceeded rates of relaxation, which
drove net closure of the ring over time (Fig. 3-fig. supplement 1A’-C’).

Ratcheting has not previously been implicated in cell extrusion (Kuipers et al., 2014) but is
well-known to drive cell deformation in early embryogenesis (Martin et al., 2008; Rauzi et al.,
2010). However in embryos, pulses are apical, last only 1-2 min, and reshape cells without removing
them. By contrast in enterocyte extrusion, pulses are basal, last 30-60 min, and are associated with delamination.

For an extruding cell to be shed from the epithelium, the junctional ring must not only close but also travel apically toward the lumen. We compared apical travel of the ring to that of the cell nucleus for the extrusion in Fig. 3A. The ring advanced slowly, with stuttering, apical-and-basal movements that produced net apical progress over 5 h of ring closure (Fig. 3D). By contrast, the nucleus shot out of the epithelium in only 15 min (Figs. 3D-E; Video 9, t=150-165 min) and continued to penetrate deeper into the lumen over the next 1.6 h (Figs. 3D-E; Video 9, t=165-263 min). After reaching maximum depth, the nucleus recoiled and came to rest on the apical epithelium (Figs. 3D-E; Video 9, t=263-443 min). These distinct kinetics suggest that the ring and the nucleus use different mechanisms for apical travel.

Altogether, these analyses provide first morphometric insights on homeostatic cell extrusions in real time. They demonstrate the ability of our platform to reveal novel extrusion behaviors, such as ratcheting, and to enable direct comparison of concurrent subcellular events, such as ring and nuclear travel. Through these abilities, our platform opens the door to dynamic and quantitative understanding of cell extrusion during organ turnover.

**Stem cell division: Mitotic orientation in real time**

Tissue homeostasis requires the replacement of extruded cells by new cells. In the midgut, new cells are generated through stem cell divisions, and terminal daughters are typically post-mitotic. Although time-lapse imaging has unique potential to reveal division behaviors (Park et al., 2016), the divisions of midgut cells have been challenging to capture. To date, live divisions have been reported in only one study, which examined pathogen-stimulated midguts *ex vivo* (Montagne and Gonzalez-Gaitan, 2014).

We surveyed our movies of near-native midguts for physiological divisions. Thirty-nine mitoses were identified in 11 independent movies, which had a combined duration of 122 hours. The average mitosis lasted 43 ± 11 min (Fig. 3G-H; Fig. 3-source data 1, Video 11). Together, these measurements imply a mitotic index of 0.28% (see Methods for calculation), which is less than the 1-2% mitotic index obtained from counts of phospho-histone H3* cells in fixed midguts (Jin et al., 2017; Kolahgar et al., 2015; Montagne and Gonzalez-Gaitan, 2014). No divisions were identified in 14 additional movies. Within individual movies, division rates did not trend upward or downward over time. This absence of drift suggests that division rates were reduced not by cumulative imaging
stress, but rather by elements that were already present when imaging began or that occurred stochastically. To lessen this inhibitory effect, adjustments to the media formulation would be one attractive approach.

Each mitosis was exhibited by a unique cell. The vast majority of these were presumably stem cells. However, a recently described cell type, the enteroendocrine precursor cell, accounts for <5% of mitoses (Chen et al., 2018). Our movies lacked markers to distinguish stem cells from enteroendocrine precursors, so the latter may have been responsible for some observed mitoses.

We investigated how mitotic cells dynamically orient in 3D space. In general, epithelial divisions can be considered in two orthogonal frames of reference: horizontal-vertical and longitudinal-circumferential. Horizontal-vertical orientation is defined by the epithelial plane (Fig. 4A) and, in development, serves to determine daughter fates (Cayouette and Raff, 2003; Dong et al., 2012; El-Hashash et al., 2011; Guo and Ohlstein, 2015; Williams et al., 2011). Longitudinal-circumferential orientation is defined by organ shape (Fig. 4F) and determines whether the organ grows longer or wider (Mochizuki et al., 2014; Schnatwinkel and Niswander, 2013; Tang et al., 2011). In epithelial development, well-understood mechanisms orient cell divisions for proper morphogenesis. However in epithelial homeostasis, the existence of analogous orientation mechanisms is a subject of debate.

To shed light on this topic, we investigated whether native mitoses in the midgut exhibited bias in horizontal-vertical or longitudinal-circumferential orientations. While performing these two analyses, we noticed an unexpected, third frame of reference: neighbor enteroblasts (Fig. 4I). Below, we describe real-time mitotic orientation in these three reference frames.

First, we considered horizontal-vertical orientation (Fig. 4A-E, Fig. 4-source data 1). Horizontal-vertical orientations at cytokinesis ranged broadly (1.6°-72°), but were biased toward horizontal. Of 10 dividing cells, 4 were <5° and 7 were <45° (Fig. 4B). These findings are consistent with prior analyses of dividing cells in fixed midguts (Goulas et al., 2012; Ohlstein and Spradling, 2007).

Do horizontal-vertical orientations stay constant throughout division? Analyzing the same 10 cells from metaphase to telophase, we found that orientations were, as a group, also biased toward horizontal; of 41 measurements, 7 were <5° and 33 were <45° (Fig. 4C). Interestingly however, this stable, population-level trend belied the dynamic re-orientations of individual cells. Tracking single cells over time, we found that 8 of the 10 cells re-oriented by ≥15° at least once, and 4 re-oriented by ≥30° (Fig. 4D-E; Videos 12, 13). Re-orientations even occurred repeatedly during a single mitosis; 3 cells re-oriented by ≥15° two or three times. These frequent, sometimes dramatic, re-orientations
were not triggered by peristaltic contractions as no temporal correlation was observed between the
two types of events. The ability of mitotic stem cells to dynamically re-orient, a feature uniquely vis-
ible in live imaging, carries implications for how measurements of spindle angles in fixed midguts are
interpreted.

Second, we considered longitudinal-circumferential orientation (Fig. 4F-H). Measuring 38
cells at cytokinesis, we found that 20 cells were ≤45° and 18 cells were >45° (Fig. 4F-H). Hence,
longitudinal-circumferential orientations are unbiased.

The nature of our movies precluded us from examining a potential exception to this lack of
bias: divisions at midgut compartment boundaries. Spradling and colleagues have reported that
compartmentalization of the midgut into distinct, stereotyped regions is reinforced by clonal parti-
tioning (Marianes and Spradling, 2013). Daughter cells generally remain in the same compartment
as their mother stem cell, and stem cell clones do not cross most compartment boundaries (Marianes
and Spradling, 2013). Since compartment boundaries are circumferential, a possible explanation for
clonal partitioning is that boundary-localized divisions are oriented circumferentially. However, our
movies lacked live boundary markers, so the small minority of divisions that may have occurred at
boundaries could not be distinguished from the large majority of divisions that occurred within
compartments. Further study will be needed to determine whether boundary-localized divisions rep-
resent a special case of circumferential bias.

Finally, we observed that a third, local reference frame formed when two enteroblasts flanked
a dividing cell (Fig. 4I-K). In this three-cell arrangement, divisions occurred nearly parallel to the
two neighbor enteroblasts (4 of 18 divisions; Fig. 4J, K, Fig. 4-source data 1). By contrast, divisions
had a broad range of orientations if only one neighbor enteroblast was present (11 of 18 divisions;
Fig. 4K). When trapped between two enteroblasts, daughter cells at cytokinesis hurled into and for-
cibly collided with the enteroblast nuclei (Fig. 4J; Video 14, t=15-22.5 min). These observations
suggest that physical contact between stem cells and enteroblasts is a spatial cue that orients the mi-
totic spindle.

In summary, these analyses provide first views of how live stem cells orient their divisions
within the midgut’s tubular epithelium. They also reveal mitotic behaviors, such as frequent hori-
zontal-vertical re-orientations, that are undetectable in fixed samples. Examining three reference
frames, we found three orientation patterns: (1) Biased horizontal orientations. In future work, a

crucial question will be whether, and if so how, horizontal orientations promote symmetric daughter
fates (Goulas et al., 2012; Guo and Ohlstein, 2015; Kohlmaier et al., 2015; Montagne and González-Gaitan, 2014; Sallé et al., 2017). (2) Unbiased longitudinal-circumferential orientations. This balanced distribution may help maintain constant organ shape over time. (3) Local orientation by two enteroblasts. This unanticipated finding supports the notion that stem cell-enteroblast adherens junctions, which are unusually pronounced (Ohlstein and Spradling, 2006), could orient the divisions of midgut stem cells, akin to other Drosophila stem cells (Inaba et al., 2010; Le Borgne et al., 2002; Lu et al., 2001). In our analysis, all mitoses occurred in the midgut’s R4a-b (P1-2) region that is exposed by the cuticular window; whether divisions in other regions, or at region boundaries, behave similarly is an open question. Looking forward, these findings provide a basis to directly investigate midgut division orientation and to probe the relationship between orientation and fate.

A quantitative threshold of Notch activation distinguishes stem cells and enteroblasts

Along with cell division and loss, cell differentiation is the third core behavior of tissue renewal. In the Drosophila adult midgut, differentiation in the enteroblast-enterocyte lineage is controlled by Delta-Notch. Delta ligand, which is expressed predominantly in stem cells, activates Notch receptor on stem (or stem-like) cells. At low levels, Notch activity is compatible with stemness. However at higher levels, it triggers enteroblast differentiation (Bardin et al., 2010; Biteau and Jasper, 2014; Kohlmaier et al., 2015; Michelli and Perrimon, 2006; Ohlstein and Spradling, 2006; 2007; Perdigoto et al., 2011; Zeng and Hou, 2015).

Fate sensor midguts (Fig. 2) enable Notch activity to be measured live. GBE- Su(H)-GFP:nls provides a sensitive readout of Notch transcriptional activation (de Navascués et al., 2012; Furriols and Bray, 2001; Guisoni et al., 2017; Housden et al., 2014), while ubi-his2av::mRFP provides a stable reference signal. We used these two markers to establish a normalized metric of Notch activity. First, to account for differences between movies, we normalized the values of GFP and RFP intensities within a given movie to a 0-to-1 scale. Second, to account for tissue depth and other artifacts within a single movie, we used these normalized GFP and RFP intensities to calculate the ratio of GFP:RFP for each egf-his2ab::CFP progenitor cell at each time point. This two-part calculation of real-time GFP:RFP enables Notch activity to be compared over time and between cells, even in different movies.

We asked whether real-time GFP:RFPs are consistent with conventional indicators of enteroblast differentiation. The numeric values of GFP:RFPs, which ranged from 0.0-1.8, generally fit with subjective evaluations of GFP intensities (Figs. 5A-B). In addition, population-level distribu-
tions of GFP:RFP were similar at different imaging depths, over time within a single movie, and
across different movies. Furthermore, eg^e cells with large nuclei (≥200 μm^3) often exhibited high
GFP:RFPs, while cells with low GFP:RFP typically had small nuclei (Fig. 5B). This association of
high GFP:RFPs with large but not small enteroblast nuclei fits with prior observations that en-
doreplication is characteristic of late enteroblasts (Jiang et al., 2009; Kohlmaier et al., 2015;
Lucchetta and Ohlstein, 2017; Perdigoto et al., 2011; Xiang et al., 2017; Zhai et al., 2017). Alto-
gether, these findings support the use of GFP:RFPs as a metric of Notch activation.

Having validated this metric, we used GFP:RFPs to measure Notch activity in stem cells and
enteroblasts. Genetic modulation of Notch signaling had revealed that stem and enteroblast identi-
ties are characterized by low and high activation (Perdigoto et al., 2011), but actual levels of Notch
signaling were not quantified. We wondered whether GFP:RFPs could quantitatively distinguish
stem cells and enteroblasts in real time.

Examining GFP:RFPs for all eg^e progenitor cells in two fate sensor movies (29,102 values;
251 cells), we found that their distribution is—suggestively—bimodal. A local minimum at
GFP:RFP=0.17 separates a sharp left peak (GFP:RFP=0.015) and a broad right peak
(GFP:RFP=0.528) (Fig. 5C, Fig. 5-source data 1). An appealing interpretation of this bimodality is
that the left peak represents stem cells and the right peak represents enteroblasts.

To directly test this interpretation, we cross-correlated Notch activity with mitotic behavior.
Mitosis is near-exclusive to stem cells, so cells that went through mitosis during imaging were identi-
fiable as stem cells, independent of GFP:RFP. The GFP:RFPs of these cells in the time points prior to
their observed mitoses were used to create a ‘benchmark’ collection of GFP:RFPs from known stem
cells (1,294 GFP:RFPs; 18 cells).

The benchmark collection of stem cell GFP:RFPs was compared to all progenitor
GFP:RFPs. If the left peak of the eg^e distribution represents stem cells, then its GFP:RFP profile
should resemble the profile of the ‘benchmark’ stem cells. Indeed, the two profiles nearly matched
(Fig. 5C’). Furthermore, 99.61% of GFP:RFPs for benchmark stem cells were less than the 0.17
threshold. This correspondence implies that stem cells populate the left peak and enteroblasts the
right peak. Supporting this interpretation, the number of data points in the left and right peaks have
a proportion of 4:3, which resembles the proportions of stem cells to enteroblasts that have been re-
ported previously using fixed tissues (Guisoni et al., 2017; O’Brien et al., 2011).
Based on these findings, we conclude that GFP:RFP=0.17 marks a threshold level of Notch activation that functionally distinguishes stem cells from enteroblasts. The precise value of 0.17 is likely specific to our particular microscope and imaging parameters, and a different microscope system would require re-assessing the enteroblast threshold through measurements of normalized GBE-Su(H)-GFP:nls intensities. Nonetheless, our findings argue that when Notch activity reaches a specific, critical level, cells transition from a stem-like to an enteroblast state.

**Real-time kinetics of enteroblast transitions**

A fundamental aspect of fate transitions is the time over which they occur. Fast transitions would allow cells to respond nimbly to acute challenges, whereas slow transitions would allow cells to receive and integrate a large number of fate-influencing signals. In this manner, the kinetics of fate transitions can define how an organ responds to changing external environments.

Midgut fate transitions have not been measured directly to date. For enteroblasts, an upper limit of two days can be inferred from observations that enteroblasts are present in stem cell clones two days post-induction (de Navascués et al., 2012; Ohlstein and Spradling, 2007). However, in developing tissues, activation of Notch target genes can occur in minutes-to-hours (Corson et al., 2017; Couturier et al., 2012; Gomez-Lamarca et al., 2018; Housden et al., 2013; Vilas-Boas et al., 2011). This precedent raises the possibility that Notch-mediated enteroblast transitions in the midgut could be considerably faster than two days.

To examine enteroblast transitions directly, we measured the rate of *GBE- Su(H)-GFP:nls* activation in movies of fate sensor midguts. A cell was scored as undergoing an enteroblast transition if GFP:RFP persistently increased from below to above the 0.17 threshold. From 95 cells with initial GFP:RFP<0.17 in two movies, 5 such transitions were identified. We analyzed kinetics for 4 of these, each of which occurred in a cell that was born before imaging started (Fig. 5D-E, Video 15).

We found that enteroblast transitions (Fig. 5D-E, gray background shading) occurred over multiple hours (2.4-6.9 h)—faster than the 2-day upper limit implied by clones, and slower than the minutes observed in some other tissues. Higher initial GFP:RFPs did not correlate with shorter transition times. Whether the imaging protocol itself affected these kinetics is difficult to ascertain, but cumulative imaging stresses were likely not a factor since transitions that occurred later in imaging sessions did not have consistently slower or faster increases of GFP:RFP.

Intriguingly, we also observed *eg* cells in which GFP:RFP fell from above to below 0.17 over several hours. These events might suggest that some nascent enteroblasts revert to a stem-like
state, at least in terms of Notch activity. If so, then enteroblast specification, as marked by loss of
mitotic capacity, occurs before commitment, in which terminal fate becomes irreversible. This two-
step process is consistent with our observed, multi-hour timescale of Notch activation: During early
transition stages, a prolonged period of low-level Notch activity likely results in prolonged expression
of high affinity Notch target genes. These targets, which are currently unknown, could serve to ini-
tialize a bistable switch that culminates in irreversible fate commitment (Bray and Gomez-Lamarca,
2018; Ferrell and Xiong, 2001). By lengthening the time between specification and commitment,
slower activation may provide nascent enteroblasts with more opportunities to ‘backtrack’ if the tis-
sue environment changes.

Contacts between newborn siblings are variable and dynamic

In order to activate Notch, a prospective enteroblast must physically contact a Delta-
expressing cell. In principle, newborn sibling cells would be ideally suited to engage in Notch-Delta
interactions with each other (Guisoni et al., 2017): Newborn cells express both Notch and Delta
(Bardin et al., 2010; Ohlstein and Spradling, 2007), and cytokinesis leaves sibling cells juxtaposed.
Sibling-sibling Notch activation requires that the two siblings stay in contact long enough to over-
come the time delays inherent to Delta-Notch lateral inhibition (Barad et al., 2010; Du et al., 2017;
Guisoni et al., 2017). However, fixed-gut studies of twin spot clones imply that after cytokinesis,
some sibling pairs become separated (O’Brien et al., 2011). If sibling contacts can be transient, then
the relationship between contact dynamics and Notch activation kinetics becomes crucial to entero-
blast specification.

To investigate contact dynamics, we sought to visualize them directly by incorporating a
membrane-localized YFP into our fate sensor line, which already contained nuclear-localized CFP,
GFP, and RFP (Fig. 2A-B). However, we were unable to parse the YFP signal without sacrificing
sensitivity in the critical GBE-Su(H)-GFP:nls channel. As an alternative, we evaluated whether con-
tact between siblings could be inferred from the distance separating their nuclei. To directly compare
cell-cell contact and inter-nuclear distance, we used movies of midguts in which progenitor cell
boundaries were visualized by LifeactGFP and nuclei by His2av::RFP (Fig. 6-fig. supplement 1, Fig.
6-fig. supplement-source data 1). This analysis revealed two strong correlations: progenitors with
inter-nuclear distance <6.0 μm were nearly always in contact, and progenitors with inter-nuclear dis-
tance >15.5 μm were nearly always separated. Inter-nuclear distances between 6.0-15.5 μm did not
correlate with either contact or separation. Based on these findings, we designated three classifica-
tions: inferred contact (inter-nuclear distance <6.0 μm), indeterminate (inter-nuclear distance ≥6.0 and ≤15.5 μm), and inferred separation (inter-nuclear distance >15.5 μm).

We used these classifications to examine the contact dynamics of sibling pairs in movies of fate sensor midguts. Analyzing 18 sibling pairs with known birth times, we found they exhibited a broad diversity of contact behaviors (Fig. 6A, Fig. 6-source data 1). At one extreme were high-contact pairs, which generally stayed in place after cytokinesis (pairs A-C, Fig. 6A). At the other extreme were low-contact pairs, which separated soon after cytokinesis (pairs N-R, Fig. 6A). Eight of 18 pairs separated at least one hour; two pairs separated and contacted repeatedly; and six pairs appeared to separate permanently. Notably, these dynamic, variable contact behaviors could not have been deduced from static images. Our observation that sibling cells routinely lose contact suggests that a substantial proportion of true sibling pairs may be missed by conventional fixed-gut assays that consider only contacting pairs.

Does sibling contact correlate with real-time Notch activation? To the contrary, both high- and low-contact siblings generally maintained low GFP:RFPs (Fig 6B-C, Videos 16, 17). Of the 36 individual siblings we tracked (Fig. 6A), only one showed persistent activation of Notch (Cell 1 of Pair L, Fig. 6D; Video 18). This particular cell was in likely contact with its sibling for at least one hour, perhaps longer, before its GFP:RFP began to increase (Fig. 6D, t=4.0 h). During the subsequent 6.2 h, GFP:RFP climbed to the enteroblast threshold even after the two siblings likely lost contact (Fig. 6D, t=9.1 h). (Note that in Fig. 6D, a collision of the Fig. 6D siblings with a mature enteroblast caused GFP:RFP measurements to artifactually spike between t=2.6-3.5 h (Cell 1) and t=1.5-3.6 h (Cell 2). See Fig. 6D caption and Video 18.) All other sibling cells remained stem-like, with no persistent Notch activation, until the end of imaging. Had imaging continued, it is possible that additional siblings might have transitioned to enteroblasts. Unfortunately, the influence of sibling contacts on such hypothetical fates cannot be assessed. Nonetheless, a simple model in which sibling-sibling contact causes rapid, asymmetric Notch activation, akin to Drosophila sensory organ precursor cells (Schweisguth, 2015), is not supported by our live data.

A delay in Notch activation?

Activation of Notch in new cells did not occur immediately, but rather multiple hours after birth. In Fig. 6D, the Notch-activating sibling was 10 hours old when it reached the enteroblast threshold. In Fig. 5D-E, cells had already been imaged for 7-12 hours when they reached the enteroblast threshold; since all these four cells were born before imaging started, their elapsed times after
birth were even longer. Because midgut cell divisions are asynchronous and stochastic, this post-birth delay in Notch activation would have been difficult to uncover without the time-resolved tracking of single cells.

Our finding that Notch activation is delayed raises at least three discussion points. First, it may explain why fewer enteroblast outcomes were observed using live versus fixed approaches. In our live movies, only 1 of 18 sibling pairs exhibited asymmetric Notch activation (Fig. 6D). By contrast in prior, fixed studies that also used 2-day midguts, 20-30% of twin-spot sibling clones exhibited asymmetric, stem-enteroblast fates (Chen et al., 2015; O’Brien et al., 2011). A major difference in the two approaches is timescale: hours for live imaging, days-weeks for twin-spot clones. With Notch activation delayed, some newborn cells that appeared stem-like during an hours-long movie might have activated Notch later and become an enteroblast or enterocyte in a days-old twin-spot clone. In this manner, the delay in Notch activation may have caused enteroblast fates to be underestimated in live movies.

Second, an important caveat is that we do not presently know whether Notch activation is comparably delayed in intact, unperturbed animals. Some potential confounding factors, such as the time required for GFP biosynthesis (Balleza et al., 2018; Couturier et al., 2012; Housden et al., 2013; Kawashashi and Hayashi, 2010; Vilas-Boas et al., 2011), involve timescales of minutes and are thus unlikely to be responsible for a delay of hours. However, because of technical challenges with pinpointing birth times for midgut cells in unperturbed animals, we cannot exclude that delayed activation is a consequence of the imaging protocol and not native physiological behavior.

Third, if delayed activation is physiological, then how exactly is it generated? And what are the consequences for dynamic fate control? One attractive notion is that a period of latency after birth could enable a cell to integrate a broad range of signals before choosing to either differentiate or self-renew. By allowing cells to fully process intrinsic and extrinsic fate signals, a latent, ‘waiting’ period could ensure that individual cell fates are coordinated with overall organ needs.

CONCLUSION

The *Drosophila* adult midgut is a premier model for organ renewal, but understanding the dynamics of renewal has been hampered by a lack of robust methodology for live imaging. Here, we have presented an imaging platform that captures the midgut in a near-native state within a live animal, yielding movies of exceptional visual quality and duration. In conjunction, we have described a pipeline for comprehensive, 4D movie analysis. We applied this pipeline to our movies for proof-of-
principle analyses that corroborated fixed-tissue observations and uncovered new renewal behaviors. These novel findings ranged from time-resolved, single-cell dynamics of division orientation and apical extrusion to large-scale, population-level measurements of Notch activation. The ability to simultaneously span cell- and tissue-level scales over extended imaging periods opens the door to quantitative study of the spatiotemporal complexity of tissue renewal.

Despite these advances, our platform also has limitations. The positioning of the dorsal window restricts imaging to one organ region, so region-to-region comparisons cannot be made in real time. The media that covers the open window also dilutes the circulating hemolymph, which contains molecules that signal to midgut cells. Further refinement of our media formulation might help restore division rates to native levels and extend midgut viability beyond 16 hours. Similar improvements might be achieved with reduced exposure to laser light, for instance via a spinning disk set up. Indeed, such enhancements will be needed to reach the paramount goal of capturing serial divisions of a single stem cell and tracing full lineages. Post-acquisition, our current registration algorithms cannot resolve movement-induced blurring in ~30% of raw movies; more sophisticated algorithms that correct for z-movements within a stack would render many of these movies analyzable.

In addition to midgut cell dynamics, our platform offers the opportunity to investigate other biological phenomena in the *Drosophila* abdomen. Animals ingest food during imaging, which enables real-time observation of events, such as colonization by ingested pathogens, that occur in the midgut lumen. By shifting the position of the cuticular window, the platform could also be adapted to study events in other abdominal organs, including ovary, testis, Malpighian tubule, and hindgut. We anticipate that long-term imaging of *Drosophila* adults will lead to new, dynamic understanding of the cell and tissue behaviors that govern the form and physiology of mature organs.
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Fig. 1. Extended imaging of the midgut in live *Drosophila* adults.

(A) Adult female midgut *in situ*, sagittal view. White highlight indicates region (R4a-b, also known as P1-2 (Buchon et al., 2013a; Marianes and Spradling, 2013)) of the midgut that will be exposed for imaging. (B-C) The midgut is accessed through a small cuticular window in the back of a live animal. B top, Schematic of imaging apparatus. The animal is affixed to a modified petri dish.
‘mount’. The chamber of the mount contains media. The underside of the mount supports a feeder
tube. See Fig. 1-fig. supplement 1 A-B and Fig. 1-fig. supplement 2. B bottom, Dorsal (left) and
ventral (right) views of an animal in the mount. In the left panel, the exposed midgut is outlined by
the magenta dotted line. Scale bars: 0.25 mm (left), 0.5 mm (right). See Video 4. C, Steps to prepare
the midgut for imaging. See Video 1 tutorial.

(D-F) Registration macros are applied post-acquisition to correct blurring from tissue movements.

D, Before registration, blurring and duplications (arrowheads) are evident. Panel is a raw z-series
projection of one movie time point. E, During registration, two ImageJ plugins are applied in series.
(1) StackReg corrects for tissue movement during z-stack acquisition at a single time point. (2) Cor-
correct 3D Drift corrects for global volume movements over multiple time points. F, After registration,
blurring and duplications are negligible. Cyan, all nuclei \(ubi-bis2av::mRFP\); yellow, stem cells and
enteroblasts \(eg\times Lifeact\text{GFP}\). Scale bars, 20 \(\mu\text{m}\). See Video 6.
Fig. 2. Comprehensive, fate-specific tracking and analysis of individual cells.

(A-B) ‘Fate sensor’ midguts enable live identification of cell types. A, Stack projection of a single time point from a 10-hour movie (Video 7). Nuclei are distinguishable for four midgut cell types: stem cells (red pseudocolor), enteroblasts (yellow-green pseudocolor), enterocytes (gray, polypliod), and enteroendocrine cells (gray, diploid). Inset shows the zoom region depicted in B. B, Genetic design of fate sensor line (esg>his2b::CFP, GBE-Su(H)-GFP:nls; ubi-his2av::mRFP). Cell types are distinguished by combinatorial expression of three fluorescent, nuclear-localized markers: enterocytes/enteroendocrine cells (His2ab::mRFP only), stem cells (His2ab::mRFP, His2b::CFP), and enteroblasts (His2ab::mRFP, His2b::CFP, GFP:nls). All scale bars, 10 μm.

(C-D) Workflow to identify, track, and analyze cells in volumetric movies. C, From raw, multi-channel z-stacks, nuclei are digitally separated into stem cell, enteroblast, and enterocyte/enteroendocrine populations using channel masks in ImageJ. D, The three population sets are rendered in 4D in Imaris. Segmentation is performed on each population to identify individual nuclei. Enterendocrine nuclei are separated from enterocyte nuclei by a size filter. Positions of individual nuclei are correlated between time points to track single cells over time.
Fig. 3. Real-time kinetics of enterocyte extrusion and stem cell mitosis.

(A-E) Morphometric analysis of a single-enterocyte extrusion. A, Time-lapse sequence (top) and schematic (bottom) shows planar view of an extrusion event. The basal region of the extruding cell (tan pseudocolor) is outlined by a six-sided ‘ring’ of E-cadherin::YFP (inverted gray, ubi-DE-cadherin::YFP). Over time, the basal ring closes to a point, and the six neighbor cells (green in schematic) draw into a rosette. Top panels are stack projections. Cyan (ubi-bis2av::mRFP) labels all nuclei. See Video 8. B, Spatial ‘footprint’ of the E-cadherin::YFP ring in the epithelial plane over time (violet-yellow color scale). The ring remains six-sided throughout closure. C, Ring closure occurs via ratcheted constrictions. During ring closure, pulses of constriction (shaded background) are interrupted by pulses of relaxation (unshaded background). See Fig. 3-fig, supplement 1. D, Kinetics of apical travel. Displacements of the junctional ring (purple) and the cell nucleus (red) are shown over time for the extrusion in Panel A. The ring (purple) advances incrementally via small, apical-and-basal movements. The nucleus (red) ejects rapidly into the lumen, then recoils. Apical nuclear
travel starts at t=150 min and ends at t=263 min (dotted vertical lines). E, Orthoview of the same 
extusion as A. Multicolored line shows path of nucleus over time (violet-yellow color scale). Magenta 
box denotes the E-cadherin::YFP ring, which is visible in this time point (t=285 min) as a density 
of YFP at the apical surface. Inverted gray, E-cadherin::YFP (ubi-DE-cadherin::YFP); cyan, all nuclei 
(ubi-bis2av::mRFP). See Video 9. F, Durations of apical nuclear travel for 18 single-enterocyte extru-
sions from 6 movies. Apical travel lasted 37-112 min with a mean of 64 ± 18 min (SD). 
(G-H) Kinetics of stem cell mitoses. G, Time-lapse sequence of a mitotic event. Green, actin 
(eg-LifectGFP); yellow, E-cadherin (ubi-DE-cadherin::YFP); red, nuclei (ubi-bis2av::mRFP). Panels 
are partial stack projections of the basal epithelium. See Video 11. H, Durations of mitosis for 39 
 cell division from 11 movies. Mitoses lasted from 30-60 min with a mean of 43 ± 11 min (SD). All 
scale bars, 10 μm.
Figure 4
Fig. 4. Real-time orientations of stem cell divisions in three reference frames.

(A-E) Horizontal-vertical orientations are horizontally biased. A, Schematic of horizontal (0°) and vertical (90°) orientations. See Fig. 4-fig. supplement 1. B, Live orientations of 10 dividing cells specifically at cytokinesis. The distribution is biased toward horizontal (<45°). Red point represents the cell in Panel D. C, Live orientations of the same 10 cells throughout mitosis. Each measurement is the orientation of one mitotic cell at one time point, from metaphase to cytokinesis (n=51 measurements). The distribution is biased toward horizontal (<45°). D, Two re-orientations in a single mitosis. Red line shows the orientation of condensed chromatin (gray, ubi-his2ab::mRFP) relative to the basal basement membrane (cyan, Concanavalin A-Alexa-647). For clarity in the 7.5 min and 15 min projections, a clipping plane was applied in the gray channel to exclude an enterocyte nucleus; this nucleus is marked by an asterisk at the left edge of the 30 min projection. Scale bar, 5 μm.

See Video 12. E, Mitotic cells frequently re-orient. Each line shows the horizontal-vertical orientations of a single mitotic cell over time. The 10 cells are the same as in Panels B and C. All lines start at metaphase (t=0 min) and continue to cytokinesis (t=30-60 min). Time intervals were either 5, 7.5, or 15 min. Colors are the same as in Panel B; red line is the cell in Panel D.

(F-H) Longitudinal-circumferential orientations are unbiased. F, Schematic of longitudinal (0°) and circumferential (90°) orientations. G-H, Live orientations of 38 dividing cells at cytokinesis. Longitudinal (≤45°) and circumferential (>45°) orientations are near-equal.

(I-K) Divisions between two flanking enteroblasts align with the enteroblast-enteroblast axis. I, Schematic of divisions contacting either two or one enteroblast(s). When two enteroblasts are present, the closer enteroblast is used for measurements (see Methods). J, Division between two enteroblasts. Orientation is nearly parallel to the axis between the enteroblast nuclei (magenta, GBE-Su(H)-GFP:nls). Gray, stem cell and enteroblast nuclei (egs>his2b::CFP). Scale bar, 10 μm. See Video 14.

K, Live orientations of divisions with two or one flanking enteroblast(s). With two enteroblasts (n=4 of 18 divisions), orientations are near-parallel to the enteroblast-enteroblast axis. With one enteroblast (n=11 of 18 divisions), orientations are broadly distributed. Orientations were measured at cytokinesis. Means ± SD are shown. Mann-Whitney test, p=0.01.
Fig. 5. Whole-population and single-cell analyses of real-time Notch activation.

(A-C) A threshold level of Notch activation distinguishes stem cells and enteroblasts. A, Single-cell measurements of the Notch reporter GBE-Su(H)-GFP:nls from live movies. Cells additionally co-express egg-his2ab::CFP (magenta) and ubi-his2ab::mRFP (gray). GBE-Su(H)-GFP:nls activation is quantified as GFP:RFP (see Methods). For the indicated cells, GFP:RFP = 0.94, 0.27, and 0.01. B, GFP:RFP values correlate with visible GFP and nuclear volume. Progenitor (egg) cells were scored by eye as either GFP-negative (top) or -positive (bottom). In cells without visible GFP, nearly all GFP:RFP values cluster between 0.0 and 0.2, and most nuclear volumes are small (<200 μm^3). In cells with visible GFP, most GFP:RFP values are spread between 0.1 and 1.4, and large nuclear volumes (≥200 μm^3), indicative of late enteroblasts, are associated with high GFP:RFPs. Blue dotted
line shows the 0.17 enteroblast threshold from Panel C. C, GFP:RFP values quantitatively distinguish stem cells and enteroblasts. Gray bars show real-time GFP:RFPs for all \textit{eg} cells in two movies (29,102 GFP:RFPs from 251 cells). Two peaks (GFP:RFP=0.015, 0.528) are separated by a local minimum (blue dotted line; GFP:RFP=0.17). Purple bars (C' inset) show real-time GFP:RFPs for ‘benchmark’ stem cells prior to an observed mitosis (1,294 GFP:RFPs from 18 pre-mitotic cells). The benchmark stem cell distribution matches the left peak of the \textit{eg} cells, and 99.6% of ‘benchmark’ GFP:RFPs are less than 0.17. Data in B and C are the aggregate of 2 movies.

(D-E) Stem-like cells transition to enteroblasts over multiple hours. D, Real-time activation of \textit{GBE-Su(H)-GFP:nls} reveals a transition from a stem-like to an enteroblast state. During a transition period lasting 6.9 h (gray background), GFP:RFP increases from a baseline of -0.049 at \(t=3.5\) h to the enteroblast threshold of 0.17 (blue dotted line) at \(t=10.4\) h. After the transition, GFP:RFP continues to increase and reaches 0.364 at \(t=15.0\) h. \textit{GBE-Su(H)-GFP:nls} shown in green (top) and inverted gray (bottom); \textit{eg>}\textit{his2ab::CFP}, magenta; \textit{ubi-}\textit{his2ab::mRFP}, gray. See Video 15. E, Kinetics of three additional enteroblast transitions. Initial baseline GFP:RFPs are <0.17. GFP:RFPs increase from baseline to 0.17 during transition periods lasting from 2.3-6.9 h (gray backgrounds: \(t=0.3-7.1\) h (top), 10.0-12.4 h (middle), 9.5-11.8 h (bottom)). Initial and final GFP:RFPs are as follows: 0.058, 0.426 (top); 0.069, 0.281 (middle); 0.022, 0.257 (bottom). All cells in Panels D and E were born before imaging started. Genotype in all panels: \textit{egGal4, UAS-his2b::CFP, Su(H)GBE-GFP:nls; ubi-}\textit{his2av::mRFP}. All scale bars are 5 μm.
Fig. 6. Dynamics of cell contact and Notch reporter activation in sibling cells after birth.

(A) Contacts between newborn siblings are highly variable. Eighteen pairs of sibling cells (rows A-R) were tracked from birth (t=0.0 h) to the end of imaging. Color shows the likelihood of sibling-sibling contact based on inter-nuclear distance (Fig. 6-supplement 1): Yellow, inferred contact (inter-nuclear distance<6.0 μm); green, indeterminate (inter-nuclear distance=6.0-15.5 μm); blue, inferred separation (inter-nuclear distance>15.5 μm). Pairs are ordered from highest (A) to lowest (P) contact. Pairs A, L, and P (red labels) are featured in Panels C, D, and B, respectively.

(B-D) Contacts between siblings do not correlate with real-time GBE-Su(H)-GFP:nls activation. Graphs show real-time contact status (background colors same as A) and GFP:RFP ratios. Sibling birth is at t=0.0 h. Red vertical lines are the time points shown in the bottom images. B, Low-contact pair P does not exhibit persistent activation of GBE-Su(H)-GFP:nls. C, High-contact Pair A does not exhibit persistent activation of GBE-Su(H)-GFP:nls. D, Indeterminate-low contact Pair L exhibits persistent activation of GBE-Su(H)-GFP:nls in one sibling. The Pair L siblings are likely in contact from t=2.6-3.6 h and are likely separated after t=9.1 h. Note that between t=2.6-3.5 h (Cell 1) and t=1.5-3.6 h (Cell 2), GFP:RFP measurements (grayed dots) are artificially high due to the
two cells colliding with a third cell that was a mature enteroblast (Video 18). Because of the intimate
proximity between the mature enteroblast and the Pair L siblings during the collision, the high GFP
signal of the enteroblast bled over into the surfaces for Cells 1 and 2. The duration of artifactual
bleed-over is indicated by gaps in the cells’ interpolated GFP:RFP lines. Genotype for all panels:
*esgGal4, UAS-his2b:CFP; Su(H)GBE-GFP:nls; ubi-his2av::mRFP*. All scale bars are 10 μm. See Vide-
eos 16-18.
FIGURE SUPPLEMENTS

Figure1-.figure supplement 1

A  Upright

B  Inverted

C  Light Sheet

D  3D view

side view

end on view
Fig. 1-fig. supplement 1. Mounts for upright, inverted, and light sheet microscopes.

(A-B) Mount for upright microscopes. A, Schematic of animal in mount on microscope stage. A', Isometric illustrations of mount components: (1) modified petri dish, (2) metal shim with cutout for Drosophila abdomen (Fig. 1-fig. supplement 2), (3) feeder tube, and (4) bottom chamber with wet Kimwipes (light blue). (Bottom chamber is not shown in A.) B, Schematic of humidity box that encloses the mount. Unassembled (B) and assembled (B') views are shown.

(C) Mount for inverted microscopes. The midgut is imaged through a glass-bottom petri dish. To elevate the animal, two spacers are glued to the bottom of the dish, and the metal shim is affixed to the spacers. Media is added to the level of the spacers.

(D) Mount for light-sheet microscopes. The barrel of a 1-ml syringe is modified to fit the metal shim. The animal and feeder tube are inside the barrel, and the dorsal surface and exposed midgut are outside the barrel. The barrel is submerged in media with one end remaining open to air. 3D, side, and end-on views are shown.
Fig. 1-fig. supplement 2. Specifications for abdomen cutouts.

The metal shim of the imaging mount includes a cutout through which the dorsal abdomen is inserted. ‘Fat’ (left) and ‘skinny’ (right) cutouts accommodate differently sized female abdomens. This diagram can be used as a CAD file for automated laser cutters.
Fig. 1-fig. supplement 3. Cell viability during extended imaging.

Cell viability during extended imaging was evaluated using the cell death stain Sytox Green. (A) Positive control. To induce cell death, midguts were dissected out of animals and cultured *ex vivo*. Sytox' cells (green) are rare at the start of culture (0 h) but became abundant after 2.5 h. (B) Appearance of a Sytox' cell during extended live imaging. In A-B, arrowheads point to the same cells before and after becoming Sytox'. Nuclei are magenta (*ubi-his2av::mRFP1*). Scale bars, 20 μm. See Video 5. (C) Timeline for appearance of Sytox' cells during extended live imaging of three midguts. Each dash marks the time at which one Sytox' midgut cell became visible. A dot marks the end of each imaging session. Yellow box in Midgut 1 marks the timepoints that are shown in Video 5 (10.6-12 h).
Fig. 3-fig. supplement 1. Enterocyte extrusion occurs via ratcheted constriction of a basal junctional ring.

(A-C) Cross-sectional area of the basal junctional ring over time for three enterocyte extrusions (blue, green, red). Pulses of ring constriction (colored background) alternate with pulses of ring stabilization or relaxation (uncolored background). (A'-C') Rates of change in ring area over time. Rings fluctuate between pulses of constriction (negative values) and pulses of stabilization (near-zero) or relaxation (positive values). Rates of constriction are generally higher than rates of relaxation. (D) Side-by-side comparison of ring area over time for the extrusions in Panels A-C. Initial area of the ring does not correlate with the overall time required for complete closure (Fig. 3-fig. supplement 1-source data 1). The blue extrusion in Panels A and D is identical to that in Fig. 3A-E and Videos 8-9.
Fig. 4-fig. supplement 1. Measurement of horizontal-vertical spindle orientation in space.

Horizontal-vertical orientation of the mitotic spindle was measured as the angle at which the presumptive spindle axis intersected a plane tangent to the basal surface of the mitotic cell. Spindle axes and basal planes were determined by examination of volumetric movies in Imaris. To establish coordinates for the spindle axis (red line), two points (red dots) were placed relative to the condensed chromatin (*ubi-his2ab::mRFP*). To establish coordinates for the basal plane (blue rectangle), three points (blue dots) were placed on the basal epithelial surface underlying the spindle. The (x,y,z) coordinates of these five points were input into a vector algebra expression to calculate the horizontal-vertical spindle angle (see Methods).
Fig. 6-supplement 1. Comparison of cell-cell contact and inter-nuclear distance for live pairs of progenitor cells.

(A) Examples of contacting and separated progenitor pairs. Contact is revealed using esg-driven LifeactGFP (green) to label the actin cytoskeleton of progenitor cells. Inter-nuclear distance (purple lines) is the distance between the centroids of the two nuclei (gray). Images are projections of single time points; however, contact and inter-nuclear distance were assessed in volumetric space. Scale bars, 10 μm. (B) Inter-nuclear distances of contacting and separated pairs. All pairs with inter-nuclear distances <6.0 μm (yellow background) are in contact (26% of all contacting pairs). Pairs with inter-nuclear distances from 6.0-15.5 μm (green background) are split between contacting and separated (73% of all contacting pairs; 58% of all separated pairs). All pairs with inter-nuclear distances >15.5 μm (blue background) are separated (42% of all separated pairs).

These three ranges are used in Fig. 6 to infer the likely contact behavior of esg⁺ sibling pairs that express only nuclear markers (inter-nuclear distance <6.0 μm, likely contact; 6.0-15.5 μm, indeterminate; >15.5 μm, likely separated). n=49 contacting and 43 separated pairs. Pairs were designated as two esg⁺ cells that were mutually closer to each other than to any other esg⁺ cell and were selected randomly from single time points of four movies. Genotype: esgGal4, UAS-LifeactGFP; ubi-his2av::mRFP.
CAPTIONS FOR SUPPLEMENTAL VIDEOS 1-18

Video 1. Narrated, step-by-step tutorial illustrates an animal being prepared for midgut imaging in the fly mount.

Video 2. Five-hour movie shows association of trachea (cyan) with the midgut tube (red). Smaller tracheal branches encircle the tube and move in concert with peristaltic contractions. A large tracheal branch (upper right) is continuous with smaller branches. The large branch does not move during peristalsis because it is not physically associated with the midgut; instead, it connects the midgut-associated branches to a spiracle (not visible in movie frame). Left video: Cyan pseudocolor, trachea (breathlessGal4, UAScyn-GFP); red pseudocolor, microtubules (SiR-tubulin). Right video: Inverted gray, breathlessGal4, UAScyn-GFP. Each time point is the projection of a confocal z-stack. Scale bar, 20 μm.

Video 3. Fifteen-hour, volumetric movie of the midgut illustrates the wide-field, high-resolution images that are acquired. Numerous physiological contractions of the midgut are evident. An enteric tracheal tube is visible in the lower left. Scale bar, 70 μm.

Video 4. After 16 hours of continuous imaging, the animal is alive and responsive.

Video 5. Cell viability during extended imaging. During death, cells become marked by the cell death stain Sytox Green, which is continuously present in the imaging media. After 11 h of imaging, an individual midgut enterocyte changes from Sytox+ (arrowhead, 10.6 h), to faintly Sytox+ (arrowhead, 11.1 h), to strongly Sytox+ (arrowhead, 12.0 h). Nuclei are magenta (ubi-his2av::mRFP). Each movie time point is the projection of a confocal z-stack. Scale bar, 20 μm.

Video 6. Movie clip of midgut before (left) and after (right) stack registration. Before registration, blurred cells from tissue movements are evident during t=20-60 min. After registration, blurring is negligible. Cyan, all nuclei (ubi-his2ab::mRFP); yellow, stem cells and enteroblasts (eg>LifeactGFP). Each time point is the projection of a confocal z-stack. Scale bar, 20 μm.

Video 7. Ten-hour movie of a ‘fate sensor’ midgut (egGal4, UAS-his2b::CFP, GBE-Su(H)-GFP:nls ubi-his2av::mRFP. See Fig. 2A-B). Nuclei are distinguishable for four midgut cell types: stem cells (red pseudocolor), enteroblasts (yellow-green pseudocolor), enterocytes (gray, polyploid), and enteroendocrine cells (gray, diploid). Each time point is the projection of a confocal z-stack. Scale bar, 20 μm.

Video 8. Twelve-hour movie of a single-enterocyte extrusion. The epithelium is oriented with its basal surface toward the microscope objective and its apical surface further away. The basal region of the extruding enterocyte (orange pseudocolor at t=0, 127.5, 285, 442.5 min) is outlined by a ‘ring’ of E-cadherin::YFP. The ring closes down to a point from t= 255-442.5 min. The intensity of the
ring fluctuates during the first half of closure and becomes consistently bright during the second half. As the ring closes, neighboring cells draw into a rosette. Meanwhile, the nucleus of the extruding cell (yellow pseudocolor) starts to drop apically at t=150 min, hits its deepest luminal position at t=262.5 min, and recoils from t=262.5-307.5 min. Cyan, all nuclei (ubi-his2av::mRFP); inverted gray, E-cadherin (ubi-DE-cadherin::YFP). Each time point is the projection of a confocal stack. Scale bar, 10 μm.

Video 9. Orthoview of same extrusion as Video 8. The nucleus of the extruding enterocyte (magenta) ejects out of the epithelium (t=150-165 min) and penetrates into the lumen (t=165-265 min). It subsequently recoils and eventually comes to rest on the apical epithelium (t=263-443 min). Multi-colored line shows the path of nuclear travel over time (violet-yellow color scale; see Fig. 3D for legend). Cyan, all nuclei (ubi-his2av::mRFP), gray, E-cadherin (ubi-DE-cadherin::YFP). Scale bar, 10 μm.

Video 10. Four-hour movie of an enteroendocrine cell extrusion. The epithelium is oriented with its basal surface toward the microscope objective and its apical surface further away. The basal region of the extruding cell (tan pseudocolor at t=0, 75 min) is outlined by a ring of E-cadherin::YFP (inverted gray signal at cell boundaries). The extruding cell is presumed to be enteroendocrine since it has a small, presumably diploid, nucleus and since it lacks expression of eag (eag>his2b::CFP is inverted gray signal in nuclei.) The E-cadherin ring closes to a point from t=0-180 min. Meanwhile, the enteroendocrine cell nucleus drops apically from t=0-143 min. Cyan, all nuclei (ubi-his2av::mRFP); inverted gray, E-cadherin (ubi-DE-cadherin::YFP) and stem/enteroblast nuclei (eag>his2b::CFP). Each time point is the projection of a confocal stack. Scale bar, 10 μm.

Video 11. Mitosis of a putative stem cell. Green, actin (eag>LifActGFP); yellow, E-cadherin (ubi-DE-cadherin::YFP); red, nuclei (ubi-his2av::mRFP). Each time point is the partial projection of a confocal stack. Scale bar, 10 μm.

Video 12. Orthoview of a mitosis with two horizontal-vertical re-orientations. The first re-orientation occurs between metaphase (24° at 7.5 min) and anaphase (60° at 15 min). The second re-orientation occurs between anaphase (62° at 22.5 min) and telophase (2° at 30 min). Gray channel, condensed chromatin (ubi-his2ab::mRFP). Red line indicates the spindle axis. Cyan line indicates the basal plane, as revealed by the basement membrane stain Concanavalin A-Alexa 647 (not shown). At each time point, the mitotic cell is shown as an orthogonal projection from the vantage of a plane that is parallel to the spindle axis and normal to the basal epithelial plane. For clarity, a clipping plane was applied in the gray channel to exclude an adjacent enterocyte nucleus. Scale bar, 5 μm.

Video 13. Orthoview of a second mitosis with two horizontal-vertical re-orientations. Top panel shows condensed chromatin of the dividing cell (ubi-his2ab::mRFP). Red line indicates the spindle axis. Cyan line indicates the basal plane, as revealed by the basement membrane stain Concanavalin A-Alexa 647 (not shown). Bottom panel reproduces the graph from Fig. 4E, with the time-resolved orientations of this particular cell in red. The first re-orientation occurs during metaphase (from 16°
at 5 min to 0° at 15 min). The second re-orientation occurs between metaphase (1° at 20 min) and anaphase (18° at 25 min). Scale bar, 5 μm.

Video 14. Division of a stem cell that contacts two enteroblasts. Division orientation aligns with the axis between the two enteroblast nuclei (magenta, GBE-Su(H)-GFP:nls). At cytokinesis (t=15-22.5 min), the new daughter nuclei hurl into the enteroblast nuclei, which recoil in response. Gray, stem cell and enteroblast nuclei (eg>his2b::CFP). Each time point is the partial projection of a confocal stack. Scale bar, 10 μm.

Video 15. Real-time enteroblast transition. In the incipient enteroblast (blue dotted circle), GBE-Su(H)-GFP:nls is initially undetectable (GFP:RFP=0.014 at t=0.0 h). Over time, its GFP intensity increases, eventually reaching the enteroblast threshold (GFP:RFP=0.18 at t=10.5 h). See Fig. 5D. Left video: Green, GBE-Su(H)-GFP:nls.; magenta, stem cell and enteroblast nuclei (eg>his2b::CFP); gray, all nuclei (ubi-his2av::mRFP). Right video: inverted gray, GBE-Su(H)-GFP:nls. Each time point is the partial projection of a confocal stack. Scale bar, 2 μm.

Video 16. A low-contact sibling pair (Pair P; Fig. 6A, B) does not activate GBE-Su(H)-GFP:nls. Following their birth at t=0.0 h, the two siblings move apart and have likely lost contact by t=1.4 h (inter-nuclear distance>15.5 μm; c.f. Fig. 6-fig. supplement 1). The mother stem cell is indicated by the blue dotted circle at t=1.0 h; the two siblings are indicated by the two blue dotted circles at t=0.0 and t=9.2 h. No GFP expression is apparent in either sibling. Left video: Green, GBE-Su(H)-GFP:nls; magenta, stem cell and enteroblast nuclei (eg>his2b::CFP); gray, all nuclei (ubi-his2av::mRFP). Right video: inverted gray, GBE-Su(H)-GFP:nls. Each time point is the partial projection of a confocal stack. Scale bar, 5 μm.

Video 17. A high-contact sibling pair (Pair A, Fig. 6A, C) does not activate GBE-Su(H)-GFP:nls. Following their birth at t=0.0 h, the two siblings remain in likely contact (inter-nuclear distance<6.0 μm; c.f. Fig. 6-fig. supplement 1) for at least 6.0 h. The mother stem cell is indicated by the blue dotted circle at t=1.2 h; the two siblings are indicated by the two blue dotted circles at t=0.0 and t=6.0 h. No GFP expression is apparent in either sibling. Left video: Green, GBE-Su(H)-GFP:nls; magenta, stem cell and enteroblast nuclei (eg>his2b::CFP); gray, all nuclei (ubi-his2av::mRFP). Right video: inverted gray, GBE-Su(H)-GFP:nls. Each time point is the partial projection of a confocal stack. Scale bar, 5 μm.

Video 18. A sibling pair exhibits asymmetric Notch activation (Pair L, Fig. 6A, D). Following their birth at t=0.0 h, the two siblings are in likely contact from t=2.6-3.6 h, are in indeterminate contact from t=3.6-9.0 h, and are likely separated after t=9.0 h. The mother stem cell is indicated by the blue dotted circle at t=1.0 h. The two siblings are indicated by the two blue dotted circles at t=0.0 and 12.2 h. A single blue dotted circle at t=10.2 h indicates when the Notch-activated sibling crosses the enteroblast threshold (GFP:RFP=0.17; c.f. Fig. 6D). This sibling exhibits nascent GFP signal at 4.0 h and increases in GFP intensity for the rest of the movie. The other sibling does not exhibit detectable GFP, but from t=1.5-3.6 h, it collides with a high-GFP enteroblast (orange dotted circle),
which causes GFP signal to ‘bleed over’ in the GFP:RFP analysis (Fig. 6D). Left video: Green, 
*GBE-Su(H)-GFP:nls*; magenta, stem cell and enteroblast nuclei (*eg>his2b::CFP*); gray, all nuclei 
(*ubi-his2av::mRFP*). Right video: inverted gray, *GBE-Su(H)-GFP:nls*. Each time point is the partial 
projection of a confocal stack. Scale bar, 5 μm.
ADDITIONAL SUPPLEMENTAL MATERIAL

Source Code File 1. Registration macros utilizing the ImageJ plugin StackReg to perform three channel stack registration over time. In this macros, the XY negative space around the image is increased by a user defined amount to account for the shifting of stack slices during the registration process. The movie is then collapsed into an RGB format and StackReg is performed on each time point using a loop function. Once completed, corrected time points are concatenated, converted back to three color hyperstacks, and then the ImageJ plugin Correct 3D Drift is applied to correct for global volume movement of the tissue over time. Macros is in *.ijm format which can be opened and viewed in ImageJ.

Source Files
Figure 1_source data 1
Figure 3_source data 1
Figure 3_figure supplement 1_source data 1
Figure 4_source data 1
Figure 5_source data 1
Figure 6_source data 1
Figure 6_figure supplement 1_source data 1
### Key resources table

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<th>Designation</th>
<th>Source or reference</th>
<th>Identifiers</th>
<th>Additional information</th>
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</table>
**Drosophila** husbandry

Fly stocks obtained from other sources:

*esgGAL4* (Kyoto DGRC)
*ubi-his2av::mRFP* (BL23650)
*breathlessGal4, UAScryt-GFP* (Mark Metzstein)
*UAS-LifeactGFP* (BL35544)
*UAS-his2b::CFP* (Yoshihiro Inoue) (Miyauchi et al., 2013)
*GBE-Su(H)-GFP:ns* (Joaquin de Navascués) (de Navascués et al., 2012)
*act5c-spaghetti squash::GFP* (Denise Montell)
*ubi-E-cadherin::YFP* (Denise Montell) (Cai et al., 2014)

Generated stocks:

*act5c-spaghetti squash::GFP, ubi-his2av::mRFP*
*esgGal4, UAS-his2b::CFP, GBE-Su(H)-GFP:ns, ubi-his2av::mRFP*
*esgGAL4, UAS-his2b::CFP, GBE-Su(H)-GFP:ns/Cyo, ubi-his2av::mRFP*
*esgGal4, UAS-LifeactGFP, ubi-his2av::mRFP*
*esGAL4, GBE-Su(H)-GFP:ns, UAS-his2ab::mRFP*

Adult female *Drosophila* 2.5 days post-eclosion were used in all movies except Video 3, which used a female 7 days post-eclosion. Females were collected 0-4 h post-eclosion, placed in vials with males, and maintained at 25 °C. Flies were fed a diet of standard cornmeal molasses food supplemented with yeast paste (1 g/1.4 ml H2O).

Fly mounts for extended live imaging on upright, inverted, and light-sheet microscopes

We designed three types of fly mounts that enable dorsal exposure of the midgut while stabilizing the live intact animal. For upright and inverted microscopes, our design is a modification of a previously published mount for imaging of adult *Drosophila* brains (Seelig et al., 2010).

**Upright mount:** To prepare the upright mount (Fig. 1-fig, supplement 1A-B), a stainless steel shim of 0.001 in thickness (Trinity Brand Industries, 612H-1) was cut into 19 x 13 mm rectangles. From these rectangles, abdomen-sized cutouts were excised either by hand using an 18-gauge PrecisionGlide needle (Becton Dickinson, 305196), or by laser cutting using a micro laser cutting system (see Fig. 1-fig, supplement 2 for CAD file). In addition, we prepared 60-mm petri dishes (Fisher, FB0875713A) with a hole 10 mm in diameter drilled into the bottom. Each shim was glued onto the base of a 60-mm petri dish with clear silicone glue (DAP, 00688) and allowed to dry overnight.

The mount includes a feeder tube to provide the animal with liquid nutrients during imaging. We found that the feeder tube was essential for prolonged survival of the animal. Feeder tubes were made from 20-μl capillary tubes (Sigma-Aldrich, P0799), which were cut into 38-mm sections. Using a tungsten wire with a small hook bent at the end, a bit of cotton (Fisher Scientific, 22-456-880) was pulled through one end of the capillary tubing to form a feeder wick. Attachment of the feeder tube to the mount is described below.
A protective bottom chamber (Fig. 1-fig. supplement 1A’) enclosed the ventral side of the animal during imaging to prevent desiccation. To create the chamber, a 3-mm wide channel was drilled down the wall of a 35-mm petri dish (Olympus Plastics, 32-103). Kimwipes (4-ply rounds, Fisher Scientific, 06-666) were cut and placed in the bottom of the humidity chamber to be soaked with water before use.

**Inverted mount:** The inverted mount (Fig. 1-fig. supplement 1C) was similar to the upright mount, but used a glass bottom petri dish with two 1-mm spacers glued approximately 10 mm apart. Spacers were made from cut pieces of glass microscope slides (63720-05, Electron Microscopy Sciences) and were adhered with silicone glue. The same metal shim was used as with the upright mount, but was not affixed to the dish until the animal was glued and its gut stabilized (see below). Once the animal was prepared, the mounting shim was positioned with animal’s dorsal side toward the glass bottom of the dish and glued to the spacers using Kwik-Sil adhesive silicone glue (World Precision Instruments, 60002).

**Light-sheet mount:** Zeiss light sheet systems require a submersible chamber. To create such a chamber, we used the barrel of a 1-ml syringe in which one end was open to air (Fig. 1-fig. supplement 1D). A 5-mm x 8-mm square was cut into the side of the syringe barrel, and a metal shim with abdominal cutout was affixed to the square window using silicone glue. A second window was cut into the opposite side of the barrel to provide physical access for mounting the animal and feeder tube inside. Once the animal and feeder tube were in place, the access window was sealed using a second metal shim and Kwik-Sil glue (World Precision Instruments, 60002). The bottom end of the syringe was sealed with dental wax (Surgident, 50092189) and the barrel was submerged in media in the Zeiss sample chamber. In this manner, the midgut was bathed in media during imaging while the animal’s head and ventral surface remained in an open-air environment.

**Composition of imaging media and agarose**

Media for midgut imaging was based on prior recipes for *Drosophila* organ culture *ex vivo* (Morris and Spradling, 2011; Zartman et al., 2013). Schneider’s Insect Media (Sigma-Aldrich, S0146) was supplemented with 5% FBS (Sigma-Aldrich, F4135), 5% fly extract (DGRC) (Currie et al., 1988), 100 µg/mL human insulin (Sigma-Aldrich, 10516) and 0.5% penicillin-streptomycin (Invitrogen, 15140). (Without antibiotics, the imaging media became visibly contaminated after several hours of imaging.) Insulin was added fresh on the day of imaging.

Low-melting point agarose was used to stabilize the midgut during imaging. To make the agarose, 2-Hydroxyethylagarose (Sigma-Aldrich, A4018) was mixed with Schneider’s to make a 6% w/v slurry. The slurry was heated to 65 °C to melt the agarose, mixed thoroughly and separated into 25 µL aliquots that were stored at 4 °C. The day of imaging, aliquots were heated to 65 °C, mixed 1:1 with a 2x concentration of imaging media, and applied to midguts as described below.

Where indicated in the text, various fluorescent dyes were added to the imaging media to visualize particular cell structures or conditions: (1) Concanavalin A-Alexa647 (Invitrogen, C21421) was used to stain the basement membrane. A stock solution of 5 mg/mL Concanavalin A in 0.1 M sodium bicarbonate was diluted 1:200 in 1x imaging media to obtain a final working concentration of 25 µg/mL. A drop of this media was placed on the dorsal cuticle prior to cutting the cuticle win-
Agarose and subsequently added media did not contain Concanavalin A. (2) SiR-Tubulin (Cytoskeleton, CY-SC002) was used to stain microtubules. SiR-Tubulin was added to 1x imaging media for a final working concentration of 0.5 μM. A drop of this media was placed on the dorsal cuticle prior to cutting the cuticle window. Agarose and subsequently added media did not contain SiR-tubulin. (3) Sytox Green (ThermoFischer, S7020) was used to mark dying cells. A stock solution of 5mM Sytox Green in DMSO was diluted 1:5,000 in 1x imaging media to a final concentration of 1μM. Sytox Green-containing media was placed over the agarose immediately prior to imaging.

Animal preparation

A narrated tutorial video (Video 1) provides step-by-step instructions for mounting the animal and exposing the midgut. Wings were broken off near the hinge using forceps. Flies were placed in a microfuge tube on ice for at least 1 h before being glued dorsal side down to the fly mount (Fig. 1-fig. supplement 1) using KWI-SIL glue. To optimize access to region R4 of the gut, the fly was tilted toward its left side when glued to the mount. For long-term survival of the animal, its right-side spiracles were kept open (Video 1). After the glue had dried, the feeder tube, filled with 10% sucrose (w/v) in H₂O, was secured to the fly mount with dental wax (Surgident, 50092189) and positioned such that the cotton wick was in reach of the animal’s proboscis. The protective bottom chamber was attached to the bottom of the petri dish with masking tape (Fig. 1-fig. supplement 1A’).

To expose the midgut, a window was cut into the dorsal cuticle as follows: First, a drop of imaging media was placed onto the dorsal cuticle. Next, portions of cuticular segments A1 and A2 were excised using Dumont #55 forceps. In the majority of animals, this excision exposed the looped midgut region R4a-b/P1-2 (Buchon et al., 2013b; Marianes and Spradling, 2013). The loop was gently coaxled using forceps to protrude slightly out of the window. The imaging media was temporarily removed, and a drop of the agarose mixture (described above) was applied to the exposed loop and allowed to solidify. Once the agarose had hardened, a drop of media was added on top of the agarose to avoid desiccation. Between steps, the setup was placed on ice to minimize animal movement. The bottom of a 60-mm petri dish was used to cover the mounted animal until ready for placement on the microscope.

We explored the alternative of cutting a window in the ventral, rather than dorsal, cuticle, but we found a ventral window to be unsuitable for long-term viability. When animals were mounted ventrally, the feeder tube could not be positioned correctly and the spiracles could not be left unoccluded. In addition, the pliable nature of the ventral cuticle often resulted in unpredictable tearing during cutting.

Microscopy

An upright Leica SP5 multi-photon confocal microscope and a 20x water immersion objective (Leica HCX APO L 20x NA 1.0) were used to acquire the movies that were analyzed in this study. The microscope was controlled via a Leica CTR6500 controller card on a Z420 (Hewlett Packard) workstation with 16GB memory and a Xeon CPU E5-1620 (Intel) running Windows 7 Pro and Leica Application Suite Advanced Fluorescence (LAS AF, v.2.7.3.9723). In addition, an inverted Leica SP8 confocal microscope with a 20x oil immersion objective (Leica HC PL APO
IMM CORR CS2 NA 0.75) and a Zeiss light sheet Z.1 with a 20x water immersion objective (Zeiss
light sheet detection optics 20X NA 1.0) were used to test the fly mounts for these microscope set-
ups. For upright and inverted setups, a humidity box was assembled around the lens and the spec-
ten to prevent desiccation (Fig. 1-fig. supplement 1B). The humidity box was formed from a pi-
pette box lid with a hole for the lens and an inlet tube for humidified air. The box was connected to
a 500-ml Pecon humidification bottle containing distilled water, and humidified, ambient air was
piped into the box via a Pecon CTI-Controller 3700. In addition, for upright setups, the Kimwipes
in the protective bottom chamber were saturated with distilled water. For upright setups, 2-3 ml of
imaging media were added to the sample, spanning the distance between the exposed midgut and
the water immersion objective. Movies were captured at room temperature (20-25 °C). Confocal
stacks were acquired with a z-step of 2.98 μm and typically contained ~35 slices. For ex vivo imaging
(Fig. 1-fig. supplement 3A), the upright Leica SP5 multi-photon confocal microscope was used with
a 20x oil immersion objective (HC PL APO 20x/IMM N.A. 0.7).

Ex vivo imaging

To provide a positive control for Sytox Green dead cell staining (Fig. 1-fig. supplement 3A),
we generated dying midgut cells by dissecting midguts and culturing them ex vivo for 2.5 h in
Schneider’s media that contained 1μM Sytox Green (ThermoFisher, S7020). An 8-well Secure-Seal
spacer sticker (ThermoFisher S24737) was used to form ‘wells’ on a microscope slide (Fischer Elec
tronic Microscopy Sciences 63720-05). One midgut and 7 μl of Sytox-containing media were placed
in each well. A coverslip (Fisher Scientific 12-545-81) was placed on top of each well. Midgut were
imaged immediately and 2.5 h after mounting.

Movie registration and cell masking in Fiji

After acquisition, movies were processed on a Mac Pro computer (OS X 10.8.5) with a 3.2
GHz quad-core Intel Xeon processor and 20GB memory. LIF files (*.lif) from LAS AF were upload-
ed into Fiji as a hyperstack for registration. To correct for X-Y drift, movies were converted to RGB
files and processed with the Fiji plugin StackReg (Arganda-Carreras et al., 2006). To correct for
global volume movements, movies were processed with the Fiji plugin Correct 3D Drift (Parslow et
al., 2014).

After registration, movies were evaluated for midgut viability. Viability was deemed to be
compromised when any one of the following behaviors were observed: (1) wholesale dimming of
fluorescent signals; (2) enterocyte extrusions en masse (>12 cells at once); (3) loss of ordered entero-
cyte packing; (4) appearance of multiple pyknotic nuclei; (5) widespread flattening and spreading of
progenitor cells. Once any of these criteria were observed, the remainder of the movie was not used
for further analysis.

For identification of individual cells, intensity thresholding of the ubiquitously expressed nu-
clear marker ubi-his2av::RFP was used to segment out individual cell nuclei. In fate sensor movies
(c.f. Fig. 2), intensity thresholding for CFP (stem cells and enteroblasts; eg>his2b::CFP) and GFP
(enteroblasts; GBE-Su(H)-GFP::nls) was applied to define masks of nuclei within each channel. Using
Fiji’s image calculator function, these masks were used to isolate the His2av::RFP-marked nuclei for
individual cell types. Specifically, after registration (Fig. 1E), channel masks were generated in Image
1079  J to digitally isolate stem cells (CFP channel minus GFP channel), enteroblasts (GFP channel), and
1080  mature enterocytes and enteroendocrine cells (RFP channel minus CFP channel). To digitally isolate
1081  enterocytes and enteroendocrine cells, whose populations were defined by the absence of
1082  *eg>his2av::CFP*, the His2b::CFP-marked progenitor nuclei were eliminated using the subtraction
1083  function in Fiji’s image calculator.
1084  Masked nuclei for these three populations were added to the raw hyperstack as unique chan-
1085  nels for use in Bitplane Imaris (see below). The two types of mature cells, enterocytes and enteroen-
1086  docrine cells, were then distinguished by a size filter in Imaris; nuclei ≤113 μm$^3$ were classified as
1087  enteroendocrine, whereas nuclei >113 μm$^3$ were classified as enterocyte. To maintain metadata struc-
1088  ture for 4D Imaris analysis (see below), final movies were exported to OME-TIFF format (*.ome.tif)
1089  using the BioFormats plugin in Fiji.
1090
1091  **Cell identification and tracking in Imaris**
1092  To perform cell tracking, Fiji processed stacks of midgut movies were opened in Bitplane
1093  Imaris from OME-TIFF format (*.ome.tif) files. Once converted to an Imaris *.ims file, the 4D vol-
1094  umes were visually inspected using the Surpass module to verify the accuracy of image processing
1095  and file conversion. Cell segmentation was then performed by applying the Surface Recognition
1096  Wizard module to the masked cell channels generated in Fiji (see above). Final products were visually
1097  compared to raw channels to confirm cell-type recognition.
1098  Cell surfaces were tracked using the Brownian motion tracking algorithm. Automated Imaris
1099  tracking accurately identified ~70-90% of cells, depending on the frequency of organ movements in
1100  the movie. Visual inspection was used to correct errors. Once cell recognition was verified for all cells
1101  and time points, individual cell statistics were exported as either a Microsoft Excel file or a comma-
1102  separated-value file. The data were then imported into Mathematica or MATLAB for quantitative
1103  analysis.
1104  For Figures 5 and 6 and their associated data, a modification of the above protocol was used.
1105  To identify cells that transitioned over time from a stem-like state (GFP:RFP≤0.17) to an enteroblast
1106  state (GFP:RFP>0.17), cells expressing *eg>his2b::CFP* were identified in Imaris. Their GBE-Su(H)-
1107  GFP:nls intensities and nuclear volume were determined at each time point. Cells exhibiting increasing
1108  GFP intensities were identified and selected for further analysis.

1109  **Spatiotemporal analyses of enterocyte extrusion**
1110  **Analyses of E-cadherin::YFP ring**: Extruding enterocytes were identified by visual inspec-
1111  tion. To measure dynamics of the E-cadherin::YFP ring (Fig. 3B-E), Fiji-processed movies were
1112  opened from OME-TIFF files (*.ome.tif) in Bitplane Imaris and viewed as 3D volumes using the
1113  Surpass module. Vertices of the E-cadherin::YFP ring that outlined the extruding cell were identified
1114  by visual inspection at each movie time point. The Measurement Points tool was used to place a poly-
1115  gon-mode measurement point at each vertex. In addition, a plane representing the basal epithelium
1116  was defined by selecting three Measurement Points on the basal epithelial surface underlying the ex-
1117  truding cell. To identify the position of the basal surface, we used either the basement membrane
1118  stain Conconavalin-A-Alexa647 or the background fluorescence of enterocyte cytoplasts when
movies were digitally overexposed. The spatial coordinates of all these measurement points were exported as comma-separated values and imported into MATLAB.

To map the ‘footprint’ of the ring in the epithelial plane (Fig. 3B), the coordinates of the ring vertices were connected with a line, and the resulting polygon was projected onto the basal plane for each time point. The polygon was color-coded according to its time point in the movie.

To calculate the cross-sectional area of the ring (Fig. 3C), the centroid of the polygon was triangulated using its vertices. The area of each component triangle was calculated as half of the cross product of the two vectors formed by the centroid and the two adjacent vertices. The area of the ring was calculated as the sum of the areas of each component triangle. Ring areas were calculated for each movie time point.

To determine the apical-basal position of the E-cadherin::YFP ring, we calculated the orthogonal distance from the centroid of the ring to the basal plane. This distance was calculated as the dot product of two vectors: the unit normal vector of one of the basal measurement points, and a vector from the centroid to basal measurement point that was used as the origin of the unit normal vector.

**Analyses of the extruding nucleus:** We defined the duration of nuclear extrusion (Fig. 3F) as the length of time that the extruding cell’s nucleus was moving apically. To determine this duration, Fiji-processed movies were opened from OME-TIFF files (*.ome.tif) in Bitplane Imaris and viewed as 3D volumes using the Surpass module. Nuclei of extruded enterocytes were digitally isolated via clipping planes and viewed in cross section. Nuclei of enterocytes surrounding the extruding cell were used to establish the baseline level of the epithelium. The duration of nuclear extrusion was measured from the time point for which apical movement of the nucleus was first apparent to the time point of maximal apical displacement from the baseline. The centroid of the nucleus was calculated from surface-recognized objects in Imaris. The distance of the nucleus from the basal surface (Fig. 3D) was calculated as the orthogonal distance from the centroid of the nucleus to the basal plane, as defined by the basal epithelium reference points described above.

**Spatiotemporal analyses of stem cell mitoses**

**Mitotic duration:** Mitoses were identified by visual inspection of maximum intensity z-projections in Fiji and confirmed in Bitplane Imaris using the Surpass module for 3D visualization. To calculate the durations of individual mitoses (Fig. 3H), we designated the start point as the initiation of nuclear condensation in the mother cell and the end point as the decondensation of the two sets of daughter chromosomes.

**Mitotic index:** To calculate mitotic index, we used 9 movies of *ubi-his2av::mRFP*-expressing midguts that each contained at least one identifiable division. Movies were processed in Fiji, and nuclei were identified and tracked in Imaris as described above. Mitotic index was calculated as $T_M/T_{SC}$, where $T_M$ is the sum of the durations of 39 individual mitoses in the 11 movies, and $T_{SC}$ is the sum of the durations of ‘screen time’ for all the stem cells in the same movies. Stem cell ‘screen time’ was calculated as the product of the number of stem cells at $t=0$ in a particular movie and the duration of that movie. (On occasion, stem cells disappeared or appeared over the course of a movie; however, these events were infrequent and are not included in our calculations.) To determine the number of
stem cells in a movie at t=0, we used one of two approaches. For midguts that expressed
\textit{egh2av}:CFP and \textit{GBE-Su(H)-GFP::nls} in addition to \textit{ubi-his2av}:mRFP (2 of 9 movies), stem cells
were identified as CFP+ cells with GFP::RFP<0.17 (c.f. Fig. 5), and the number of stem cells was
counted following Imaris surface recognition as described above. For midguts in which stem cells
were not identifiable through specific markers (7 of 9 movies), the number of stem cells was estima-
ted as 20% of total, \textit{ubi-his2av}:mRFP-expressing cells (de Navascués et al., 2012; O’Brien et al.,
2011).

\textbf{Horizontal-vertical orientation:} To measure the real-time horizontal-vertical orientations of
dividing cells (Fig. 4A-E). Fiji-processed movies were opened as OME-TIFF files (*.ome.tif) in Birt-
plane Imaris. For each mitotic cell, the positions of the spindle poles and of the basal epithelial sur-
face were determined at each time point between the start and end of mitosis (Fig. 4-fig. supplement
1). Spindle pole positions were inferred from the morphology of the condensed chromosomes and
marked using the Measurement Points tool. A plane representing the basal epithelium was defined
using the Measurement Points tool to place three points on the basal epithelial surface underlying
the spindle. To identify the position of the basal surface, we used either the basement membrane
stain Concanavalin-A-Alexa647 or the background fluorescent signal of enterocyte cytoplasmics made
visible when movies were digitally overexposed.

The coordinates of spindle poles and basal planes in 3D space were exported as Excel files
and opened in Mathematica. To calculate the spindle angle, we defined two vectors: the ‘spindle
pole vector’, which was the difference between the coordinates of the two spindle poles, and the ‘ba-
sal plane vector’, which was the cross product of two vectors determined from the three points defin-
ing the basal plane. The spindle angle was calculated as the dot product of the spindle pole vector
and the basal plane vector.

\textbf{Longitudinal-circumferential orientation:} To measure longitudinal-circumferential orienta-
tion of dividing cells (Fig. 4F-H), movies were analyzed as maximum-intensity projections in Fiji.
Longitudinal and circumferential axes were determined for each mitotic cell by visual inspection,
based on the local morphology of the midgut tube and the orientation of the ellipsoid nuclei of sur-
rounding enterocytes. Division orientation was measured at the time point when we observed de-
condensation of the daughter chromosomes, an event signifying the end of mitosis. To calculate lon-
gitudinal-horizontal orientation, we used the Fiji Angle Tool, which measures an angle defined by
two vectors formed from three points. One vector was defined by the difference between the posi-
tions of the two daughter cells, and the other vector was defined by the longitudinal axis of the mid-
gut tube.

\textbf{Orientation relative to neighboring enteroblasts:} We identified mitoses in which the divid-
ing cell contacted either one enteroblast or two enteroblasts using visual inspection. To determine
the spatial coordinates of the mitotic cells and the enteroblasts, Fiji-processed movies were opened as
OME-TIFF files (*.ome.tif) in Bitplane Imaris. Nuclei were recognized using the Surface Recogni-
tion Wizard. The 3D coordinates of the relevant cells were exported into MATLAB.

Division orientation relative to neighboring enteroblasts (Fig. 4I-K) were calculated as fol-
low: For mitotic cells contacting one enteroblast, we designated the daughter cell closer to the en-
teroblast as ‘D1’ and the other daughter cell as ‘D2’. We defined an ‘enteroblast-D1 vector’ as the
difference between the coordinates of the enteroblast and D1. We defined a ‘D1-D2 vector’ as the
difference between the coordinates of D1 and D2. To calculate the division angle, we computed the
dot product of the enteroblast-D1 vector and the D1-D2 vector.

For mitotic cells contacting two enteroblasts, we designated the reference enteroblast as the
enteroblast whose nucleus was closer to the mother stem cell nucleus prior to division. D1 and D2
daughters were determined relative to that enteroblast following the procedure detailed above.

Mitotic cells that contacted enteroblasts were excluded from analyses of horizontal-vertical
and planar orientations.

**Quantitative assessment of Notch reporter activation**

Activation of the Notch reporter **GBE-Su(H)-GFP:nls** was measured in movies of fate sensor
midguts (esgGal4, UAS-his2b::CFP, GBE-Su(H)-GFP:nls; ubi-his2av::mRFP) (Figs. 5 and 6). As de-
scribed above, Fiji-processed movies were opened as OME-TIFF files (*.ome.tif) in Bitplane Imaris,
and surface recognition was performed to identify individual cell nuclei using the Add New Surfaces
function in the Surpass Module. To quantify **GBE-Su(H)-GFP:nls** activation, we calculated the
normalized ratio of GFP:nls and His2av::mRFP intensities as follows: (1) To generate normalized
intensity values, raw intensity values for GFP and RFP of single cells at each individual time point
were determined from cell nuclei. These raw intensities were exported to MATLAB and divided by
the maximum intensity in that movie to yield normalized intensities. (2) The ratio of normalized
GFP:RFP intensities was calculated for each cell at each time point. The resulting real-time, normal-
ized GFP:RFPs enabled quantitative comparison of **GBE-Su(H)-GFP:nls** expression between differ-
et cells, at different times, or across different movies.

**Calculating inter-nuclear distances of progenitor pairs**

To perform the initial analysis of inter-nuclear distances for contacting and non-contacting
progenitor pairs (Fig. 6—fig. supplement 1), we used movies of midguts with genotype esgGal4, UAS-
LifeactGFP; ubi-His2av::RFP. For this analysis, two *esg* cells were designated as a pair if they were
mutually closer to each other than to any other *esg* cell. Pairs were selected randomly from single
time points in four separate movies. Movies were examined in 4D using the Surpass Module in
Imaris, and *esg* pairs were identified as either contacting or non-contacting based on their Life-
actGFP signal. To determine the inter-nuclear distance of a pair, the \((x,y,z)\) coordinates for centroid
of each nucleus was determined based on surface recognition in Imaris. The distance \(D\) between the
two centroids was calculated using the equation \(D = \sqrt{((x_2-x_1)^2 + (y_2-y_1)^2 + (z_2-z_1)^2)}\).

To determine the inter-nuclear distances for sibling pairs with known birth times (Fig. 6), we
used movies of ‘fate sensor’ midguts (esgGal4, UAS-his2b::CFP, GBE-Su(H)-GFP:nls; ubi-
his2av::mRFP). Following a stem cell division, the inter-nuclear distance of the two siblings at each
movie time point was calculated as described above.
BIBLIOGRAPHY


