Raeppli: a whole-tissue labeling tool for live imaging of *Drosophila* development

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In Fig. 1, the stop cassette should have been removed in Fig. 1B, ii and iii. The correct figure appears below.

The authors apologise to readers for this mistake.
RESEARCH ARTICLE

Raeppli: a whole-tissue labeling tool for live imaging of Drosophila development

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ABSTRACT

Observation of how cells divide, grow, migrate and form different parts of a developing organism is crucial for understanding developmental programs. Here, we describe a multicolor imaging tool named Raeppli (after the colorful confetti used at the carnival in Basel). Raeppli allows whole-tissue labeling such that the descendants of the majority of cells in a single organ are labeled and can be followed simultaneously relative to one another. We tested the use of Raeppli in the Drosophila melanogaster wing imaginal disc. Induction of Raeppli during larval stages irreversibly labels >90% of the cells with one of four spectrally separable, bright fluorescent proteins with low bias of selection. To understand the global growth characteristics of imaginal discs better, we induced Raeppli at various stages of development, imaged multiple fixed discs at the end of their larval development and estimated the size of their pouch primordium at those developmental stages. We also imaged the same wing disc through the larval cuticle at different stages of its development; the clones marked by Raeppli provide landmarks that can be correlated between multiple time points. Finally, we used Raeppli for continuous live imaging of prepupal eversion of the wing disc.

KEY WORDS: Drosophila, Live imaging, Lineage tracing, Multicolor labeling, Organogenesis, Whole-tissue labeling

INTRODUCTION

The study of the development of a model organism over the entire development period or even of a single organ as it forms is hampered by the long time span of these processes. Therefore, it is technically difficult to image the behavior of individual cells of a growing organ over the entire length of its development. One approach that successfully overcomes this limitation is lineage tracing, in which the cells of a primordium are labeled at the early stages of organogenesis and analyzed in the final structure (Kretzschmar and Watt, 2012). However, in current technologies, only a small proportion of cells of the organ are labeled with a limited repertoire of reporters. Ideally, labeling a majority of the cells with different colors that could be detected during development of the organ would maximize the information acquired per single sample and provide an overview of tissue dynamics. We call this approach ‘whole-tissue labeling’, in contrast to cell labeling.

The wing imaginal disc is one of the most studied models of organogenesis. The primordium of the Drosophila melanogaster wing is a group of ~25-50 cells set aside during embryogenesis. During the following 5 days of larval development, the primordium grows to form a sac-like structure of ~31,000 cells, called the wing imaginal disc (Madhavan and Schneiderman, 1977; Martin et al., 2009; Worley et al., 2013). During metamorphosis, most of the larval cells undergo histolysis and the wing imaginal discs evert to form the adult wing and notum structures (Cohen et al., 1993; Merriam, 1978; Milán et al., 1996). The whole process takes approximately 10 days. Despite Drosophila being one of the faster developing model organisms, it is not possible to monitor the entire development of a wing with current methods.

Conventional cell-labeling methods involving the generation of mitotic clones, mosaic analysis with a repressible cell marker (MARC) and flip out clones have been extensively applied to wing imaginal disc development and have provided a general overview of disc development (González-Gaitán et al., 1994; Lee et al., 2000; Milán et al., 1996; Weigmann and Cohen, 1999; Johnston and Sanders, 2003). Many clones have been generated in multiple separate discs and the average of multiple discs was used to build an overall picture of imaginal disc growth. This overview is very useful, yet these conventional cell-labeling methods provide limited information about the development of an individual disc. Conventional techniques only label a limited proportion of cells in an organ with one or two different markers, which does not provide important information about what is occurring in neighboring unlabeled cells.

A landmark in cell labeling has been achieved with the Brainbow method, which uses the CRE site-specific recombinase to select one of three possible fluorescent proteins in a transgene to mark cells (Livet et al., 2007). The progeny of those cells inherit the expression of the chosen fluorescent protein. Using multiple copies of this construct, Livet et al. accomplished combinatorial labeling resulting in cells labeled with a spectrum and diverse hues of colors. This method has been adapted in Drosophila melanogaster with Flybow and dBrainbow (Hadjieconomou et al., 2011; Hampel et al., 2011). Although Flybow and dBrainbow are very powerful cell-labeling tools, their low efficiency of recombination (1%) excludes them in the presently available forms as whole tissue-labeling tools. Moreover, these techniques require fixation for the visualization of all the markers in their repertoire. Therefore, color detection is lethal and an impediment to continuous live imaging. More recently, the Lollibow system has been developed, which allows in vivo imaging without fixation; however, the recombination frequency is still low at 15%, which does not allow whole-tissue labeling (Boulina et al., 2013). Another recent method, TIE-DYE uses multiple flip-out constructs to mark the cells of a primordium efficiently (Worley et al., 2013). This technique too relies on fixing and staining the samples for detection, which limits in vivo imaging.
We have developed a new lineage-tracing tool called Raeppli (after the colorful confetti used at the Basel carnival). Raeppli marks 90% of the cells in a primordium with combinations of four spectrally separable, bright fluorescent proteins. The high efficiency of cell labeling with fluorescent proteins, together with combinatorial marking of the progeny of primordial cells, meets the criteria for whole-tissue labeling. We used whole-tissue labeling to measure the primordium sizes at different developmental times and to estimate cell division rates. Moreover, whole-tissue labeling, in combination with a newly developed cooling device to anesthetize larvae, allowed us to image the same wing disc as it developed during the larval stages. We followed the same clones through several developmental time points, and measured the growth of each one of those clones within the same wing disc. Finally, we used Raeppli for high temporal resolution live imaging of the wing disc eversion in prepupal stages.

RESULTS
Design of Raeppli
There are three main prerequisites for whole-tissue labeling: Multiple color combinations that can be distinguished, high efficiency of recombination and low bias of fluorescent protein choice. These three requirements ensure the labeling of the cells of a primordium with diverse color combinations and simultaneous detection of their progeny. Raeppli was designed and constructed with those parameters in mind.

Raeppli is a transgenic construct that contains the sequences necessary for marking cells with one of four possible fluorescent proteins that can be combinatorially used to provide multiple color combinations: TagBFP (Subach et al., 2008), mTFP1 (Ai et al., 2006), mOrange (Shaner et al., 2004) [or E2-Orange (Strack et al., 2009) for nuclear constructs] and mKate2 (Shcherbo et al., 2009) (Fig. 1A). These fluorescent proteins were chosen for their

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Fig. 1. Schematics of Raeppli constructs and Raeppli activation. (A) The features of the Raeppli construct from left to right. The constructs contain 5X UAS and LexO sites that can act on a basal hsp70 promoter (dark green ellipse) for expression of chosen fluorescent protein. UAS and LexO sites are flanked by Lox2272 or LoxP sites, respectively. Cre protein can excise one of the enhancers in a mutually exclusive manner. There is a single attB site (gray arrow) downstream of enhancers and promoter, which is available for recombination with one of the attP sites (blue arrows). A full hsp70 promoter (light green ellipse) regulates the expression of the integrase gene. The expression of integrase is blocked by the presence of a stop cassette consisting of yellow gene with stop codon (black box) flanked by FRT sites (red triangles). Bacterial vector backbone sequences are placed upstream of attP sites and fluorescent proteins to increase the distance between attP sites and fluorescent proteins to increase the distance between attP sites and the attB site. Fluorescent protein genes, each with a stop codon are arranged in a linear fashion each preceded by an attP site. The illustrated order applies to the NLS construct. The CAAX construct is ordered TagBFP, mKate2, mOrange, mTFP1. (B) (i) After the expression of the flipase gene, Flp removes the stop cassette and makes expression of integrase through full heat shock promoter possible. (ii) Heat shock causes the expression of the integrase gene by activating the full heat shock promoter. (iii) Integrase protein recombines the attB site with one of the attP sites of the construct and removes the intervening region. (iv) The chosen fluorescent protein can be expressed by the action of LexA or Gal4 proteins. Each fluorescent protein gene is followed by a stop codon. Therefore, the only expressed fluorescent protein is the one brought closest to the promoter.
brightness and their spectral properties that allow the emission from each of the fluorescent proteins to be detected separately through sequential scanning (supplementary material Fig. S1). The fluorescent proteins used in the Raeppli constructs can be detected separately from the GFP and far red channels; therefore, Raeppli is compatible with many other established imaging tools. We generated two types of Raeppli constructs that can be used to mark different cellular structures. In the first construct, the fluorescent proteins are targeted to the nucleus by a nuclear localization sequence (NLS), which is useful to analyze growth characteristics and position of the clones (Raeppli-NLS). In the second construct, the fluorescent proteins are targeted to membranes by a Ras farnesylation sequence (CAAX box), which is useful to analyze cell and clone shape changes (Raeppli-CAAX).

High efficiency of recombination was obtained by using the phiC31 integrase system for color choice. PhiC31 integrase efficiently recombines sequences containing attP sites with sequences containing attL sites. Although this integrase system is traditionally used to insert sequences that contain an attB site into an attP site localized in the genome, efficient intramolecular excision has been reported in constructs containing attB and attP sites in cis (Bischof et al., 2007; Lu et al., 2011; Sangiorgi et al., 2008; Thorpe and Smith, 1998). The recombination between attB and attP sites results in attL and attR sites, which cannot be used as a template for the reverse reaction in the absence of specific cofactors (Thorpe and Smith, 1998; Thorpe et al., 2000). Hence, the color choice of Raeppli is irreversible and stable.

In the Raeppli constructs, the coding sequence for each fluorescent protein is downstream of an identical attP site that may be recombined to the single attB site of the construct. The recombination event selects a single fluorescent protein for expression. As the distance between two site-specific recombination sites influences the recombination frequency, the attP site of the first fluorescent protein, which is the closest to the attB site, may be more often selected. In order to minimize such a bias, we designed the construct to place the attP sites and fluorescent proteins as far away as possible from the single attB site. Our reasoning was that the greater the distance between the attB site and the attP sites, the less likely the distance between consecutive attP sites would cause a bias in the selection.

The expression of the selected fluorescent protein can be driven by either of the two binary systems: Gal4/UAS and LexA/LexO (Brand and Perrimon, 1993; Yagi et al., 2010). To increase the versatility of the system, the LexO and UAS sites in the construct are flanked by LoxP and Lox2272 Cre site-specific recombinase recognition sites, respectively (Brand and Dymecki, 2004). Without Cre-mediated excision, the construct can be used either with UAS/Gal4 or LexO/LexA systems. Expression of Cre causes recombination either between LoxP sites, excising the LexO site, or between Lox2272 sites, excising the UAS site, in a mutually exclusive manner (Fig. 1A; supplementary material Fig. S3). Thus, it is possible to restrict the sensitivity of Raeppli to only one of the binary systems by crossing the flies to a Cre source. This allows Raeppli transgenics to be selectively expressed by either the LexA or Gal4 source. Thus, a ubiquitously expressed Gal4 or LexA source can be used to detect Raeppli whereas the other driver can be used to manipulate the sample spatially and temporally independently of detection of Raeppli (e.g. by overexpressing RNAi, dominant-negative or gain-of-function transgenics).

**The activation of Raeppli**

Two ordered events must occur for the selection and expression of fluorescent proteins in Raeppli constructs: expression of integrase for site-specific recombination (color choice; Fig. 1Bi-iii) and expression of fluorescent protein by one of the two binary systems (detection; Fig. 1Biv). The Integrase source is under the control of the full heat-shock-protein70 (hsp70) promoter; however, to suppress low level of expression of integrase, a stop cassette (yellow coding sequence with a stop codon) that is flanked by FRT sites was placed between the full hsp70 promoter and the integrase coding sequences. Therefore, the first step in the activation of integrase expression is the excision of the stop cassette. The stop cassette can be excised by the Flippase (Fp) site-specific recombinase, which can be expressed from either hs-Flp or UAS-Flp transgenes (Fig. 1Bi,ii). After the stop cassette is excised, integrase expression is induced by the action of either the full hsp70 promoter or the distant UAS enhancer on the promoter. Integrase promotes site-specific recombination between the single attB site downstream of the enhancer sites and minimal hsp70 promoter and one of the four attP sites upstream of each fluorescent protein-encoding gene. The recombination event excises the intervening region and brings one of the fluorescent protein open reading frames under the control of the enhancer sites. Importantly, the Integrase source and white eye color marker are excised during this recombination. Excision of the integrase gene limits the exposure of the cells to Integrase, lowering potential toxicity towards the genome and making the color choice irreversible (in addition to the irreversibility of the attB attP recombination event). Excision of white serves as a useful internal control for the stability of Raeppli transgenic lines (Fig. 1Biii).

The end product of these orderly events is a transgene that expresses a single fluorescent protein under the control of either Gal4 or LexA (Fig. 1Biv). Heat shocking larvae that contain a single copy of Raeppli-NLS and hs-FLP and Tub-Gal4 transgenes results in a choice of one of the four fluorescent proteins in the primordial cells and ubiquitous expression of the chosen fluorescent protein. These cells divide to form clones that are stably marked by the color of the chosen fluorescent protein (Fig. 2A). In a similar set-up, the presence of two copies of Raeppli-NLS can result in ten different colors and increases the probability of labeling neighboring cells differently from each other (Fig. 2C).

**Raeppli labels tissues efficiently and with low color choice bias**

We quantified color selection bias and the labeling efficiency of Raeppli constructs in two different systems. In order to test the bias of fluorescent protein selection, we induced one copy of Raeppli-NLS during embryogenesis with Tub-Gal4; UAS-Flp and analyzed the resulting clones in the larval epidermis, which is composed of large, non-dividing cells (Fig. 2B,B’). The non-dividing nature of the system ensures that each fluorescently labeled cell results from an independent color choice event. We quantified the color choice of over 150 cells per larva in five larvae for a total of 1062 cells. The frequency of choice was similar for each fluorescent protein with a mild bias towards the E2-Orange, which is the first fluorescent protein in the construct, chosen in 32% of the cases.

To analyze further the color bias of Raeppli, we crossed hsFlp1.22; Tub-Gal4 flies with flies that have two copies of Raeppli-NLS or Raeppli-CAAX transgenes. The larvae were heat shocked once, at different time points during development. The heat shock provided a transient source of Flippase that excised the stop cassette and subsequently induced expression of the integrase gene. Between the time of induction and the time of detection, these cells divided and generated clones that formed different regions of the organ. Most of the cells in wing disc epithelium have five or six neighbors (Classen et al., 2005); thus, to mark primordial cells differently from their...
neighbors in wing disc, we used two copies of Raeppli, which generates ten colors (Fig. 2C). We quantified the frequency of choice of each of the ten color combinations that the two copies of Raeppli provide. Each of the color combinations had a prevalence around the expected 10% with the lowest chosen color combination being 8.2% and the highest being 11.8% in a total of 254 quantified clones (Fig. 2C′). To test whether this position bias was due to the design of Raeppli, we generated a version of Raeppli that contains eight fluorescent proteins, four nuclear fluorescent proteins followed by four membrane-tagged versions, assuming that a putative position bias would be accentuated in this configuration (supplementary material Fig. S2A). This transgene was activated by Tub-Gal4; UAS-FLP and the resulting larval epidermis was imaged (supplementary material Fig. S2B). We quantified the color choice of >500 cells per larva for a minimum of three larvae. The frequency of choice was similar for each fluorescent protein, with a mild bias towards the first and last fluorescent protein coding sequences (supplementary material Fig. S2C). We then tested whether there was position effect variation on color choice. We conducted the same analysis for three independent insertions of eight fluorescent protein constructs. We found no overt effect of insertion position on color choice (supplementary material Fig. S2C).

For testing the efficiency of labeling, we measured the ratio of the labeled area to the full area of the wing disc in samples containing hsFlp1.22; Tub-Gal4 and two copies of Raeppli-NLS or Raeppli-CAAX transgenes, that were heat shocked at different time points, in a total of 21 discs. At all time points, >90% of the primordium was labeled with clones of diverse color combinations (Fig. 3I). A similar >90% efficiency value was found for a single copy of Raeppli (see Fig. 2A as an example).

These data show that the design of Raeppli ensures high efficiency and relatively unbiased selection of fluorescent proteins.

Temporal activation of Raeppli

We used the whole-tissue imaging approach to analyze organogenesis. We first analyzed the growth characteristics of the wing disc pouch in fixed samples: the cell lineages were labeled by Raeppli starting from different time points during larval development. We crossed hsFlp1.22; Tub-Gal4 flies with flies that have two copies of Raeppli-NLS or Raeppli-CAAX transgenes (Fig. 3A-D and 3E-H, respectively). The resulting larvae were heat shocked once, at different ages. Primordial cells labeled at the time of heat shock will give rise to clones that will retain the initial color combination. Information about the cell division pattern and rate in the wing disc was extracted from these clones. After the heat shock, >90% of the cells in the primordium expressed different fluorescent protein combinations and the clone sizes were generally uniform within the same sample, indicating a uniform pattern of proliferation, as reported previously (Milán et al., 1996). As expected, the average size of clones correlated with how long the disc had grown after Raeppli induction; discs containing Raeppli transgenes induced at earlier time points in development gave rise to smaller clones than those induced at later time points.
to a small number of large clones (Fig. 3A,B,E,F); discs containing Raeppli transgenes induced at later time points resulted in a large number of small clones (Fig. 3C,D,G,H). To analyze growth rates in wing discs, we counted the number of clones in the pouch area of discs that were heat shocked once at 24-48, 48-72 or 72-96 hours, respectively, after egg laying. Heat shock at 96-120 hours resulted in too many clones to quantify them reliably individually. The number of clones per pouch indicates that the progeny of on average 12 cells in 24-48 hours, 26 cells at 48-72 hours and 211 cells at 72-96 hours form the pouch at wandering third instar stage. This result corresponds to about one cell division per 24 hours between the time windows 24-48 hours and 48-72 hours and one cell division per 8 hours between the time windows 48-72 hours and 72-96 hours, which suggests a boost in cell proliferation rate that happens in the beginning of third instar in the pouch area, but these data may be biased due to lack of cell division in first instar stage which lasts until 40 hours after egg laying (Madhavan and Schneiderman, 1977). The division rate we observe between 72 and 96 hours is consistent with previous reports (González-Gaitán et al., 1994; Martín et al., 2009; Milán et al., 1996; Wartlick et al., 2011).

We then analyzed the cell division pattern in the eye antennal disc, which has a differential cell division rate imposed by the posterior to anterior advance of the morphogenetic furrow; the cells posterior of the furrow stop dividing and differentiate to form the ommatidia, whereas the cells anterior to it continue to divide. The larval eye antennal discs of the heat-shocked larvae containing two copies of Raeppli-NLS showed two different clone patterns: very small clones corresponding to the cells posterior to the morphogenetic furrow at the time of heat shock, and big clones corresponding to the cells anterior to the morphogenetic furrow at the time of induction (Fig. 3K, compare green inset with red). Thus, in an epithelium with unequal cell division rates, Raeppli can be used to highlight the difference in cell division rates and infer information about the time when the change of division rates occur.

In our experiments we have noticed that the basal expression of hs-Flp provides enough Flippase to sporadically induce a color choice without heat shock in ~10% of the cells. The premature induction of Raeppli by hs-Flp before heat shock could cause a limitation in experiments whereby a tighter temporal control of clone induction is necessary (e.g. analysis of difference in clone growth within the same tissue). For such uses, we tested whether UAS-Flp could provide a tighter temporal control of Raeppli induction. When we compared the induction of color choice with UAS-Flp with early-expressing Gal4 drivers (En-Gal4, Hh-Gal4) to the induction with later-expressing Gal4 drivers (Nubbin-Gal4, Ap-Gal4), we observed big clones only with the early-expressing Gal4 drivers (supplementary material Fig. S4A). Consistent with this, we obtained a much tighter temporal control with a recently published clone induction system using Tub-Gal80ts; Actin-Gal4, UAS-Flp (Fernández-Hernández et al., 2013). Gal80ts blocks Gal4 activity at the permissive temperature of 18°C and can be relieved by changing the temperature to a restrictive temperature of 29°C. We incubated

Fig. 3. Heat shock activation of Raeppli provides information about growth rate of clones. (A-H) Third instar larval wing discs from larvae with two copies of Raeppli-NLS (A-D) or Raeppli-CAAX (E-H) transgenes, hsFlp1.22 and Tub-Gal4. The larvae are heat shocked at time points indicated below the panels as AEL (hours after egg laying). The time in hours that passed after the heat shock (hours AHS) until the imaging is indicated above the panels. (I) Quantification of the average area labeled by combinations of fluorescent proteins in samples heat shocked once at different time points. Number of discs quantified are indicated in parentheses. (J) Quantification of average number of clones. Error bars represent s.d. in I and J. (K) Third instar larval eye-antennal discs from larvae with two copies of Raeppli-NLS. The cells posterior to the morphogenetic furrow at the time of heat shock (white arrowhead) have exited the cell cycle and give rise to single cell clones (green box inset). The cells anterior to the morphogenetic furrow give rise to multicellular clones (red box inset). Position of morphogenetic furrow at the time of dissection is indicated with red arrowhead.
the larvae with two copies of Raeppli-NLS and Tub-Gal80\textsuperscript{Ts}; Actin-Gal4, UAS-Flp at 18°C until mid-third instar and switched to 29°C overnight to induce Raeppli. In this set-up we observed only small clones in analyzed discs (supplementary material Fig. S4B).

**Live imaging with Raeppli**

Fixed samples only provide a static picture of the developmental process from which we infer patterns of cell division. A more informative view of growth requires live imaging of the same sample at different stages of development. This approach allows analysis of the growth of each of the primordial cells as well as comparison of growth patterns between different regions of the growing organ. Snap shots of the growing wing disc can be taken through the cuticle in anesthetized larvae using confocal microscopy (Nienhaus et al., 2012). Without the use of whole-tissue labeling, it is not possible to assign clonal relationships among the cells to improve our understanding of the contribution of each primordial cell to the final organ. Whole-tissue labeling marks the clones so that they can be traced at different time points. A larva with two copies of Raeppli-NLS and hsFlp, Tub-Gal4 was heat-shocked at the first instar stage to induce color choice. This larva was anesthetized on ice and the right wing disc of the larva was imaged at different time points in development (Fig. 4A-C from earlier to later time points). By fixing and staining the disc with the lineage restriction boundary markers Patched (Ptc) and Wingless (Wg) at the last time point, it was possible to map the position of the clones with respect to lineage boundaries (Fig. 4C\textsuperscript{′}). We quantified the growth in surface area of 35 different clones, corresponding to 98% of the disc area, at different time points (Fig. 4D-G). The surface area of clones grew on average 2.4 (±0.8) times between 48 hours and 86 hours and 1.4 times (±0.3) between 86 hours and 92 hours. Tracing each of the identified clones through time provided individual growth parameters at different regions of the same wing disc (Fig. 4G). Overall placement of clones was traced too. Cell migration and reorganization are rare occurrences in wing disc development (Baena-López et al., 2005). Indeed, by live imaging the clones in the same disc, we observed that the shape and relative position of most clones did not change much between 48 and 96 hours. However, we did identify a large-scale clonal rearrangement in clone 12, which was a continuous clone that spans regions in both ventral and dorsal compartments at 48 hours after heat shock (Fig. 4D). At 86 hours after heat shock, it was constricted by clone 18 (Fig. 4E) and at 92 hours after heat shock it was bisected and the ventral portion of clone 12 was not connected to the dorsal portion (Fig. 4F).

Next, we used Raeppli for continuous imaging of the wing disc eversion process in early pupal development (supplementary material Movies 1, 2; Fig. 5A,B). Within the first 10 hours of the pupal development, the wing disc everts and opens up to show the whole adult wing pattern (Fristrom and Fristrom, 1993). By using the clone colors provided by Raeppli as regional markers, it was possible to analyze the movement patterns of different regions of the wing. We heat shocked larvae with two copies of Raeppli-NLS and hsFlp, Tub-Gal4 at different larval stages. Pupae from larvae heat shocked at early development had large clones that were useful to map general tissue movement. We identified that the wing blade rotates before opening fully after eversion (Fig. 5A). Pupae from

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**Fig. 4. Live imaging of a wing imaginal disc through the cuticle.** (A, B) Wing disc of a larva with two copies of Raeppli-NLS, hsFlp1.22, Tub-Gal4, heat shocked at first instar larval stage. The same disc is imaged through the cuticle at different time points of its development. (C, C\textsuperscript{′}) The disc is dissected, fixed and stained for a combination of Wg- and Ptc-specific antibodies at the latest time point. Wg and Ptc proteins mark the dorsoventral and anteroposterior boundaries, respectively. No bleed-through is observed between the Raeppli detection channels and the antibody staining channel. (D-F) The clones from A-C are numbered and their boundaries are traced for ease of tracking. (G) The surface areas of the clones are measured and their fold change between the first and last time point is plotted. x-axis corresponds to the clone identifier number. Data points are colored the same as the clone for ease of finding the clone. Scale bar: 40 μm.
larvae heat shocked later in development had smaller clones that were useful to analyze cell rearrangements and clone shape changes. A previous report analyzing the cell movements in single regions of the pupal disc proposed intercalation and cell division as important driving forces for wing morphogenesis (Taylor and Adler, 2008). Indeed, we identified an increase in cell number in multiple clones (Fig. 5B′ as an example region) and cell intercalation in regions later forming a groove (Fig. 5B″ as an example region). More detailed whole-tissue imaging using Raeppli could be useful to correlate these movements, in order to get a deeper understanding of collective cell movement ultimately resulting in the final wing morphology.

**DISCUSSION**
Raeppli provides a new experimental paradigm, which we name ‘whole-tissue labeling’. The low bias, the usage of four spectrally separable fluorescent proteins and the high efficiency of recombination make it possible to label most cells with different color combinations. Whole-tissue labeling can be used to label diverse aspects of development, such as remodeling, cell fate specification and mapping of cell projections. We demonstrated the use of whole-tissue labeling in organogenesis, with emphasis on the wing disc as a model. The possibility of analyzing the cell division patterns and the growth rates of essentially all the cells maximizes information acquired from a single sample. Imaging the development of all the clones simultaneously enables analysis of their behavior with regard to their neighbors in the primordium.

An ideal whole-tissue labeling tool would label each cell of the tissue individually at all times. In theory, this would be feasible with combinatorial labeling, but in practice it becomes challenging with increasing cell numbers: higher cell number necessitates more colors and hues to mark the clones distinctly, which requires increasing genetic complexity (more copies of *Raeppli* in the genotype) and causes challenges of detecting subtle differences in color hues. We
opted to use two copies of Raeppli for most of our experiments, as
this provides enough color combinations to map clones in the wing
disc. It is also possible to use three copies of Raeppli in wing discs
simultaneously without any alterations in our detection parameters
(Fisun Hamaratoglu, personal communication). The use of more
copies of Raeppli transgenes increases the color combinations that
are acquired. In fact, it is possible to label polyploid cells, such as
the larval cuticle cells, individually with many different color
hues by induction of Raeppli with heat shock after the endocycles
(data not shown).

Growth characteristics of wing discs has been measured before by
generating marked mitotic clones or flip-out clones (Baena-López
et al., 2005; García-Bellido and Merriam, 1971; González-Gaitán et
al., 1994; Martin et al., 2009; Milán et al., 1996; Johnston and
Sanders, 2003). Because the clones are randomly generated, each
disc had a different set of cells marked and the information from
multiple discs had to be averaged. The efficiency of Raeppli
induction permits the growth of a majority of the cells that are
present in the same disc to be traced. This approach can be
especially useful for analyzing the growth of the disc in mutant
backgrounds and for comparing the growth rates of diverse regions
in the disc. Moreover, Raeppli can be used in combination with local
perturbations, such as overexpression or loss of function, in clones
of cells. The possibility of detecting Raeppli through either LexO or
UAS sites makes it possible to perturb the developing organ with the
other binary system. Therefore, the effects of mutations and the
response of the whole tissue can be mapped with whole-tissue
labeling. Furthermore, it is also possible to image the development of
a mutant phenotype via live imaging of the same disc. Effects of
the mutations can be further characterized with antibody staining, in
addition to Raeppli detection.

In conclusion, Raeppli is a new addition to the expanding imaging
toolbox for developmental biology. There are many cell labeling
tools that are very useful for determining single cell properties or
mapping axon projections, by labeling a lower percentage of cells
in the tissue. Raeppli is more suited for approaches that benefit from
marking the whole tissue, such as live imaging and measuring whole
tissue behavior, and thus provides a complementary method for
imaging development.

MATERIALS AND METHODS

Cloning

Minimal attP GGAGTAGTGCCCCAACTGGGGTAACCTTTGAGTTCT-
CTCAGTGGGGGCGTAGG site was cloned between
Minimal attP GGAGTAGTGCCCCAACTGGGGTAACCTTTGAGTTCT -
SpeI and EcoRI RI. The reverse primers of those sequences harbor a
TCTCCTGA) sequences were cloned in the same plasmid between
NLS (GACCCAAAAAAGAAGAGAAAGGTA -
attP sites of pBluescript II SK. NLS (GACCCAAAAAAGAAGAGAAAGGTA-
GATCCTAAAGAGAGAAGGTAGATGCCTCAAAAAAAGAGAGA-
AAGGTATGA) or CAAX (GGAGGGAGGAGAGATCTAGCCTGAACCCT-
CCTGATAGTGCCCGCTGCTGAGCTGCAAGTGTGTCG-
NotI and subcloned into a plasmid that harbors another such
tagged fluorescent protein coding sequence cut with SpeI and EcoRI.
The resulting two fluorescent protein-harboring vectors were cloned together by
reiterating of the same strategy.

The backbone of Raeppli was cloned in a modified pUASTattB vector, where
the UAS site is replaced by UAS and LexO sites that are floxed to
mutually exclusively excise either UAS or LexO (pUASTLOattB vector)
(supplementary material Fig. S3). The LexO/UAS-hsp70 minimal
promoter-containing fragment in the pUASTLOattB vector was cut out of
the vector and cloned upstream of the attB site. Full hsp70 promoter
flip-out integrase phiC31 fragment was subcloned into this backbone and
the four tagged fluorescent protein coding sequences each harboring
minimal attP sites upstream are subcloned into this backbone with NotI
and EcoRI.

Genetics

The Raeppli constructs contain P-element end repeats for transgenesis. We
generated transgenic fly lines in multiple loci in the genome by using
standard P element transgenesis methods in y w flies. The insertion
sites were mapped through splinkerette PCR method (Potter and Luo, 2010)
(supplementary material Table S1). Stocks were maintained at 25°C on
standard fly food. Tub-Gal4, hsFLP, En-Gal4, Hh-Gal4, Nubbin-Gal4, UAS-
FLP flies were obtained from the Bloomington Stock Center. hsFlp;
mr232LexALHG flies were obtained from Konrad Basler (University of
Zurich). Unless otherwise indicated, overnight collected embryos were aged
for a day and resulting larvae were heat shocked for 2 hours at 37°C in a
water bath.

Imaging

With the exception of in vivo imaging, the samples were dissected and fixed
for 20 minutes in PBS with 4% paraformaldehyde (Electron Microscopy
Sciences). The samples were washed thoroughly and mounted in Vectashield
(Vector Laboratories). Samples were imaged with a Leica SP5 confocal
microscope with LAS AF software using the laser lines 405 nm (for
TagBFP), 458 nm (for TFP1), 514 nm (for mOrange2 or E2Orange), 594
nm (for mKate2) and 633 nm (for Alexa 680) sequentially. Suggested
photomultiplier tube (PMT) windows are indicated in supplementary
material Fig. S1B. An HCX PLAN APO CS 20× dry objective was used
(numerical aperture 0.70, working distance 0.59 mm). Samples were scanned
with 1-µm-thick slices and with 1 airy pinhole. Images were analyzed using Fiji
(Schindelin et al., 2012). Images in Fig 4 were smoothened with Fiji for better visualization. Areas were measured by
drawing region of interests in Fiji and using the measure tool.

For immunostaining, mouse anti-Ptc [mAb Apal, Developmental Studies
Hybridoma Bank (DSHB), University of Iowa] and mouse anti-Wg (mAb
4D4, DSHB, University of Iowa) primary antibodies were used with Alexa
680-conjugated donkey anti-mouse secondary antibody (A-21057, Life
Technologies).

For live imaging, larvae were chilled with ice and kept anesthetized at a
low temperature on an ice chamber during imaging (typically less than
30 minutes). Between imaging sessions the larvae were kept at room
temperature on grape juice plates supplemented with fly food.

Live imaging of pupae was carried out on a coverslip glued over a small
hole cut in a small Petri dish. The chamber was humidified with a piece of
filter paper soaked in water. White prepupae were oriented laterally on the
coverslip in a pool of halocarbon oil. Samples were imaged every 10
minutes for supplementary material Movie 1 and 15 minutes for
supplementary material Movie 2 with Leica SP5 system. 3D reconstruction
was performed with Imaris software. The movie in Fig. 5B is 2D projected
with mean signal intensities.

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Competing interests

The authors declare no competing financial interests.

Author contributions

O.K. and E.C. designed the Raeppli strategy, cloned Raeppli constructs and
generated the transgenic fly lines. O.K. conducted experimental analysis with the
help of E.C. for Fig. 2B and supplementary material Fig. S2, with the help of A.S.D.
for Fig. 4 and supplementary material Fig. S1A and with the help of A.P.-S. for
Fig. 5B, O.K., A.P.-S. and M.A. wrote the manuscript in interaction with all the
contributing authors.
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Supplementary material
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