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Towards automatic image analysis and assessment of the multicellular apoptosis process

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Abstract

Apoptotic programmed cell death (PCD) is a fundamental aspect of developmental maturation. However, the authors' understanding of apoptosis, especially in the multi-cell regime, is incomplete because of the difficulty of identifying dying cells by conventional strategies. Real-time *in vivo* microscopy of *Drosophila*, an excellent model system for studying the PCD during development, has been used to uncover plausible collective apoptosis at the tissue level, although the dynamic regulation of the process remains to be deciphered. In this work, the authors have developed an image-analysis program that can quantitatively analyse time-lapse microscopy of live tissues undergoing apoptosis with a fluorescent nuclear marker, and subsequently extract the spatiotemporal patterns of multicellular response. The program can process a large number of cells ($>10^3$) automatically tracked across sets of image frames. It is applied to characterise the apoptosis of *Drosophila* wing epithelium at eclosion. Using the natural anatomic structures as reference, the authors identify dynamic patterns in the progression of PCD within the *Drosophila* tissues. The results not only confirm the previously observed collective multi-cell behaviour from a quantitative perspective, but also reveal a plausible role played by the anatomic structures, such as the wing veins, in the PCD propagation across the *Drosophila* wing.

1 Introduction

In this paper, we present an image-analysis application, developed on the existing image-processing methods, to quantify spatiotemporal features of the multicellular apoptosis behaviour. Apoptosis, a natural form of programmed cell death (PCD), is an orderly cellular process whereby damaged or unnecessary cells are destroyed and removed in a programmed manner [1, 2]. Apoptosis is essential for normal physiology, such as development, maintenance and aging among metazoans. *Drosophila melanogaster* is an excellent model organism for the study of developmental apoptosis because it is genetically tractable, and the essential apoptosis mechanisms are conserved in this organism [1–3]. Apoptotic removal of unnecessary cells is employed throughout tissue remodelling and organ development of *Drosophila* [4, 5]. In this study, we focus on the developmental process of *Drosophila* wings regulated by apoptosis. The mature wings of a *Drosophila* fruit fly are formed when the fly undergoes metamorphosis that transforms it into the adult insect [6]. During metamorphosis, the epithelial cells secrete the wing cuticle, an elastic transparent protein matrix constituting

the outer layer of the adult wing (Fig. 1a). At eclosion, which is the ‘hatching’ stage of the adult fly from the pupal case, the epithelial cells undergo apoptosis and are subsequently removed, leaving the wing structure transparent, except at the veins (Fig. 1b) [6].

The apoptosis process has been studied using quantitative experimentation, particularly at single-cell level [7–9]. Nevertheless, how the communal PCD is coordinated at multicellular level is largely unclear to date. Real-time *in vivo* microscopy is proven to be an efficient method to visualise apoptotic cellular response. Time-lapse fluorescence microscopy of the apoptotic *Drosophila* epithelial cells during eclosion of wing development has been used to demonstrate plausible collective apoptotic progression occurring at multicellular level [10]. In those experiments, a nuclear DsRed reporter driven by vestigial-Gal4 (denoted as *vg:DsRed*), where vestigial is a transcription factor, was successfully designed to monitor the cellular apoptotic activity. Specifically, in viable cells, the *vg:DsRed* protein is localised to the nucleus. When a cell undergoes apoptosis, the integrity of the nuclear envelope is compromised and the fluorescent protein is released into the cytosol. Therefore the loss of the nuclear localised fluorescent signal of *vg:DsRed* marks the apoptotic PCD in single cells. This criterion was implemented through subjective inspection and counting to qualitatively delineate the collective ‘wave’ of PCD [10]. However, the method of subjective counting for the purpose of PCD assay is labour intensive and low-throughput.

A variety of computer-aided image processing techniques have been applied to microscopy images to detect apoptotic activity in single cells [11–16]. Nevertheless, these studies primarily analyse single still images, instead of tracking progression of cells over an extended time period. In addition, the existing cellular image processing programs are developed for processing a relatively small number of cells per frame (<100), acquired at a high magnification. Specifically, the CellProfiler is designed to handle small numbers of cells of a particular morphology, without providing the function of quantification of spatial patterns in distributed tissues [17]. The toolbox Dead Easy Caspase does not allow for tracking of individual cells over a period of time, or characterisation of the macroscopic patterns in a population of cells [13]. In summary, the available image-processing programs can fulfil partial requirements for an efficient study of apoptosis occurring in a small number of cells. However, they lack the capabilities to characterise dynamic and/or communal behaviour of a cell population over an extended period of time.

In this work, we have studied an application of image analysis techniques to the automatic quantification of spatiotemporal evolution of the apoptosis process at the tissue level, measured by *in vivo* time-lapse fluorescence microscopy. The image-analysis program can identify and segment fluorescence-labelled nuclei of single cells from image sequences. Subsequently, each nucleus is temporally tracked and checked for morphological changes that reveal the onset of apoptosis. The program allows for analysis of stacks of images, each containing a large number of cells ($>10^3$). With the use of reference maps, plausible characteristics of spatiotemporal patterns of the progression of apoptosis across a tissue can be detected. The program is implemented using the macro language of ImageJ (version 1.49b), a versatile image processing freeware widely utilised by the biomedical community (<http://rsbweb.nih.gov/ij/>). The program can be accessed at http://utdallas.edu/~lan.ma/Macro_FlyApoptosis.zip. Notably, FRET-based indicator of the activity of caspase-3, the

central protein executing apoptotic cell death, has been applied to enable quantitative visualisation of the collective propagation of PCD in *Drosophila* tissues *in vivo* [18, 19]. Therefore our method of cell tracking along with the monitoring of nuclear morphology serves as one of the ways to quantitatively depict multi-cell apoptotic events. Recently, collective regulation of apoptosis has been observed in various tissue remodelling events, such as the replacement of larval abdominal epidermics in *Drosophila* and the neural tube closure in mammals [18–20]. It is observed that the PCD regulation in cell populations is dynamically orchestrated in time and in space. We propose that our method, together with the proper imaging systems, has great potential for quantitatively portraying the spatiotemporally coordinated apoptotic process at the tissue level in general.

2 Results and discussion

2.1 Overview of program

This present image processing and assessment program is developed for, but not limited to, the analysis of time-lapse microscopy data of *Drosophila* wing epithelium with a fluorescent nuclear marker. The time-lapse image sequences of *Drosophila* wings are acquired at 30-second intervals at eclosion *in vivo*, when the wing epithelium is progressively eliminated through PCD [6, 21]. The video microscopy analysed here starts from the initiation of epithelial apoptosis, and ends before the cellular debris gets rapidly pushed towards the wing veins (Movie 1, at <http://utdallas.edu/~lan.ma/Movie1.tif>).

We seek to develop an ImageJ-based program that can automatically assess multicellular response in a high-throughput fashion. To this end, we have designed a two-phase image analysis program. The workflow of the program is illustrated in Fig. 2, where the numbered arrows refer to the output at each step, starting from the original microscopy sequence as input (Fig. 2, arrow 1). The workflow is outlined as follows. In Phase One, the image sequence is initially checked for data quality and consistency through a preprocessing step, including removal of obstructed frames, registration of misaligned frames implemented by the StackReg plugin [22] and a sharpening step implemented by the built-in ImageJ Unsharp Mask filter for image amplification. Typically a radius $R = 5\text{pixel}$ and a weight $W = 0.6$ provide enough sharpening. For registration, simple translations or rigid body rotations are utilised in order to avoid the introduction of rounding errors in pixel intensity, associated with non-rigid transformations [23]. Note that the wing veins are not affected by the apoptosis process, and thus are masked and excluded from further analysis. The preprocessed images (Fig. 2, arrow 2) are then subject to the step of ‘fluorescence localisation’, where the fluorescent signal is classified into nuclear, cytosolic, or background localisation. The resulting data is a two-colour map showing the nucleus and the cytosolic leakage (Fig. 2, arrow 3). Meanwhile, the preprocessed image sequence undergoes a cell-tracking algorithm [24], whose output is time-series of the coordinates at local maximum intensity of isolated fluorescent areas, corresponding to location of single cells.

In Phase Two, morphology measurements of the nuclear areas detected in Phase One are conducted first, and the changes of the nuclear morphology are used for determination of the onset of apoptosis. For example, the fact that nuclei get rounder and smaller with time of apoptosis onset is applied [16, 25–27]. The morphology information (Fig. 2, arrow 4) is then

combined with the temporally linked coordinates to determine the progression of apoptosis in each cell, by the step of ‘characterisation of single-cell apoptosis stage’. The resultant data (Fig. 2, arrow 5) is a temporal sequence of apoptosis maps showing the position of each cell and its apoptosis status (viable or apoptotic). To identify plausible communal patterns of multicellular PCD in the wing, each apoptosis map is divided into equivalent sub-regions according to the anatomical quadrants of the wing. In each sub-region, the fraction of apoptotic cells is calculated to quantify the degree of apoptosis at multicellular level. The two-dimensional distribution of the degree of apoptosis is finally plotted as a temporal sequence of colour maps, to visualise the spatiotemporal evolution of PCD across the wing (Fig. 2, arrow 6). In the following subsections, we will discuss in greater detail the steps after preprocessing.

2.2 Fluorescence localisation

In live cells, the fluorescent reporter protein *vg:DsRed* is confined within the intact nucleus. When the integrity of the nuclear envelope is disrupted in an apoptotic cell, the reporter protein gradually diffuses into the cytosol. Therefore the loss of the nuclear localisation of the fluorescent signal is used as a visual criterion to represent the onset of cellular apoptosis as previously published [10]. The purpose of Phase One (Fig. 2, Phase 1) of the program is to segment the images according to the cellular location of the fluorescent signal.

In terms of fluorescent appearance of a cell during apoptosis, the intact nucleus has relatively high fluorescence intensity and a sharp edge. In contrast, the cytosolic fluorescence diffused from dismantled nuclear membrane has relatively low intensity with a dull outer edge. Therefore the edge information together with the pixel intensity is utilised to segment and classify the nuclear- and cytosolic-localised fluorescent signal as follows.

First, a Canny–Deriche edge detection plugin is applied to the preprocessed images to detect the position of the nuclear envelope of live cells. Second, an intensity threshold, implemented by a hysteresis connection threshold plugin, is determined under the guidance of the detected nuclear edges. Specifically, a map of nuclear edges is overlaid on the corresponding preprocessed image as a visual guidance, then the user can select an appropriate cut-off threshold to ensure that only the pixels above the threshold as well as encircled by the nuclear edge are selected as intact nuclei. Third, in the case of touching nuclei, a further segmentation mask is applied to separate the two nuclei. In particular, the domain occupied by a single cell and the position of its cellular border can be estimated using a watershed algorithm [28].

Once the intact nuclei are identified, the nuclear pixels are removed from the preprocessed image. Then the cytosolic signal is classified by the same three steps, namely edge detection, intensity thresholding and watershed segmentation. The remaining pixels are considered as background. The results of Phase One are stored as a temporal sequence of two-colour maps that classifies the location of the nuclear protein and that of the cytosolic leakage, if any (see an example in Fig. 3b).

It is noteworthy that the microscopy images used here are captured to analyse the tissue-level patterns, where the magnification is compromised, leaving the intact nuclei roughly

visible. Therefore the challenge is to discern nuclear signal from cytosolic signal based on images with relatively low sub-cellular resolution. Practically, we find that the sequential segmentation of the nuclear and cytosolic signal of single cells can help detect the relatively dimmer signal of the cytosolic reporter protein with higher accuracy as well as efficiency, after the bright pixels of nuclei are removed. Although hysteresis thresholding in combination with edge detection is used here to segment nuclei and cytosol, more sophisticated methods with a higher degree of automation may be alternatively applied. These include the model-based method [29], supervised learning [30], unsupervised mining [31] and enhanced watershed algorithm together with interaction model [32].

2.3 Characterisation of single-cell apoptosis stage

In the first step of Phase Two, the apoptosis status at single-cell level is determined. That is, each cell is identified to be viable or dying (apoptotic). We find it is challenging to precisely determine the initial time point of cytosolic leakage if we solely rely on the fluorescence localisation, because the identification of little amount of cytosolic leakage at the early apoptosis stage is subject to significant noise (data not shown). Rather, we need to take into account the morphological changes in the shape and size of the nucleus, which have proven effective in identifying cellular apoptosis [11, 12, 16, 25, 26]. Comparing with using the FRET-based indicator of the caspase-3 activity in previous studies of collective PCD [18, 19], the usage of the *vg:DsRed* marker here requires the extra characterisation and assessment of nuclear morphology for the determination of cellular apoptosis activity. However the advantage is that our method allows for concurrent identification of the location of individual cells.

During the temporal evolution of apoptosis, three cellular stages with distinct morphological features can be defined. First, the viable nucleus is intact with stable size, normal mean fluorescence intensity and regular shape (denoted as ‘Vi’). In the second stage, the visual appearance of the nucleus is characterised by shrinking size, increased mean fluorescence intensity and regular shape and is considered to be in a compacted stage (denoted as ‘Co’), during which a cell prepares for nuclear condensation of apoptosis [26, 33]. In the final stage, the cell has a larger fluorescent area and a decreased average intensity because of cytosolic dispersion, and is considered apoptotic (denoted as ‘Ap’) [12, 16, 26]. That is, the *vg:DsRed* protein loses nuclear localisation in the Ap stage.

To classify cells into the three stages, we need to quantify necessary morphological features. In this regard, Filippi-Chiela *et al.* describe two morphological parameters, the nuclear area (*A*) and the nuclear irregularity index (NII) [12]. The parameter NII is a compound measure [12], using features of aspect ratio (*Asp*), area box (*Arbx*), radius ratio (*Rr*) and roundness (*Rou*), and is computed as: $NII = Asp - Arbx + Rr + Rou$. With the images used here, we find that a third parameter, namely the mean fluorescent intensity of nucleus (*I*), is helpful in efficiently identifying the three stages. The reason is because compacted chromatin in the Co stage results in increased concentration of the reporter protein and thus enhanced mean fluorescent signal, in contrast to the Ap stage.

Furthermore, instead of setting independent parametric criterion for each stage, we only need to characterise the Co stage, which has the most prominent nuclear features to

recognise, and in addition temporally separates the Vi and Ap stages. For a cell, once the frame of the Co stage is determined, the preceding and succeeding frames can be identified as Vi and Ap stage, respectively. Specifically the Co stage of a cell has a combinatorial feature with decreased nuclear area (A), highest nuclear fluorescence intensity (I) and regular shape (NII) [12, 16, 26]. Practical considerations to exclude outliers that are not cellular, such as fluorescent areas that are too small to be a nucleus and areas with saturating pixel intensity, pose additional lower bounds for A and NII and an upper bound for the mean intensity. The logic rule to determine the Co stage is expressed as

$$(I_{min} \leq I \leq I_{max}) \text{AND} (A_{min} \leq A \leq A_{max}) \text{AND} (NII_{min} \leq NII \leq NII_{max}) \quad (1)$$

The threshold values are obtained semi-automatically by the program based on the distributions of I , A and NII of a sampled cell population, so that the false positive and false negative rates of the detection of the Co stage are minimised to a predefined limit. In particular, the threshold values are optimised in an iterative fashion as follows. (1) The initial threshold values for I , A and NII are selected using their respective histograms to cover the range for all cells. (2) Via a macro named ‘Apoptosis Analysis’, a user assesses the performance of the program with the parameters, by manually comparing the apoptosis map of a selected sub-region of 10–20 cells to the preprocessed images. (3) The subjectively determined mislabelled cells are analysed by a macro named ‘Parameter Learning’, to generate histograms of their I , A and NII . The false-positive (FP) and false-negative (FN) populations are analysed separately, such that the thresholds are improved to eliminate these two types of errors. Specifically, one standard deviation from the mean of the FP or FN population is, respectively, excluded from or included in the improved range. Steps (2)–(3) are conducted iteratively to evaluate the improved parameters, until a performance satisfying given FP and FN objectives is obtained.

2.4 Temporal apoptosis analysis of single cells

Using the positions found by the cell-tracking algorithm, the progression of the single-cell apoptosis stage can be followed over time. Most of the cells start as viable and progressively go through the three stages, namely Vi, Co and Ap. In Fig. 4, time trajectories of the three morphological parameters of five representative cells are shown. Note that the trajectories and colour maps are marked in green, red, or yellow if the cell is in the Vi, Co, or Ap stage, respectively (Fig. 4).

We assume that the entrance of a cell into the Co stage is irreversible, meaning that once a viable cell transits into the Co stage, it has to move into the Ap stage in later frames. Therefore for a few cells fluctuating in and out of the Co stage because of noise, the first time and the last time the nucleus enters the Co stage are defined as the beginning and the end of the Co stage, respectively. We speculate that the main source of the noise is the loss of focus when a cell shifts in and out of the focal plane.

At the beginning of the image sequence, a group of cells are already in the Ap stage and do not go through the Co stage during the sequence. To distinguish this class of cells, especially from the non-cell fluorescent regions, a two-step processing is conducted. In step one, the time trajectory is screened to ensure that a Co stage does not appear at any point. In step

two, the parameters I , A , NII of the fluorescent areas are measured and classified based on a criterion of the Ap stage. Specifically, the mean intensity is lower, the fluorescent area is larger and the shape is more irregular compared with live cells. Note that in step two, the morphology of the area of the nucleus plus cytosolic leakage is analysed. Again, the practical consideration of A , I and NII to exclude non-cellular outliers is taken into account, leading to the same logic format as (1) for the Ap stage, albeit with different threshold values.

The result of the temporal analysis is stored as a series of colour maps, showing the apoptosis stage (live or apoptotic) of each cell in each frame, where cells in the Vi or Co stage are denoted as alive whereas cells in the Ap stage are denoted as apoptotic. An example of identified live and apoptotic cells in a single frame is shown in Fig. 3c.

2.5 Quantification of multicellular spatial patterns

This work aims to quantify the patterns of PCD progression at the tissue level. To this end, the apoptosis activity is further analysed according to the natural reference system of the wing anatomy, such as the veins, as we hypothesise that they play a role in regulating the dynamics of apoptosis. For instance, the wing veins do not go through PCD, while the apoptotic cellular debris is absorbed by the veins [6, 21]. Therefore our conjecture is that the anatomical structures of *Drosophila* wing may affect the collective apoptosis, either through the delivery of chemical signals, or providing hydrostatic impact on the epithelium.

To extract spatial patterns according to our hypothesis, a wing is divided into major quadrants based on salient anatomical features, including the veins and the edge of the wing. Each quadrant is sliced into equally spaced sub-regions according to the relative distance towards the anatomic structures selected as the boundary. As shown in Fig. 5, different structures can be selected as boundaries of a quadrant to generate different distance reference maps. A same reference map is applied to all the frames to monitor the temporal evolution of plausible collective patterns. On each slice, a value representing the percentage of apoptotic single cells out of the population residing in this domain is assigned. The wing region containing the slices with the valued degree of apoptosis is finally plotted as an apoptosis colour map.

The above algorithm of spatial pattern recognition can quantify the propagation of apoptosis in relationship to the anatomical structures over an extended time span. Rows b–f of Fig. 6 show the examples of identified macroscopic patterns of apoptosis across the wing tissue in Movie 1, based on the five reference maps in Fig. 5. Note that an anatomical structure is either marked in magenta or white if it is or is not a quadrant boundary in a reference map, respectively. Using the time-lapse apoptosis maps, we can recognise spatiotemporal patterns of apoptosis response, even though there exists some degree of stochasticity where the propagation along a certain direction may fluctuate back and forth between neighbouring slices.

In particular, in row *b* of Fig. 6, a wing blade is divided into four quadrants via the veins and the outer edge of the wing, and the degree of apoptosis is quantified in slices along the longitudinal direction of a wing. The resultant time-lapse maps show a trend of communal

apoptosis response starting along slices near the quadrant boundaries, which then propagates towards the quadrant centre. Such a pattern suggests that plausibly a biochemical signal triggering the initiation of apoptosis is released from the veins and the outer edge, and subsequently diffuses towards the centre of the quadrant, so that the closer a cell resides to a vein, the earlier it senses the extracellular signal and starts apoptosis. Note that in this regard, the central longitudinal vein appears to have a lesser effect than the other two veins, in that the cells close to it undergo PCD in a delayed time scale than those close to the other veins. This collective propagation starting from the anatomical structures is indeed supported by row c of Fig. 6, where the thick central vein is excluded from the reference map. In row d of Fig. 6, the outer edge is excluded from the reference map, and the corresponding pattern confirms that the central vein has a lesser effect in inducing apoptosis compared with the other two veins and the edge. Thus we speculate that the pro-apoptosis biochemical signal may be more diluted in, or released more slowly from the central vein. In rows e and f of Fig. 6, the slices are positioned in incremental distances to the outer edge or the line orthogonal to the longitudinal axis of the wing at the upper left-hand side corner of the image, respectively. The resultant maps not only confirm the propagation pattern from the edge towards the centre of the wing as observed above, but also a concomitant pattern from the two ends towards the midline along the longitudinal axis of the wing. This longitudinal pattern could reflect apoptosis provoked by mechanical forces associated with the cuticle fusion process, which appears to propagate from the outer edge of the wing. The same collective patterns of apoptosis are reproducible from the analysis of a second set of microscopy data (see Movie 2, at <http://utdallas.edu/~lan.ma/Movie2.tif>, and the apoptosis map of Movie2 http://utdallas.edu/~lan.ma/Movie2_ApopMap.zip).

2.6 Validation of program

To evaluate the performance of the program, we validate if the apoptosis status of single cells can be detected reliably. Specifically, we randomly select five sub-regions of the image sequence, each containing ~ 200 cells, and validate the sub-regions separately. For each sub-region, the microscopy images after preprocessing are compared with the apoptosis colour maps produced by the program. First, the frame(s) of the Co stage of every single cell in the preprocessed images is subjectively determined, which should be the frame(s) immediately before the nuclear localisation of the fluorescent signal becoming compromised. Then for each frame, the program-detected apoptosis status of individual cells ('Co' in particular) in the sub-regions are checked against the subjective results of apoptosis status that have been enumerated cell by cell [13, 15, 16]. Subsequently, the FP and FN populations with respect to the detection of the single-cell apoptosis status are collected for each frame throughout the sequence. Finally, the FP and FN rates averaged across all frames of the five sub-regions are calculated.

Fig. 7 demonstrate the resulting histograms of the morphological parameters I , A and NII of the cells that are examined during the program validation. The histograms are classified into cells whose apoptosis status is correctly detected (Fig. 7, left-hand side column), FP cells (Fig. 7, right-hand side column, red bars) and FN cells (Fig. 7, right-hand side column, magenta bars). Fig. 7 shows that the number of correctly detected cells is significantly higher than that of the FP as well as FN cells.

Indeed, Table 1 shows that the FP rate and FN rate averaged for the image sequence have low values of 0.71% and 4.43%, respectively. Sensitivity, the probability of actual positives which are correctly identified by our image-analysis program, has a high value of 95.7%. Therefore the detection of apoptotic cells by the program is reasonably reliable.

3 Conclusion

As a step towards automatic high-throughput quantification of the progression of apoptosis at the tissue level, we have developed an image-processing program, implemented by ImageJ, to identify the spatiotemporal patterns among apoptotic cell groups. The program is designed for analysing time-lapse microscopy of tissues with a fluorescent reporter. In Phase One, each image is segmented to classify the fluorescent signal into nuclear or cytosolic localisation. Meanwhile, the individual cells are tracked over time. In Phase Two, the morphology characteristics of the classified areas are measured and employed, in combination with the tracking information, to determine the apoptotic progression in each cell. In addition, the local degrees of apoptosis, quantified as the fractions of apoptotic cells on reference grids, are represented by a colour map, allowing for the visualisation of the spatiotemporal patterns of apoptosis across cell groups. Application of this program to the live imaging data of apoptosis in *Drosophila* tissues supports the collective propagation of PCD at the multicellular level from a quantitative perspective. Furthermore, the results indicate that the *Drosophila* anatomical structures may play a regulatory role in inducing the spatial patterns of PCD. Finally, we expect that the program can be applied to quantify the dynamic patterns of other collective PCD processes, including the tissue regression of the salivary gland in *Drosophila* and the mammalian PCD during neural tube closure (NTC) [18–20], provided the transgenic organisms with adequate nuclear markers are developed. In particular, the fkh protein [34] and the LMO family [35] could, respectively, serve as the candidate nuclear proteins for fluorescence labeling to monitor the *in vivo* PCD in *Drosophila* salivary gland and mammalian NTC. Taken together, in this study we have developed a quantitative tool for annotation of the collective patterns of apoptosis at the tissue level.

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Fig. 1. Anatomical structures

a Drosophila wing before

b Drosophila wing after

Removal of epithelium via PCD, with top view (left-hand side) and cross-section (right-hand side) of the wing. The outer cuticle layer, the inner epithelial layer and the veins are, respectively, shown in blue, green and red. The transparent cuticle is only partially displayed, to allow visualisation of the epithelium underneath

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Fig. 2.
Workflow of the image-analysis program

Fig. 3. Comparison of the two-colour map of detected nuclear and cytosolic areas *b*, or live and apoptotic cells *c* with the original fluorescence microscopy image *a*

In *b*, the nuclear and cytosolic area is, respectively, coloured in magenta and blue. In *c*, the live and apoptotic cell is, respectively, coloured in green and red. The non-cell areas are coloured in cyan. Three insets including 31 cells are correspondingly shown in *a* and the colour maps *b* and *c* for comparison. Note that the wing veins in *b* and *c* are masked without processing

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Fig. 4. Temporal change of the three morphology parameters, A (area), I (mean intensity) and NII (nuclear irregularity index) of five example cells and detection of their apoptotic status
 A is in unit of pixels, I has arbitrary unit and NII is unitless. *a* original image at tissue level, where the five cells are labeled. In the top panels of *b–f*, temporal trajectories of A, I and NII of the cells are plotted. In the bottom panels of *b–f*, the original images (zoomed in) and the detected apoptosis status of the cells at three distinct stages, namely Vi (viable, nucleus coloured in green), Co (compacted, nucleus coloured in red), Ap (apoptotic, nucleus plus cytosolic leakage coloured in yellow), are illustrated. The time interval between consecutive frames is 30 s

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Fig. 5. Five different distance reference maps of a wing

Distance reference map is generated by slicing each quadrant of a wing according to the distance from selected anatomical reference boundaries, where *a* three longitudinal veins and edge of the wing, *b* three veins, *c* two thin veins and edge of the wing, *d* edge of the wing, *e* the line orthogonal to the longitudinal axis of the wing at the upper left-hand side corner of the image, respectively, are selected as reference boundaries. The selected anatomical reference structures are coloured in red, whereas the excluded anatomical structures are coloured in white

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Fig. 6. Detected apoptosis spatiotemporal patterns using the five reference maps in Fig. 5. Frame 1, 11, 21 and 28 of the original movie is listed in row a

According detected spatial patterns of apoptosis response at the wing tissue level using different distance grids are shown in rows *b–f*, where the reference structures selected in distance grid are coloured in magenta, and the anatomic structures excluded from distance grid are coloured in white. On each strip of the distance grid, the number of apoptotic cells is normalised to the total number of cells. The resulting fractions of apoptotic cells across all

strips, valued between 0 and 1, are plotted in each frame as a colour map using the colour scale given at the bottom of the figure. The time interval between consecutive frames is 30 s. For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.

Fig. 7. Histograms of the morphological parameters I, A and NII of 28668 cells in total

The left-hand side column shows the histograms of the cells whose apoptosis status (Co) are correctly detected. The right-hand side column shows the histograms of the FP (red) and FN (magenta) cells. The units for A, I and NII are the same as Fig. 4

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Table 1

Validation of our multi-cell apoptosis image-analysis program

No. of cells	No. of frames	FP, %	FN, %	Sensitivity, %
28668	30	0.71	4.43	95.7

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