Live Cell Cycle Analysis of *Drosophila* Tissues using the Attune Acoustic Focusing Cytometer and Vybrant DyeCycle Violet DNA stain

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Abstract

A protocol for cell cycle analysis of live *Drosophila* tissues using the Attune Acoustic Focusing Cytometer is described. This protocol simultaneously provides information about relative cell size, cell number, DNA content and cell type via lineage tracing or tissue specific expression of fluorescent proteins *in vivo*.

Flow cytometry has been widely used to obtain information about DNA content in a population of cells, to infer relative percentages in different cell cycle phases. This technique has been successfully extended to the mitotic tissues of the model organism *Drosophila melanogaster* for genetic studies of cell cycle regulation *in vivo*. When coupled with cell-type specific fluorescent protein expression and genetic manipulations, one can obtain detailed information about effects on cell number, cell size and cell cycle phasing *in vivo*. However this live-cell method has relied on the use of the cell permeable Hoechst 33342 DNA-intercalating dye, limiting users to flow cytometers equipped with a UV laser. We have modified this protocol to use a newer live-cell DNA dye, Vybrant DyeCycle Violet, compatible with the more common violet 405nm laser. The protocol presented here allows for efficient cell cycle analysis coupled with cell type, relative cell size and cell number information, in a variety of *Drosophila* tissues. This protocol extends the useful cell cycle analysis technique for live *Drosophila* tissues to a small benchtop analyzer, the Attune Acoustic Focusing Cytometer, which can be run and maintained on a single-lab scale.

Keywords

Cell cycle; Flow cytometry; *Drosophila*; Gal4/UAS; metamorphosis

Introduction

Flow cytometry can be used for measurements of cell viability, relative cell size, DNA content and fluorescent protein expression in live cell populations. Due to the replication of nuclear DNA during S-phase, information about DNA content in a population of cells can be used to infer relative percentages in different cell cycle phases [1–3]. This method has become a cornerstone of cell cycle analysis in model systems from yeast to mammals.

The fruit fly *Drosophila melanogaster* has become an excellent model system for genetic *in vivo* analyses of cell cycle regulation. The extensive genetic tools available in flies allow for

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elegant tissue specific and temporally regulated manipulations of cell cycle regulators along with *in vivo* fluorescent protein-based lineage tracing [4–6]. Flow cytometry has been used to study DNA content in a number of *Drosophila* cell types, including endoreplicating cells and cultured mitotic cells [7,8]. An important advance for *in vivo* cell cycle studies was made by de la Cruz and Edgar, with the development of a protocol for flow cytometric analysis of live diploid *Drosophila* imaginal discs [9,10], a protocol which has been used and adapted by many labs. This technique, when coupled with genetic *in vivo* lineage tracing via inducible fluorescent protein expression and tissue specific labeling, allows one to obtain information about gene manipulation effects on overall cell doubling time, cell size and to determine precise timing of cell cycle phases in vivo [9,11]. However this method has thus far relied on the use of the cell permeable Hoechst 33342 DNA-intercalating dye to stain and quantify DNA in live cells, which has limited users to flow cytometers with a UV laser capable of exciting the Hoechst dye. These are generally found only in sorters (ie. BD FACS Vantage™, BD FACSAria™) or expensive multicolor benchtop systems (ie. BD LSR™), usually requiring support by institutional flow core facilities.

We have modified the Hoechst-based protocol to use a new live-cell DNA dye from Invitrogen, Vybrant DyeCycle Violet. This dye is compatible with a violet 405 nm laser, more common in smaller benchtop analyzers and available in the small self-contained benchtop analyzer, the Attune Acoustic Focusing Cytometer. Here we present a detailed protocol for cell cycle analysis that can be coupled with cell type, cell size, cell number and lineage analysis in a variety of *Drosophila* tissues during various stages of development using DyeCycle Violet and the Attune. This protocol expands the number of cytometers suitable for such analysis with *Drosophila* tissues and provides examples of how this type of live cell cycle analysis can be modified for additional tissue types and developmental stages.

Protocol Text

1) Fly Husbandry

1.1) Cross flies of desired genotypes in narrow plastic vials with 10mL Yeast-Glucose Medium[3] or other protein-rich media of your choice. The extensive *Drosophila* transgenic tools available for tissue specific expression and lineage tracing with *in vivo* fluorescent protein expression are described in detail elsewhere[4,5]. Transfer parents to fresh vials daily to obtain series of 24 hour progeny collections, or for more precise staging, collect embryos on agar plates and transfer newly hatched larva to vials as described[10]. To ensure uniform conditions across experiments and avoid overcrowding, the total number of F1 progeny in a single vial should be no more than 100. Depending on the genetic design of the experiment, keep vials in an appropriate incubator until the desired developmental stage.

1.2) Collect experimental animals: you will need mature larvae at the 3rd larval instar (L3) at about 110–120 hours of development for larval dissections or white prepupa (WPP) for accurate pupal staging. Dramatic cell cycle changes, including a switch to a non-proliferative state occur at distinct timepoints during metamorphosis[12,13]. Therefore correct staging of pupa (within 1 hour of development) is essential for reproducible cell cycle data during metamorphosis. WPP corresponds to 0 hours after pupa formation (0h APF) as shown in Figure 1 panel I. Collect WPP in a 35mm Petri dish on a folded Kimwipe wet with 2.5 ml of water to maintain some humidity. Age pupa until desired stage (hours APF) at appropriate temperature. Note that development proceeds 1.2 times faster at 29°C and 2.2 times more slowly at 18°C than at the standard 25°C[14].

1.3) Heat-shock animals to activate heat-shock flipase (hs-flp)-induced recombination for lineage-tracing clones if needed[9,11]. For the heat-shock of larva, immerse vials in a water bath (set at 37°C) and ensure larvae are fully submerged. For the heat-shock of pupa, seal the Petri dish with Parafilm and fully submerge it into the water-bath. Be sure to remove Parafilm after the heat-shock, to allow sufficient oxygen exchange for animal survival. Duration of the heat-shock depends on activity of the flipase transgene used and number of clones desired. We routinely use 7–8 minutes for inducing "flipout" lineage tracing clones[15] in larvae with the hs-flp transgene on the first chromosome and 2–4 minutes for heat shock of pupae in 35mm dishes, while the hs-flp transgene on the second chromosome requires a 20 min. heat shock to obtain similar numbers of clones.

The rate of cell division and timing of dissection determines clone size in each tissue. Under normal conditions, we find that clones induced in the larval wing with a 20 minute heat shock (using the hs-flp transgene on the second chromosome) and dissected 48 hours later, contain approximately 20–30 well separated clones with sizes ranging from 10 cells per clone to two cells per clone, with an average around four cells per clone. In contrast, a 7 minute heat-shock with the same hs-flp transgene of pupae in a petri dish at 0 hours APF dissected 36 hours later, yields well separated clones (approximately 15–25) ranging from one to four cells with an average of 2.2 cells per clone. Such data can be used to determine the average cell doubling time for the tissue under study.

2.) Dissection

2.1) Gently transfer the larvae/pupae to a clear glass dissection dish containing 1X PBS.

2.2) Using a dissecting microscope and ample light, gently grasp a larva by the abdomen with a sharp Inox #5 forcep, cut the anterior third with micro-scissors, and invert the anterior third by pushing the mouth area inwards toward the posterior while holding the larval cuticle steady (Fig. 1A). Carefully remove opaque fat body and any remaining gut to increase visibility of desired tissues, such as wing, eye or brain. Dissect the desired tissues; cleanly removing wings from trachea and cuticle with forceps, eyes from mouth-hooks, or brain from eyes (Fig. 1G).

2.3) For dissecting pupa; use a forcep to gently grasp a pupa by the anterior tip of the pupal case where there is an air bubble, to avoid damaging the pupa inside (Fig. 1C). Forceps or micro-scissors in the opposing hand can be used to help position the floating pupa for proper grasping. Cleanly cut through the posterior tip of the pupa with micro-scissors. This cross-sectional cut should be made at a slight angle toward the dorsal part of the animal, to release the internal pressure without forcing the histolyzed fat into the desired tissues in the thorax and head. Cut carefully along the dorsal median, avoiding the wings and the anterior eye-brain complex. Carefully remove the pupa from the pupal case by grasping the posterior edge of the pupal epidermis and pulling it out of the pupal case (Fig. 1D). Poke a small hole in the cuticle anterior to the eye-brain complex with forceps. Using a glass Pasteur pipette with a rubber bulb, gently wash PBS dissection solution through the partially dissected pupa to remove histolyzed fat and any remaining gut tissue. This also effectively lubricates the glass pipette with lipids and proteins, which reduces the incidence of dissected tissues from sticking to the interior of the glass pipette later.

2.4) To obtain pupal wings, the cuticle enveloping the wing and the wing itself must be pried from the pupa (Fig. 1E). This can be done by holding the pupa down by the head with one forcep while using another forcep to either grasp the most posterior tip of the wing cuticle and pulling out or maneuvering the forcep under the wing near the hinge before dislodging it from the side of the carcass (Fig. 1E). The wing can then be cut or torn at the hinge to remove it from the body and put in a pile away from the carcasses and other material.

2.5) To obtain pupal eyes or brain, wash the pupa until the eye-brain complex becomes dislodged and free from the pupa (Fig. 1F, H). Using a forcep, gently pull the pupal eye away from the brain and save the desired tissue.

2.6) Do not take longer than 45 minutes to dissect each sample, as results becomes less accurate the longer cells are left in PBS after opening the animals.

3.) Tissue Dissociation and DNA Staining

3.1) Using a lubricated pasteur pipette, carefully transfer the dissected tissues into 0.5mL of Live DNA Stain Solution in a 5mL polystyrene tube (Fig. 1G). Transfer as little PBS as possible (less than 300μL) into the DNA Stain Solution, as dilution of trypsin will inhibit tissue dissociation.

3.2) Incubate tubes with shaking at 23°C at 500rpm (we use an Eppendorf Thermomixer™).

3.3) Incubate for 70 minutes, then vortex gently for 5 seconds at a medium speed (setting of 5). Excessive vortexing will cause cell disruption and form debris, while under-vortexing will lead to cell clumps. Return the samples to shaking at 23°C at 500rpm for an additional 15 to 20 minutes before vortexing them once more for 5 seconds at speed 5.

3.4) For brains and pupal eyes older than 36hAPF, a modified protocol is required. Dissociate tissues in DNA Stain Solution at 23°C at 500rpm for 45–60 minutes in 1.7ml microcentrifuge tubes. Then one-by-one manually dissociate large chunks of tissue by transferring to dissecting dish along with about 50 μl DNA Stain Solution and rapid pipetting up and down with a manually drawn pasteur pipette [16] (of approximately 100–150 μm opening). Transfer each dissociated specimen with the rest of the 0.5 ml of DNA Stain Solution into a 5mL tube. Incubate an additional 45–30 minutes (up to 90 minutes of total incubation time) at 23°C with shaking at 500rpm until clumps are no longer visible.

3.5) Fully dissociated samples should have very few large visible chunks of tissue, although note that the acellular transparent pupal wing cuticle will not dissociate.

4.) Flow Cytometry

4.1) Turn on the Attune cytometer and open the Attune software. The cytometer lasers should be turned on via the startup menu, and a daily performance test should be performed with Performance Tracking Beads with adequate scores before each day of data acquisition. Fluid levels should be at least half full and waste emptied before the performance test. Details for startup, proper calibration and operation of the Attune can be found in the Attune User Guide (http://tools.invitrogen.com/content/sfs/manuals/cms_082577.pdf)

4.2) Design a new template by creating 4 dot plots and 2 histograms in a blank workspace with the parameters indicated in 4.3, or select a previously designed

template suitable for the experiment, and indicate the number of samples to be collected. We have provided Attune templates (files in .get format) for live *Drosophila* cell cycle analysis with Vybrant DyeCycle Violet and GFP or RFP expression in Appendix I.

4.3)   The templates provided include four dot plots and two histograms with the parameters indicated in Fig. 2. Channels for detection include: FSC and SSC – forward scatter and side scatter indicating cell size and membrane complexity, VL1 height (H), width (W) and area (A)-indicating Violet laser excitation of DyeCycle Violet staining the DNA, and BL1-A – Blue laser Channel 1 Area, indicating GFP fluorescence. The provided template for RFP analysis is identical to the template for GFP analysis, except that it uses the Blue laser channel 3 (BL3-A), which is optimized for RFP detection.

4.4)   The dot plots in the templates have gates to restrict analysis to the desired populations and to limit debris and cell clumps from analysis (Fig. 2). Gate 1 excludes debris and clumps based on SSC vs. FSC (Fig. 2A). Gate 2 excludes unstained cells, sub G1 apoptotic cells and cell clumps based upon identification of singlets (VL1Area vs. VL1Width) (Fig. 2B). Gate 3 is a derived gate, encompassing cells within gates 1 and 2, and identifies GFP (or RFP) negative cells vs. DNA content based on VL1Height (Fig. 2C). Gate 4 is a derived gate, encompassing cells within gates 1 and 2, and identifies GFP (or RFP) positive cells vs. DNA content (Fig. 2C). Dot plots of cell size as measured by GFP (or RFP) vs. forward scatter (FSC) are used to generate Gates 5 and 6 (Fig. 2D). The two histograms plot DNA content on the X-axis and cell count on the Y-axis for the populations defined by Gate 3 and Gate 4. (Fig. 2E and F) Histograms can also be included for cell size if desired (not shown). For cell size histograms, populations should be set to Gates 5 and 6, plotting FSC on the X-axis and counts on the y-axis.

4.5)   Set recording to stop at 10,000 GFP or RFP positive events (this will be Gate 4, the derived Gate of Gate 1, Gate 2 and the GFP or RFP positive gate). Ranges from 6,000 – 10,000 GFP positive events have traditionally been the goal for publication quality profiles[9,11]. We suggest using a minimum of 15 wings or eyes, that are approximately 50% GFP positive to obtain sufficient Gate 4 events[10] for high quality profiles. However, we have successfully obtained clear data for preliminary cell cycle analysis from as few as 6 wings (Fig. 3A). Set the acquisition volume for 300μL, this will use about 460μL of the total 0.5mL sample. Table 2 shows the appropriate threshold and voltage settings provided in the template for the example in Fig. 2.

4.6)   Run samples at standard sensitivity or high sensitivity 100μL/min and record. We find little to no difference in our analysis with the different sensitivity levels. Due to the acoustic focusing of cells, this cytometer can accurately measure samples at very high flow rates. Unlike traditional cytometers, we find no data compromise by acquiring at speeds up to 1,500 events per second. The recorded data files generated by the Attune software are in .fcs format, which is compatible with most cell cycle modeling software.

5.) Data Analysis

5.1)   Histograms of cell size and DNA content for GFP (or RFP) positive and negative populations can be compared. They can be manually layered by copying and pasting using Adobe Photoshop™ software. If the y-axis is set to automatic, the Y-axis will scale with the highest count for each sample.

Overlaying two graphs with the Y-axis set to autoscale creates an overlay of histograms with the axes set for the global maximum for each population. This allows easy visual comparison for relative changes in cell cycle phasing, even when total cell numbers of GFP positive and GFP negative populations are not equivalent (example in Fig. 3B). Alternatively, a custom y-axis (y-axis manual option) can be set to a fixed value for the histograms. In this case the relative cell numbers for two populations can be compared. As the Attune uses a defined volume of sample per run, absolute quantification of cells for each run, in each gate with percentages of the total population, can be obtained from the statistics table automatically generated during the run in the workspace (example in Fig. 3F). Dot plots and histograms can also be easily layered in the Attune software, by simply dragging and dropping different samples from the sample file menu at right onto the desired graph of an open sample for comparison. However to overlay subpopulations from *within* a single sample (ie. GFP positive and GFP negative within the same sample), we use Photoshop.

5.2) Two methods can be used to find relative percentages in different phases of the cell cycle for a population. The approximation method relies upon user defined regions to delineate boundaries of G1, S, G2 and >G2 peaks. This method allows quick and easy estimations of percentages within the user-defined regions (Fig. 3E). We set our regions based upon a direct test with *Drosophila* Kc cells, labeled for S-phase using Ethynyl-deoxyUridine (EdU) incorporation. The second method uses third party modeling software for estimation of cell cycle distribution. We used ModFitLT™ (Verity Software House) software for example, in Fig. 3F.

Representative Results

Figure 2 shows representative results for a larval wing sample, expressing GFP in the posterior half of the tissue, using the provided GFP template. Similar results are obtained with the same tissue type and expression pattern for RFP using the provided RFP template (Fig. 3A). The provided templates and voltages (Table 2) are suitable for analysis of larval eyes (Fig. 3B), brains and wings, as well as pupal eyes, brains (Fig 3D) and wings. Histograms of relative cell size provided by plotting FSC for populations in Gates 5 and 6 can also be generated (Fig. 3C). Gates may need to be adjusted slightly due to differences in cell size for different tissues or differences in fluorescence intensity for different GFP or RFP transgenes. This can be done while running a small test volume of sample (50–100μL), before hitting record.

Allowing the y-axis (counts) for the cell cycle or cell size histograms to auto-scale (y-axis scale set to automatic) in the Attune software, results in histograms showing the global maximum for each population. Figure 3B–D shows representative results with GFP positive and GFP negative histograms with y-axis set to the global maximum for each population. Setting a specific y-axis maximum (y-axis scale set to manual with a user defined value), allows for comparison of absolute numbers between populations, which can be useful for comparing proliferation rates, but makes it difficult to compare cell cycle phasing in populations when numbers of GFP positive cells vs. GFP negative cells are vastly different.

Fig 3B shows cell cycle profiles for GFP positive and negative cells in larval eyes, expressing GFP in the posterior using the GMR-Gal4, UAS-GFP transgenes. The overlay reveals the differences in cell cycle phasing of the posterior GFP expressing cells. Fig 3C shows the relative change in cell size between GFP positive and negative cells in B, by plotting cell size as measured by FSC. Fig 3D shows a cell cycle profile from pupal brains at 46h APF. GFP positive cells are lineage tracing clones expressing the G1-S regulators

Cyclin D and E2F that disrupt quiescence and lead to S-phase entry, indicated by the red arrow and confirmed by S-phase labeling with incorporation of EdU (Fig. 3D inset). This is in contrast to the non-expressing GFP negative cells, which at this stage are normally arrested in G1 (black trace, Fig. 3D).

Most cell cycle profiles are used to obtain information about percentages of a population in different cell cycle phases. This can be approximated in the Attune software by creating user defined regions on the histograms delineating G1, S and G2 phases (Fig. 3E). The Attune software automatically generates a statistics table for each run, giving the absolute counts of cells within the user defined regions and percentages (Fig. 3F). This method works well when comparing relative changes in phase distribution between a control and experimental population. Alternatively, modeling software can be used to estimate percentages in each phase as well as apoptotic cells. We used ModFitLT™ (Verity Software House) in Fig. 3G, which can directly open the .fcs data files generated by the Attune software.

Discussion

The protocol described here allows for analysis of cell cycle, relative cell size and relative cell number in live *Drosophila* tissues at various developmental stages. When this analysis is coupled with cell-type specific fluorescent protein expression or lineage tracing, detailed information can be obtained about cellular responses to discreet cell cycle or growth perturbations. As proof of principle, we disrupted quiescence in the pupal fly brain by expressing G1-S cell cycle regulators in GFP labeled cell clones, leading to aberrant DNA replication at a developmental stage when brain cells are normally over 90% G1 arrested (Fig. 3D).

However there are some important limitations to this live cell cycle analysis method. First, one cannot distinguish between cells in the various stages of mitosis using the protocol described here. Thus, the analysis described here should be supplemented with immunofluorescence staining for a mitotic marker, such as Ser10-phosphorylated Histone H3[18] to quantify mitotic index in the tissues of interest. In addition the method described here cannot distinguish between cells in G1 vs. a G0 arrest, as both states have the same DNA content. Since DyeCycle Violet is taken up by live cells, identification of cells in later apoptotic stages is also limited. Lastly, it is important to note that measurements of cell size based upon forward scatter are relative cell size measurements rather than absolute quantification of size. For absolute quantification of cell size we recommend a volumetric approach such as that used in a Coulter Counter (Beckman Coulter).

When cell cycle phase and size data is coupled with lineage tracing to measure cell doubling time, detailed information about the cell cycle and growth can be calculated, such as the length of the G1, S and G2 phases and cell growth rate[9,11]. This coupled with discreet genetic manipulations of cell cycle regulators *in vivo* can provide detailed information about cell cycle and growth regulation for quantitative cell cycle, cell growth and tissue morphogenesis modeling.

Acknowledgments

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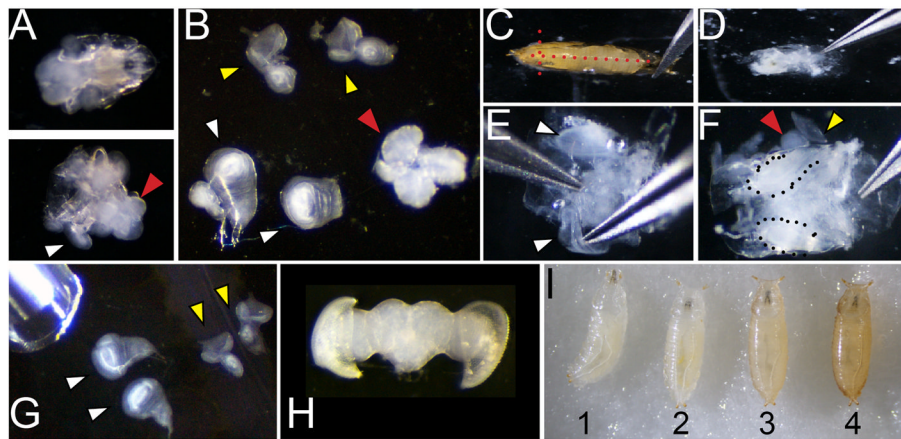
Figure 1. Dissection and staging of larval and pupal tissues
A. Dissection of larvae (of genotype w^{1118} shown) at 110 hours of development showing the anterior third before (top) and after (bottom) inversion. White arrowhead indicates a wing attached to the larval cuticle and a red arrowhead indicates the position of the brain B. Wings (white arrowhead; note wing at left is attached to a leg disc, wing at right is torn at the dorsal notum), eyes (yellow) and brain (red) removed from the larvae. C. Dissection of pupa showing positions of cuts with forceps placed at the airspace anterior to the head (red dotted lines) D. Pupa removed from cuticle before washing to remove fat and gut. E. Cleaned pupa showing process of lifting wings for removal F. Cleaned pupa showing wings (dotted) and partially removed eye-brain complex (red and yellow arrowheads). G. Dissected larval wings and eyes showing pipette for transfer. H. Dissected post-mitotic pupal eye-brain complex. I. Correct staging of animals at the larval/prepupa transition; animal 1 is still moving and is too young; animal 2 is at the correct stage to collect, 0hAPF (after pupa formation) it is white and immobile; animal 3 is too old to collect, the darkening of cuticle is evident by 1hAPF; animal 4 is too old to collect already tanned at 2hAPF.
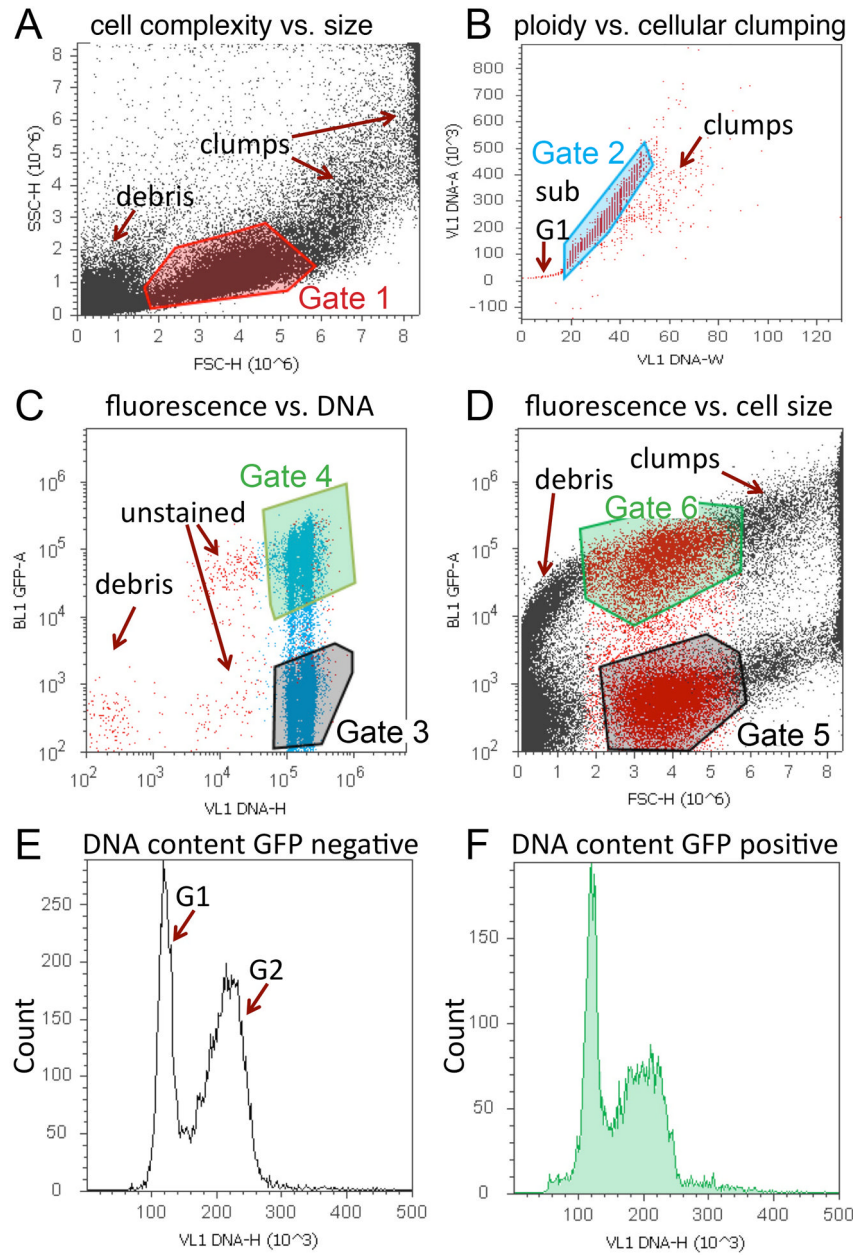
Figure 2. Representative cell cycle analysis of tissues expressing GFP using Vybrant DyeCycle Violet and the Attune

A representative workspace, containing 4 dot plots and 2 histograms is shown for a larval wing sample expressing GFP and Cyclin D in the posterior wing, driven by an *engrailed*-Gal4 transgene. A. Dot plot of cell size with Gate 1 to exclude debris and clumps. B. Dot plot to discriminate singlets with Gate 2 to exclude unstained cells, sub-G1 DNA content (apoptosis), and clumps. C. Dot plot of GFP vs. DNA content. GFP negative (Gate 3) and positive (Gate 4) cells can be distinguished and 2N and 4N DNA content is evident based upon position along x-axis. D. Dot plot of cell size as measured by GFP vs. forward scatter (FSC) to generate Gates 5 and 6. Note that cells within Gate1 are colored red for visual assistance in determining Gates 5 and 6 for this scatter plot. E. Histogram of DNA content vs. counts for GFP or RFP negative (population set to Gate 3). F. Histogram of DNA content

vs. counts for GFP or RFP positive (population set to Gate 4). Histograms can also be included for cell size if desired. For cell size histograms, populations should be set to Gates 5 and 6.
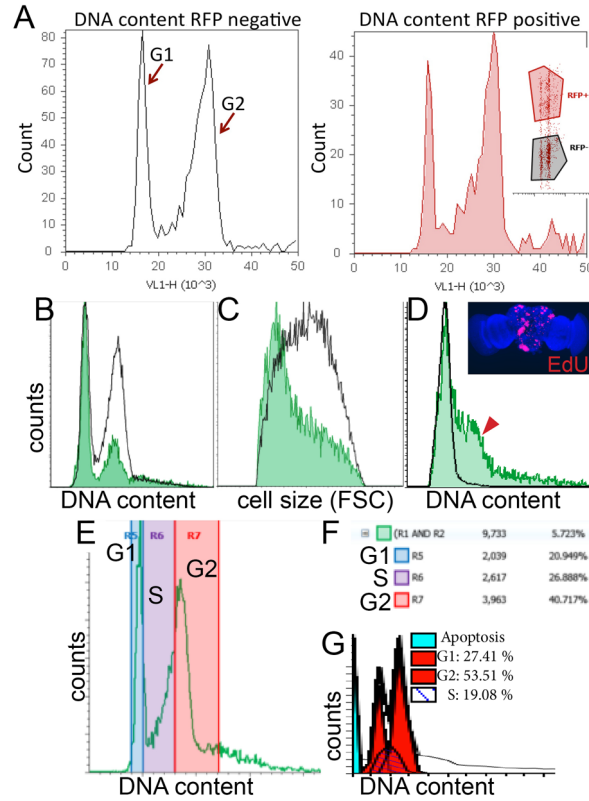
Figure 3. Representative histograms of cell cycle and cell size analysis for various developmental stages and tissues

(A) Representative histogram data is shown for comparing cell cycle in specific cell types using RFP expression with DyeCycle Violet (inset shows scatter plot of RFP fluorescence vs. DNA content). This example contains larval wings expressing RFP in the posterior, driven by an *engrailed*-Gal4 transgene. (B) Overlays of GFP positive vs. GFP negative DNA content histograms in larval eyes with GFP expression driven by the *GMR*-gal4 promoter[17], showing most GFP positive cells in G1. (C) Smaller relative cell size of *GMR* driven GFP+ cells compared to GFP- controls, as measured by forward scatter using Gates 5 and 6. (D) In the pupal brain, GFP positive cells indicate heat-shock induced lineage tracing clones via flp-out method[5] induced during the 2nd larval instar, over-expressing the G1-S cell cycle regulators Cyclin D and E2F, causing aberrant S-phase entry (red arrowhead) in the normally postmitotic G1 arrested tissue. The abnormal S-phase entry observed by flow cytometry was confirmed by incorporation of EdU, which labels cells in S-phase (inset). (E) Example of regions used to estimate relative percentages in G1, S and G2 phases. Boundaries were estimated based upon S-phase incorporation of EdU in *Drosophila* cells in a separate experiment. (F) Example of statistics table generated by Attune software showing relative percentages in the regions defined. (G) Example of cell cycle modeling of representative Attune data using ModFitLT™.

Table 1

Required reagents and instruments

Name of the reagent	Company	Catalogue number	Comments (optional)
12×75 mm Polystyrene Round- Bottom 5mL Test Tube	BD Falcon™	352058	5mL tubes
Attune® Acoustic Focusing Cytometer	Life Technologies/ Applied Biosystems®	4445315	Blue/Violet configuration
Attune® Cytometer Software (version 1.2.5)	Life Technologies/ Applied Biosystems®	*Free*	PC only
Attune™ Performance Tracking Beads (5×10^6 beads/mL)	Life Technologies/ Applied Biosystems®	4449754	For daily performance test
Dumont #5 Inox forceps	Fine Science Tools	11251–20	
Embryo dishes 30 mm × 12mm	Electron Microscopy Sciences	70543–30	Glass dissection dishes
Eppendorf® Thermomixer	Eppendorf	022670051	
Trypsin-EDTA Solution (10x)	Sigma	T4174	
Vannas-Tübingen Spring Scissors	Fine Science Tools	15003–08	Straight 5mm Cutting Edge
Vybrant® DyeCycle™ Violet Stain	Life Technologies/ Invitrogen	V35003	

Live DNA Stain Solution (10mL):

1mL 10X Ca^{2+} Mg^{2+} free PBS (pH7.2)

9mL 10X Trypsin-EDTA (Sigma)

5µL Invitrogen Vybrant® DyeCycle™ Violet (note that this is 0.25X the recommended concentration for mammalian cells. We find that higher concentrations are toxic to *Drosophila* cells.)

10X Ca^{2+} Mg^{2+} free PBS (pH7.2): 1.37M NaCl, 27 mM KCl, 100mM Na_2HPO_4 (dibasic), 20mM KH_2PO_4 (monobasic) adjusted to pH 7.2

Table 2

Threshold and voltage setting for the analysis in Fig. 2

	FSC	SSC	BL1	VL1
Threshold (x1000)	100	10	10	10
Voltage (mV)	2950	4250	1800	1150