**Response to the review comments:**

*We thank the editor and reviewers for the opportunity to correct and improve this protocol. Below we outline our response to the comments.*

**Editorial comments**

The Discussion should be expanded to include limitations of this technique.

*We have included additional discussion pointing out the limitations of this technique including; inability to resolve G0 vs. G1 phases, or G2 vs. M phases,* *limitations* *detecting apoptotic cells with DyeCycleViolet and limitations of measuring relative cell size via FSC as opposed to absolute volumetric measurements (ie. via Coulter Counter).*

Your current highlighted protocol is just over two pages. You may consider highlighting additional sections to include more detail in the video.

*We have highlighted additional sections to provide more useful detail in the video.*

**Reviewer #1**

*Summary*

The authors describe a protocol intended to study cell cycle in drosophila tissues by means of a live-DNA staining with Vybrant DyeCycle Violet in combination with the Attune Acoustic Focusing flow cytometer, making advantage of drosophila genetics techniques.

The protocol is a modification of a previously described protocol established by de la Cruz and Edgar (Drosophila Methods and Protocols, Methods in Molecular Biology, vol. 420). Although this protocol does not represent a major novelty compared to the previously published protocol, it is well detailed, and makes use of newest flow cytometry reagents available, which undeniably constitutes an improvement in terms of accessibility and throughput.

The manuscript is well written, each step of the protocol is well described, and all reagents are publicly available. As a plus, the authors provide a template for flow cytometry which can potentially be used to set up the experiment on the Attune Acoustic Focusing flow cytometer.

*Minor Concerns*

It would help the reader if the authors make particular attention to referencing precisely the figure number and the panel number throughout the text and not only in the figure legends.

*We have corrected the text to include Figure panel citations*

In Figure 1, although not critical, it would be interesting to mention the genotype of the fly strain used. As a matter of fact, this is what the authors do in Figure 2 when they describe the analysis of GMR-gal4 UAS-GFP tissue.

*We have included the genotype in this experiment*

The paragraph numbering is off after 4.4

*We have corrected this mistake.*

**Reviewer #2**

*Summary*

The paper gives an in depth protocol for flow cytometry analysis of cells from Drosophila tissues using a recently developed live stain for DNA that works with a 405nm laser. The paper provides details on how to apply this technique using the Attune Acoustic Focusing Cytometer. The protocol is of general use as it allows for flow cytometry using relatively inexpensive equipment. The authors provide useful suggestions for working with pupal discs and larval and pupal brains - tissues that have not been as well established as sources for flow cytometry studies.

*Minor Concerns*

section 1.3 - Can the authors provide some guidelines for when to perform heatshocks to induce clones of varying sizes. Presumably heat shocks are performed a set number of hours or days before dissections.

*We have included further detail on the timing of heat shocks for clonal analysis.*

Figure 2 - These are representative results from an experiment in which GFP is expressed in the posterior half of the wing. The legend should indicate the gal4 driver used.

*We have now indicated the details of this genotype – including the gal4 driver.*

Figure 3 - Can the authors provide a little more detail on the experiment shown in D: what method is used for overexpression and lineage tracing (flp-out mehtod?) when were clones induced? Can you name the gene that is overexpressed?

*We have now indicated the details of this genotype – including the responding genes and the gal4 driver.*