We would like to thank the editor and reviewers for their thoughtful comments and suggestions. We have implemented the suggestions and these have improved the clarity of the protocol and the manuscript. Below we respond to each concern with text that is in red.

Editorial comments:
General:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
We have proofread the manuscript.

2. Please include at least 6 key words or phrases.
We added one more key word.

3. Please do not include citations in the abstract.
We removed citations in the abstract.

Protocol:
1. For each protocol step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps.

We have modified the protocol steps to make sure that the steps are clear.

Specific Protocol steps:
1. 1.2: What food is used here? There is nothing in the Table of Materials.

We reference the food that we use and make clear that commercial Drosophila food will also be suitable.

2. 1.4: How exactly do you know which flies express dpp-gal4/UAS-Dpp-GFP, etc.?

We clarified these steps as follows.
1.4. To select the appropriate larvae for dissection (those expressing both Dpp-GAL4 and UAS-Dpp-GFP) first choose larvae that are non-Tb. These larvae will be normal length rather than short and fat.

1.5. Among the non-Tb larvae there will be larvae expressing CyO-GFP and larvae expressing Dpp-GFP. To select the Dpp-GFP expressing larvae view them under a fluorescence stereomicroscope. While both Dpp-GFP and CyO-

GFP expressing larve will have some GFP fluorescence, the Dpp-GFP expressing larvae can be distinguished in that the GFP is restricted to the wing discs and the fluorescence much less bright than the CyO-GFP expressing larvae. Select these less bright larvae for dissection.

References:
1. Please do not abbreviate journal titles.
We have fixed this.

Table of Materials:
1. Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.

Done.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This paper describes a protocol for ex-vivo imaging of Drosophila wing imaginal disc to visualize GFP tagged Dpp. Although significance of Dpp signal in the wing imaginal disc has been established, dynamics of Dpp ligands including secretion of ligands is less known due to lack of proper method of live-imaging. This study provides a protocol for live-imaging of Dpp, which will be useful information for the community. The paper is well written, and the most of the protocol are clearly presented. I have a few concerns that should be addressed prior to publication.

Major Concerns:
The most problematic points in this paper are that the images are collected without proper control. I suggest the authors should include two different controls. First, they should image tissues with or without expressing GFP-Dpp in an identical condition to confirm that fluorescent puncta are only visualized in tissues expressing GFP-Dpp. Second, the authors should express GFP-Dpp in disc alleles of dpp mutants (e.g. dpp[d6]/dpp[d14]) for imaging. This is important for ensuring GFP tagged Dpp is physiologically functional in ex vivo system.

We added the control showing that there is no fluorescence in dpp-Gal4 wing discs. We also show the expression of GFP (without the fusion to Dpp) under the control of the Dpp-Gal4 driver (*dpp-gal4; UAS-GFP)* to show that fluorescent GFP puncta do not appear and disappear in the periphery when GFP is not fused to Dpp. We were not able to generate flies that express Dpp-GFP in a dpp [d6]/dpp[d14] background in the time allotted for resubmission, but we prominently reference the paper that generated this Dpp-GFP fly strain and also showed that it rescues loss of Dpp (Teleman, A. A. & Cohen, S. M. Dpp gradient formation in the Drosophila wing imaginal disc. *Cell.* **103** (6), 971-980, (2000).

Minor Concerns:
It would be useful if the authors provide more details of how they processed the images to make movies in Figures 2 and 3.

We added an image export step to the end of the protocol
5.3. The images can be collected as a time series which can then be exported as AVI files (no compression, 3 frames per second) to obtain video files of the dpp release.

Reviewer #2:
Manuscript Summary:
In this manuscript, Laura Faith George and Emily Anne Bate wrote a protocol to visualize Dpp-GFP release in the wing imaginal disc of Drosophila. The manuscript is well-written and I would recommend it for publication in Jove.

Major Concerns:
My major concern deals with the videos the authors propose us. Both videos (Figure 2 and Figure 3) do not have the same resolution, therefore I would recommend to show the two videos with the same resolution. Also, I would suggest the authors to show a video (in Figure 2) where it is possible to follow (with the help of arrows) the dispersion of Dpp-GFP in puncta in through the wing disc epithelium.

We generated new videos that are magnified to the same extent and have all of the same settings. Figure 3 shows a suboptimal wing disc mounting that does not allow focus on the release events and therefore the resolution is not as good as Figure 2.

We do not expect to see the dispersion of Dpp-GFP through the wing disc epithelium because when it is intracellular, it is quenched compared to when it is released into a neutral environment. We clarify this expectation and result in the introduction. We also change the arrow to a box that disappears after the first few frames. The box directs attention to where in the image to look for appearing and disappearing Dpp-GFP puncta.

Minor Concerns:
Line 32: the appropriate reference has to be written instead of "(REF)"

We removed all references from the abstract including the rogue REF.

Lines 177-Line 184: the orientation (antero-posterior and dorso-ventral) of both discs is missing in the legend of Figure 2 and Figure 3.

We added the orientation to the figure legend and the orientation arrows to the first frames of the videos.

Reviewer #3:
This is a useful protocol explaining how to perform live imaging of Dpp-GFP in Drosophila wing discs. I have only minor points:

Minor Concerns:
1. There are several different UAS-Dpp-GFP lines around which were generated by different labs. Which one are the authors using? Or does it not matter, and they all work similarly?

We added a paragraph that includes a description of the strain and referenced Teleman, A. A. & Cohen, S. M. Dpp gradient formation in the Drosophila wing imaginal disc. *Cell.* **103** (6), 971-980, (2000).

2. The red arrow is not really at the "edge" of the Dpp expressing region, but several cells away. It is unclear whether the arrow is misplaced, or whether the wording needs fixing?

We changed the wording to better describe where we most easily observe Dpp-GFP release and we changed the arrow to a box that disappears after the first few frames of the videos. The text now reads:
"We observe Dpp-GFP fluorescence appearing and disappearing in the cell bodies and far from the cell bodies. Dpp signaling is dependent on actin-based filopodia-like structures called cytonemes that extend far from the cell body[22,23]. Therefore, it is likely that Dpp-GFP puncta that is visualized far from the Dpp-producing cell bodies in these presented videos is likely in cytonemes or at cytoneme "synapses"[15]. These puncta are most apparent close to the D/V boundary, which can be seen as the gap in the Dpp-GFP stripe."

3. I had a bunch of questions such as

-Is it known that prior to release Dpp is in an acidic vesicle? I realize lysosomes are acidic, but I don't believe Dpp is in lysosomes prior to secretion?

We added the following paragraph to address this concern:
"Drs. Aurelio Teleman and Stephen Cohen created a Dpp-GFP fusion protein which is able to rescue loss of Dpp meaning that it is biologically active and is released in a biologically relevant manner[16]. Here, we describe how we visualize Dpp release events using this Dpp-GFP. This fusion protein is particularly useful because GFP is pH sensitive such that when it is in acidic vesicles, the fluorescence is quenched[17]. Therefore, when a protein tagged with GFP is released from a vesicle into the more neutral extracellular environment, GFP fluorescence intensity increases[17]. We took advantage of the pH sensitivity of GFP to determine if Dpp-GFP resides in acidic vesicles. We imaged wing discs expressing Dpp-GFP before and after the addition of ammonium chloride which neutralizes intracellular compartments vesicles[18]. We

found a significant increase in fluorescence of puncta after the addition of ammonium chloride, suggesting that intracellular Dpp-GFP is quenched before the addition of ammonium chloride[18]. We conclude that intracellular Dpp-GFP resides in acidic membrane-bound compartments, such as vesicles, and is unquenched upon addition of ammonium chloride to neutralize the pH of intracellular compartments[18]. This makes live imaging of Dpp-GFP a useful technique to visualize the dynamics of Dpp in the Drosophila wing disc as it is released from acidic compartments into the extracellular environment. "

-How do the authors know that the Dpp-GFP spots that become visible and then disappear are not simply going in and out of the plane of focus of the confocal ?

We added the following explanation:
"We do not see Dpp-GFP puncta moving within the plane of focus in time series if we acquire in one plane of focus. We also do not see movement of Dpp-GFP puncta if we image in a z-stack. We conclude that the Dpp-GFP puncta we see using this method are release events rather than movement of vesicles intracellularly."

-What evidence is there that the spots that disappear are doing so due to internalization events, and likewise that the spots that appear are being secreted?
We added the paragraph quoted above to show that the Dpp-GFP is quenched until ammonium chloride is added to neutralize it. We also referenced the Dahal and Pradhan 2017 paper in which these experiments were first reported.

but noticed that many of these questions are answered in reference 9. They should probably be addressed explicitly in the introduction, otherwise the reader may also wonder.

Thank you for these thoughtful suggestions.