Reviewers' comments:
Reviewer #1:
Manuscript Summary:
This manuscript by Coombes et al., gives a detailed protocol for the findings recently described Nature Communications manuscript from the same group (Coombs et al., 2019). This protocol is very nicely detailed and gives a good in depth description of the process. This methods described need access to specific highend imaging equipment (eg a spinning disc microscope), and the methods described here are quite set-up specific, although efforts have been made to make it generically available to other systems. The introduction is quite brief but the method is well discussed in the discussion

Major Concerns:
-The introduction mentions mammalian systems (in the paragraph on line 55), but doesn't go into much detail and is not referenced at all. A bit more information in this paragraph, including some references for comparison to your zebrafish methods would be useful to the reader.
We added specific examples of approaches used in vitro and in vivo in mammalian systems with corresponding references and also more references regarding relevant prior zebrafish work.

- Do you look at mendelian ratios of your F1 crosses to assess whether you have a single insertion allele for each line? This would potentially be important as multiple insertions could lead to brighter lines/differential expression that might change the levels between founders. This is worth outlining in your methods.

This is a good suggestion, we added a note in the relevant step.

- Much is made in the introduction and discussion about the chemokine ligands being endogenous, but it is not specifically pointed out that driving the chemokine receptors is an overexpression system using the lyz promoter in the intro (this is touched on in the discussion on line 377). This limitation is important to consider when looking at quantification of receptor/ligand dynamics and should be pointed out more clearly early on.

We added a note in the first step where the construct is mentioned, to point this out earlier.

- The figures included are very nice, but don't really allude to the method. This is a complex experiment, and a brief timeline/flowchart would be useful for the reader. A figure showing the constructs used would be a very useful addition.

We have added the construct and representative images as well as a flow chart in new figure 2.

Minor Concerns:
- Line 100: "Lift barriers from fish tanks at around 9 AM". While it's acknowledged that this may vary between aquaria it may be useful to mention that this is shortly after the lights come on.

We amended this

-Line 105: NOTE: "In our experience, the expression results are more consistent when injecting inside the cell and discarding the injections that may not be clearly inside the cell." This is a useful note. Please can you also comment whether only one cell stage embryos are injected, or whether 2-16 cell stage would also work

We added a relevant note.

- Line 110: "It is important to check the injected eggs later in the day and remove unfertilised or dead eggs." I agree with the importance of this, but please can you add why (ie, to keep the clutch healthy)

We updated this

- Line 113: "should be visible in neutrophils". Can you comment how much transient expression you see before growing up the larvae (eg, a percentage of neutrophils/number of neutrophils labelled)

We have not quantified this as it varies between batches, but we provide an indication.

-Line 117: "Depending on the transgenesis success, which varies with each construct, you should observe a percentage of positive offspring in a subset of the crosses." Can you comment here in more detail. What types of percentages do you normally see?

We have not quantified this in detail as it varies between batches, but we provide an idea.

- Line 185: "The exposure time has to be as low as possible to minimize fluorescent exposure and maximize temporal resolution in the time-lapse." Exposure time and laser power?

We added a note: The laser power depends on the condition of the laser but has to be adjusted to a level that permits low enough exposure time for dynamic imaging. We typically take a time lapse of one stack every 30 sec.

Reviewer #2:
Manuscript Summary:
The Jove Submission by Coombs et al provides very useful guidelines on how to study neutrophil responses to tissue damage in vivo, including the chemokine receptor dynamics underlying cell migration. It describes detailed and clear protocols on how to perform these studies taking advantage of the optical transparency of zebrafish embryos.

Major Concerns:
none

Minor Concerns:
Lines 67/68: The text might suggest that genetic tractability is a feature specific for zebrafish. I suggest to rephrase this to make clear that both zebrafish and mice are genetically tractable, but in zebrafish it is less technically demanding.
We amended this phrase accordingly.
Line 101: "at around 9 AM (this may vary in different fish facilities)". I suggest to add "shortly after the onset of daylight"
We amended this phrase accordingly.
Line 103: it might be useful to mention that many researchers prefer (or are more used to) using agarose molds to align eggs
We removed this part in response to reviewer 3 to eliminate the general protocol details for microinjection of eggs. We kept the specific information, such as what to screen for, construct info and amounts injected.
Line 117: suggest to insert fluorescently-tagged before receptor.
This was added
Line 123, step 1.10 Suggested addition: after validating the neutrophil-specific expression of the transgene
This was added
Line 140 Suggest to add a step 2.5 to describe whether embryos are left to hatch naturally or to be dechorionated.
This was added
Line 144: Because neutrophils are also present at earlier stages, I suggest to insert abundant before neutrophils
This was added
Line 365/366: could useful > could be useful
This was corrected
Line 383-386: A little more detailed discussion would be useful on how it may be checked whether or not a fluorescent tag might influence the biological function of the receptor
We added a few additional lines in the discussion.

Reviewer #3:
Manuscript Summary:
The authors describe methods to monitor the dynamic of Cxcr1 chemokine receptor in the neutrophils in response to wounding in vivo. This information could be valuable to the community. The major issues are the resolution of receptor internalization is not good, and the quantification method for receptor internalization is confusing.

Major Concerns:
1. Is there any evidence showing that the chemokine receptor is expressed in neutrophils?
Yes, such data can be found in previous publication (Deng et al 2013, Journal of Leukocyte Biology, Fig. 4A). We have added a citation for this in the representative results section.
2. Better experiments and images at higher resolution are needed to demonstrate the receptor internalization.
* Because this manuscript describes live imaging of receptor dynamics in response to endogenous ligands, it is important to show the receptors are internalized using time-lapse experiments. Is it possible to wound the embryos after taking time-lapse at basal levels?
In the original paper we indeed show time-lapse movies where the internalisation can be observed as it changes in cells. We appreciate that the movies provide more clarity and show the internalisation more convincingly, so we added three movies in this paper from the original paper showing the behaviour or Cxcr1 and Cxcr2 in real time at steady state (movie 1), upon wounding (movie 2) during initial mobilisation and chemotaxis phase (movie 3). We also provide a corresponding figure 2, showing receptor distribution in steady state to versus upon

wounding. Finally, we provide a magnified image of an example of neutrophils at wounds with labelled Cxcr1 and Cxcr2 in new figure 4.

* Neutrophil morphology is complex and changed quickly, which makes it difficult to get images at high-resolution. Is it possible to fix the embryos and image neutrophils at high-resolution to provide additional information?

We added a suggestion for this as potential extension to the protocol in the discussion, as it can allow more detailed localisation analyses.

* The authors used the differences in intensity between neighboring pixels to evaluate the receptor distribution. What does "neighboring pixels" refer to? As the author mentioned, the expression of receptors on the plasma membrane (maybe be cytosol as well) should be quantified to provide convincing evidence.

We provide more explanation for the neighbouring pixel definition in a corresponding note. Neighbouring pixels refers to pixels close by, the distance is determined empirically based on the size of local peaks in intensity. For our images, with a pixel size of 0.389 μm, when the receptor showed vesicular distribution each bright dot was about 5 pixels. We therefore compared intensities of each pixel with 5 pixels apart, i.e.
if you have a horizontal line of 10 pixels, pixel 1 is compared with pixel 6, pixel 2 is compared with pixel 7 etc.

Regarding the expression of receptor at the plasma membrane, we performed this quantification in neutrophils in the original paper and compared it with the contrast-based measurement. We found that the membrane-based quantification gave consistent results with the contrast method in single cells but was less sensitive and accurate in clustered cells because it is not possible to accurately segment individual membranes in overlapping neutrophils (Supp Fig.1 of original paper). We now show these data as Figure 3 in this paper for clarity. Our recommendation here is that contrast needs to be normalised to internal reference, that need not be a membrane marker, to account for global fluctuations of contrast across samples. In our case, we were able to use the non-responding cells in the CHT as these were always present in the field of view. But we appreciate this will not always be applicable to other studies and suggest a control cell marker as alternative. To avoid misunderstanding of our point, we removed the word ‘membrane’ marker from that sentence (1823-1826), as this need not be a membrane marker. Generally, we try to suggest different options with related considerations to help readers choose the best approach for their questions and sample types.

Minor Concerns:

1. "trimeric G-proteins" should be "trimeric G proteins"

This has been amended

2. In zebrafish, embryos are not eggs.

We refer to fertilised eggs but changed ‘eggs’ to ‘embryos’ in a couple of instances where it was appropriate to say embryos.

3. It is necessary to describe the transgenic construct to show where the tag is placed because the tag might affect the protein's function. Or the author could briefly mention the strategy and refer the readers to the paper they have just published. The authors also did not explain why the tandem fluorescent timer is used.

We describe the constructs in new Fig.1 and explain the original intention of usage in the representative results section.

4. It is unnecessary to grow embryos in E3 medium. Egg water is sufficient.

We understand this to be the case for young embryos, but for older embryos, close to larva stage, E3 medium is commonly used. We added a note that young embryos can be grown initially in egg water (step 2.2).

5. I think it is not necessary to describe the details of embryo harvest and microinjection because these can be found in other published papers. However, it is needed to describe the actual amount of RNA and DNAs instead of concentration injected into the embryos. Also, it is better to inject DNA and mRNA using separate needles unless the DNA solution is RNAase free. Otherwise, RNA can be degraded when it is mixed with DNA. Additionally, it is also a good practice to inject DNA into the cell.

We agree that this is not essential and have removed some standard steps and added a citation. We have kept the more customised steps as suggested. We added a note regarding the importance of RNAse free solution for transposase mixture and mention the alternative option to inject with separate needles (step 1.3).

6. It is essential to describe how much the ventral fin is cut.

We added some more explanation in step 3.4

7. The authors generated the transgenic lines, but why fluorescently tagged receptor mRNA (e.g. Cxcr1-FT) is injected in the chemokine response assay.

We clarify now that these steps are not part of the same experiment but a side experiment to establish the behaviour of the receptor in the presence versus absence of candidate chemokine ligand.

8. In the figure legend, it is necessary to name the transgenic lines properly. For example, figure 2, what is (sfGFP) lyz:Cxcr1-FT?

We have now added an additional figure explaining the constructs and transgenic lines (new figure 2) so that the reference of sfGFP is understood in the subsequent figures.

9. There are some issues in Figure 2. 1) Blue and green color are difficult to be distinguished. Suggest not to

outline the cells, which makes it challenging to see membrane expression. Instead, color coding the areas might be sufficient.
We have now changed this figure to the Supplementary figure 1 from original paper, where the cells are shown with and without the contours in the different channels. Blue and green colours are no longer shown in the same image.
2) What is "N" referred to? Cell number or embryo number? Three are too few for any static analyses. 3) "at the wound focus": Does it refer to the wound area?
We do not use these data for statistical analyses but just to indicate what values each quantification gives from the same cells, we added a note in the legend to explain the data are not intended for statistics but to show qualitative examples of analysis. Wound focus refers to the wound area. We removed the word focus for clarity and added dotted lines to indicate the wound in the image.
4). Panel C shows images at 80 min after wounding? Is it too late? Receptor could be internalized initially but is recovered afterward. We now provide a time-lapse of the cells starting 10 mpw to indicate dynamics of receptor at an earlier stage in the same embryos, where it can be seen that Cxcr1 and Cxcr2 are different from early stage as soon as they reach the wound.
5) It is essential to show actual images in embryos injected with Cxcl8-MO. Did Cxcr1 fail to internalize in embryos injected with cxcl8-MO? Also, did neutrophils migrate to the wound in cxcl8 MO-injected embryos?
We added example images taken from Supp Video 5 of original paper in new figure 4. Cxcl8a and Cxcl8b knockdown has been reported to reduce neutrophil recruitment to wounds (de Oliveira et al. Journal of Immunology 2013 and Zuniga-Translavina J. Immunol. Res 2017). For the purpose of receptor distribution analysis, we imaged the neutrophils long enough to observe accumulation of cells at the wound.