nature portfolio

Peer Review File

Multiplexed volumetric CLEM enabled by scFvs provides new insights into the cytology of cerebellar cortex

REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

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1. One concern in this study is to choose Triton X-100 as a detergent for comparison. While Triton X-100 is commonly used to aid antibody penetration in light microscopy, it is not widely used for EM or immuno-EM studies. For EM studies, saponin is one of organic solvents which dissolve lipids from cell membranes making them permeable to antibodies. Furthermore, organic solvents can be used to fix and permeabilize cells at the same time by coagulating proteins. Saponin interacts with membrane cholesterol, selectively removing it and leaving holes in the membrane. Most researchers in the EM field choose low concentration of saponin to balance the antibody penetration and excellent membrane morphology in their immuno-EM experiments. Majority of their immuno-EM images showed great morphology of membrane at the ultrastructural level with the use of saponin. In some immuno-EM studies, Triton X-100 has been used. But those studies don’t care the cell membrane, most of them were interested in some subcellular organelles.
2. Another point of concern is the lack of clarity on how the antibodies penetrate the cell through the cell membrane in detergent-free immunofluorescence labeling. A more thorough investigation and explanation of this process are needed to provide a comprehensive understanding of the technique's efficacy.
3. The animals were perfused with the fixative (4% paraformaldehyde + 0.1% glutaraldehyde). The low concentration of glutaraldehyde (0.1%) may not be sufficient to adequately preserve the lipids in the cell membrane. However, for detergent-free immunofluorescence labeling, scFv probes or nanobody probes were incubated for 3 days (50 μm) or 7 days (120 μm). This could potentially affect the ultrastructural morphology.
4. This study lack of novelty. The use of scFv has been well-established over the years, making it a solid foundation for the volume CLEM study. However, the choice of detergent-free immunofluorescence labeling raises questions about its suitability. Considering alternative approaches, such as utilizing low concentrations of saponin, might offer a more effective option for preserving membrane morphology during immuno-EM experiments.
5. The choice of 0.3% Triton X-100 to demonstrate detergent issues in EM seems excessive. Most EM studies recommend not exceeding 0.1% Triton X-100, making it unnecessary to use a higher concentration for this purpose.

Reviewer #2 (Remarks to the Author):

This is a technology development manuscript describing the generation and implementation of an assortment of single chain antibody-based probes (scFvs) against different brain proteins to label tissue for correlative fluorescence/volumetric electron microscopy. The key advance is the nature of the labeling probes, which can diffuse deep into fixed brain tissue and penetrate cells without the need for detergent permeabilization. The authors do nice side-by-side comparisons with whole IgG antibodies to highlight this. Detergent-free labeling thus allows processing for ultrastructural analysis

by EM, with excellent membrane preservation. Furthermore, the scFv labeling reagents can be easily labeled with different fluorescent dyes allowing the authors to visualize numerous (here they show 6) different labels in the same sample using spectral unmixing confocal microscopy. Serial section EM images of the same samples were then reconstructed and aligned with the fluorescence images to achieve correlated fluorescence/ultrastructure. Overall the data were compelling, with many beautiful examples of correlated fluorescence localization with 3D ultrastructure, nicely demonstrating the power of the technique. While the manuscript primarily focuses on tool development and offers little in the way of novel biological insight, I feel the potential future impact of the technique (i.e. ability to assign neural identities to volumetric connectomics EM datasets, ultrastructural localization of channels, receptors, etc.) will have broad appeal. There are several specific points that deserve attention:

-Nowhere in the manuscript do the authors validate their labeling reagents in a knockout background. In many cases the labeling pattern is distinct, consistent with previously published work and the localization of the signal makes sense with the correlated ultrastructure (i.e. vGLUT labels presynaptic terminals), but in some cases it is more ambiguous. For example, in Fig. S2a,e the authors argue that the CB and PV scFvs label more of the target proteins in the cell nucleus, is this real signal or are these probes picking up something non-specific in the nucleus that the IgG does not?

-While many of the images are quite striking, overall the manuscript lacked any sort of quantitative analysis. Just as one example, in Fig. 1, showing a simple pixel correlation scatter plot comparing the YFP and GFP-scFv signal would give readers a better idea of how evenly the scFv is penetrating cells to label YFP.

-p. 9 ".....immunofluorescence patterns that were similar to or in some cases stronger than their parental mAbs in Crus 1 of the cerebellar cortex (Figure 2 a; Sup. Figure 2; Sup. Figure 3). In many cases the comparisons between mAb and scFv is not entirely fair since the mAb is labeled in the 488/green channel (in which brain tissue notoriously has more autofluorescence) and the scFv in the red channel e.g. NPY signal in Sup. 2b,d; PSD95 in Sup. 2f. Is the labeling really that much cleaner or is the background signal in the green channel making the mAb appear worse than it is?

-p. 9 "......found that the anti-calbindin scFv penetrated to a depth of ~150 μm in a 300-μm tissue slice". So the probe labeled throughout the entire slice?

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We thank the reviewer for pointing out the important fact that saponin is a far better detergent for electron microscopy ultrastructural studies than Triton X-100. In response to this suggestion, we did a new series of experiments analyzing twenty-two tissue blocks at various saponin concentrations, sample thicknesses, and durations of antibody incubation. Moreover, directly conjugated mAbs have become available, so we moved the results related to the penetration tests with secondary antibody labeling (original Figure 1 d and e) to Supplementary Figure 3 b and c). The new results are now presented in new Figure 1 panels f-i, new Supplementary Figure 4 (plus additional new Supplementary Figure 5 that we will describe below in point 4). The key result is that saponin at 0.05% concentration does not allow fluorescently labeled monoclonal antibodies to penetrate into the middle 500 µm of a 1-mm block even after a 1-week incubation with the labeled antibody (Figure 1 g; Supplementary Figure 4). In contrast, the fluorescently labeled scFv penetrated throughout a block in the absence of detergent (Figure 1 f; Supplementary Figure 4). In different experiments, we did examine if higher concentrations of saponin could aid in deeper penetration (see new Supplementary Figure 5) but for our purposes, even saponin at 0.05% was problematic. The reason was that we found small breaks in the plasma membranes of neuronal processes (arrows, Fig 1 i) that were not present in samples not treated with detergent (Fig 1 h). While these ultrastructural breaks are small and, for many kinds of studies, would be of no consequence, for connectomics they are serious. This seriousness is related to the requirement for automatic algorithms to segment each nerve cell process. When two adjacent objects have a

continuity between them, this is often interpreted by the algorithms erroneously as the same object. Such merge errors are far more difficult to find and correct than split errors, so avoiding them at all costs is necessary (Shapson-Coe et al. 2021; Januszewski et al. 2018). With newer techniques like multicolor 2-photon microscopy (Mahou et al. 2012; Blanc et al. 2023; Pudavar et al. 2024), lightsheet microscopy in uncleared tissue (Schmid et al. 2013), and confocal done with clearing approaches compatible with electron microscopy (Furuta et al. 2022), we think having fluorescent scFv penetration hundreds of microns into tissue blocks will be of great use in CLEM studies. We have modified the text to make these points clearer (line 150).

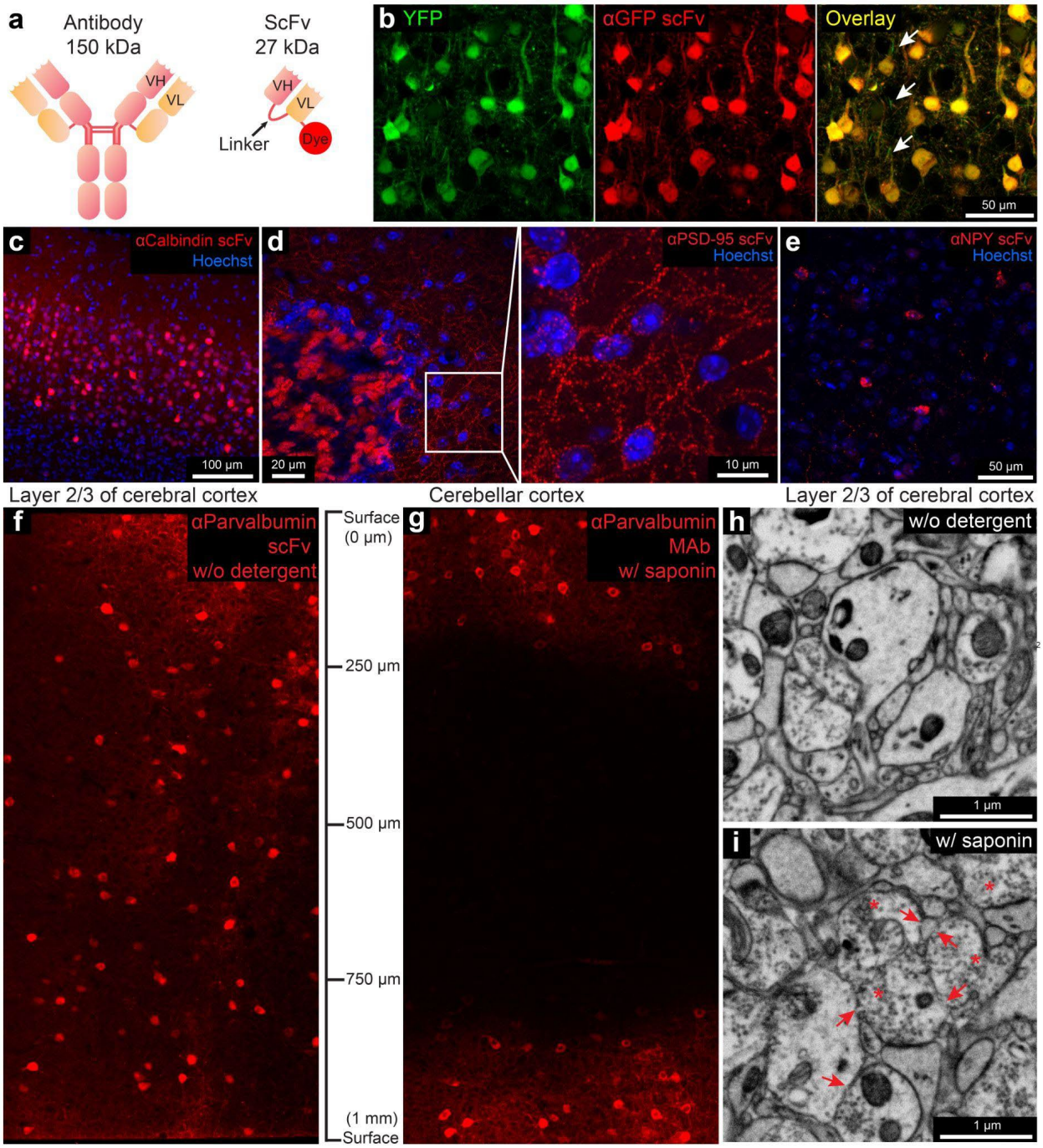
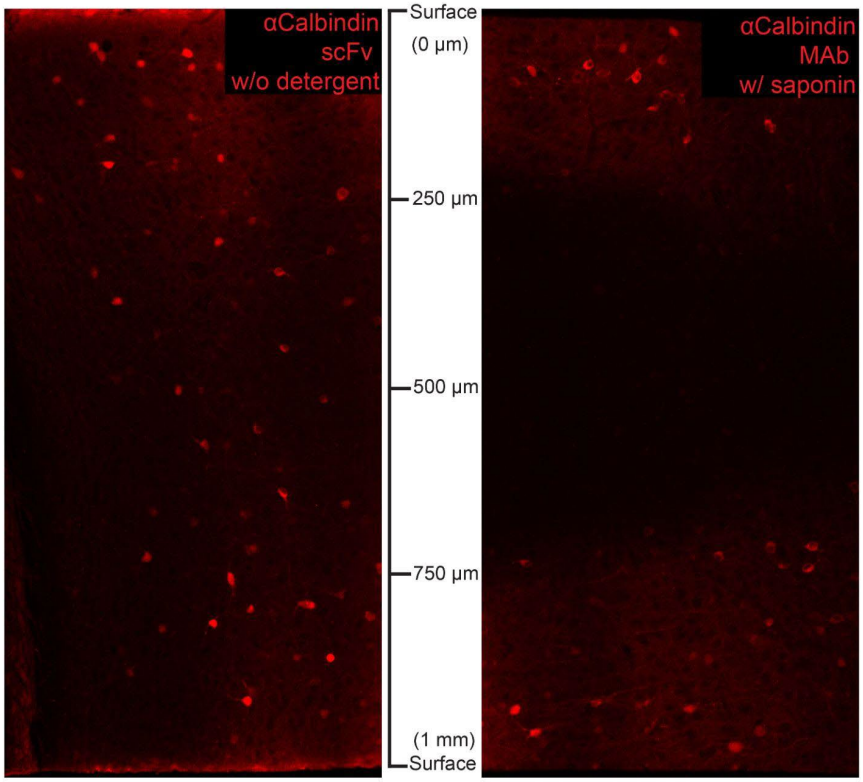


Figure 1. Fluorescent scFv probes label brain tissues without detergents to preserve electron microscopy ultrastructure.

a, Schematic representations of a full-length IgG antibody and an scFv probe with a conjugated fluorescent dye. **b**, Confocal images from the cerebral cortex of a YFP-H mouse labeled using a GFP-specific scFv probe conjugated with the red dye 5-TAMRA. Arrows show thinner neuronal processes, perhaps myelinated, that are not labeled by scFv. **c**, Layer ⅔ of the cerebral cortex labeled with a calbindin-specific scFv probe. **d**, Cerebllum cortex of Crus 1 labeled with the PSD-95 specific scFv. Right panel is the enlarged boxed inset from left. **e**, Cerebral cortex labeled with the NPY-specific scFv. **f** and **g**, Tissue penetration depth comparison of a parvalbumin-specific scFv without detergent and its parental

mAbs directly conjugated with fluorophores with 0.05% saponin on 1-mm cerebral cortex tissue sections with a 7-day incubation. **h** and **i**, Comparison of ultrastructure from samples incubated 7 days without detergent and with 0.05% saponin. Arrows indicate membrane breaks. Asterisks indicate abnormal appearing vesicle-filled axonal terminals.



Sup. Figure 4. Penetration of anti-calbidin scFv into 1-mm tissue sample.

Tissue penetration depth comparison of a calbindin-specific scFv without detergent and its parental mAbs directly conjugated with fluorophores with 0.05% saponin on 1-mm cerebral cortex tissue sections with a 7-day incubation.

2. Another point of concern is the lack of clarity on how the antibodies penetrate the cell through the cell membrane in detergent-free immunofluorescence labeling. A more thorough investigation and explanation of this process are needed to provide a comprehensive understanding of the technique's efficacy.

We agree with the reviewer on the importance of investigating how scFvs penetrate the cell membrane in the absence of detergent. We are interested in determining the mechanism as well. We think there are at least two possible mechanisms:

First, because all the immunolabeling experiments in this study were performed on brain tissue samples from animals perfused and fixed with 4% formaldehyde (prepared fresh from paraformaldehyde) + 0.1% glutaraldehyde in PBS, the cell membrane penetration by scFvs may be simply explained by the fact that formaldehyde and glutaraldehyde permeate the lipid bilayer. formaldehyde and glutaraldehyde are commonly used as chemical fixatives by crosslinking amino groups of proteins (Fischer et al. 2008). It has been known that formaldehyde also

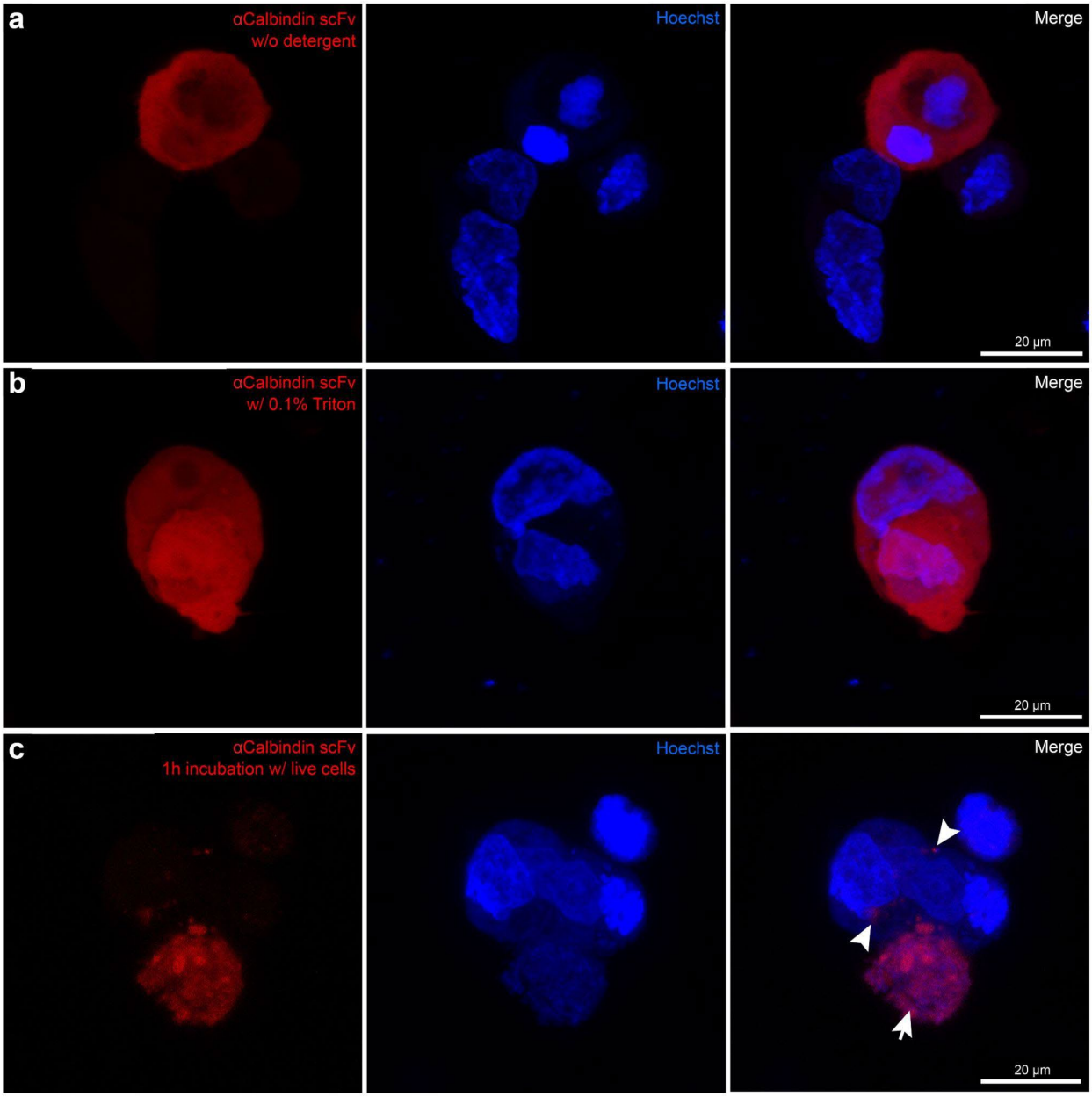
dissolves lipids (Fox et al. 1985; Kiernan 2000; Thavarajah et al. 2012). A recent study using surface plasmon resonance (SPR) (Cheng et al. 2019) showed that fixation with formaldehyde perturbed the integrity of membranes (10 ± 5% mass loss), and they showed increased permeability of sucrose. In another recent study using atomic force microscopy (Ichikawa et al. 2022), both formaldehyde and glutaraldehyde were shown to increase the size of nanoscopic protrusions on cell membranes. These protrusions were generated by membrane protein aggregates induced by crosslinking via formaldehyde or glutaraldehyde. The aggregated membrane proteins may create gaps between them and their nearby lipids providing a permeability pore. Additionally, two extracellular space-preserving fixation methods employing formaldehyde and glutaraldehyde (Fulton and Briggman 2021; Lu et al. 2023) showed that full-length antibodies can penetrate cell membranes albeit with lower diffusivity than scFvs, supporting the idea that the formaldehyde plus glutaraldehyde treated membranes do have gaps caused by the fixation.

We have tested this idea as well by using scFv immunolabeling on HEK293T cells cultured as a single layer on a petri dish with a coverglass bottom. HEK293T cells allowed us to avoid the issue of cut/fragmented cells in tissue sections where scFv could penetrate into cells via a cut surface rather than through a membrane. After transfecting the HEK293T cells with a plasmid encoding calbindin, we fixed the cells with the same fixative (4% formaldehyde + 0.1% glutaraldehyde in PBS) for 15 min and then washed with PBS. Overnight immunolabeling of the anti-calbindin scFv was then performed without or with 0.1% Triton-X. The results showed that in both conditions (without or with 0.1% Triton-X), the scFv can penetrate and label its intracellular target (we have added a new Supplementary Figure 7 a, b to show this data). This result provides evidence consistent with the idea that the cell membranes fixed with 4% formaldehyde + 0.1% glutaraldehyde allow scFvs to penetrate into intracellular spaces. Additionally, we also tested a 1-hour immunolabeling protocol using scFvs and full-size mAbs directed to transfected calbindin in COS-1 cells both without and with detergent permeabilization. Similarly, we found that the scFvs were able to penetrate COS-1 cells and label intracellular targets. However, the mAb was unable to penetrate at least at 1 hour (see new Supplementary Figure 8). In another experiment we did find that an overnight incubation with a mAb did label fixed cells that were not permeabilized with detergent. From all of these experiments we infer that due to their small size the scFvs are better to penetrate fixed cells than larger immunoprobes. We have modified the text to make these points clearer (line 169).

It is also possible that scFvs by virtue of their small size could permeate unfixed lipid bilayers. Indeed (Li et al. 2016) showed that anti-pTau nanobodies when injected into the blood of live mice could cross the blood-brain barrier and also cross neuronal cell membranes to label intracellular pTau. In (Bernard et al. 2016), after transgenically inducing expression of an anti-Otx2 scFv to express in cells of the choroid plexus cells, scFv in the CSF can cross the blood-brain barrier and neutralize Otx2 in the cortex, perhaps via transcytosis. In (Thiel et al. 2002), scFvs were shown to be able to pass through live cornea with an intact epithelium. (Im, Chung, and Jang 2017) showed that scFvs can enter live, unfixed culture cells.

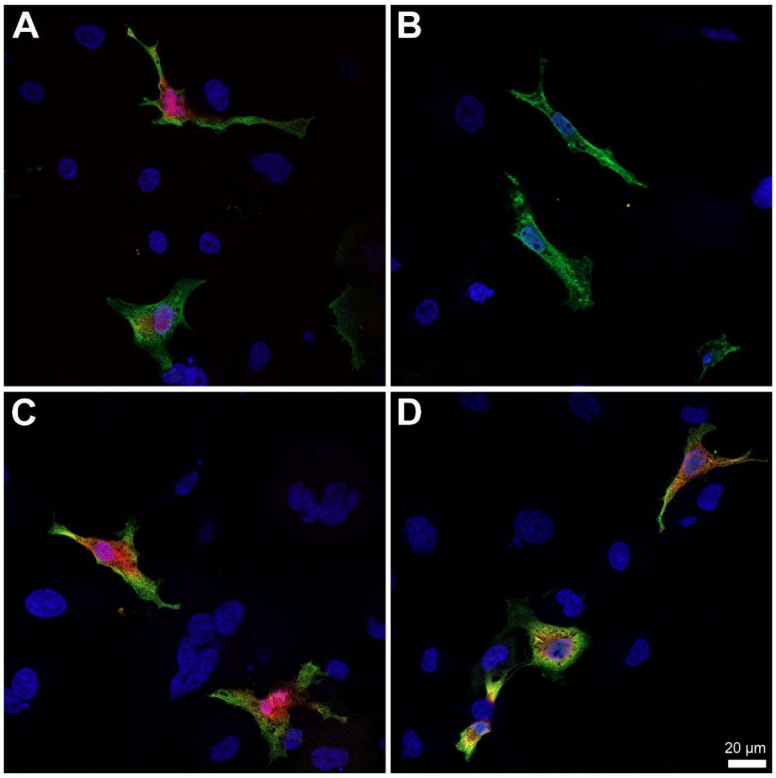
Based on these results, we were motivated to see if the scFvs we generated could cross into living cells. We attempted to immunolabel the transfected HEK293T cell for calbindin with the anti-calbindin scFv using live HEK293T cells. We found that after a one-hour incubation, the scFv could penetrate cells (Supplementary Figure 7 c, arrow). However, unlike the penetration of fixed cells described above, the labeling was more punctate. This labeling was most likely

explained by endocytosis as has been previously seen for extracellular dye molecules (see for example, (Tsuriel et al. 2015)). We have added a Supplementary Figure 7 c to show this result. Consistent with this, it has been documented that both nanobodies and scFvs can be internalized into cells via endocytosis (de Beer and Giepmans 2020; Wittrup et al. 2009; Alric et al. 2018; Kim et al. 2020). We have modified the text to make this point clearer (line 176). This is a potentially important route of entry because it provides an option to achieve immunolabeling of larger tissue samples, such as a whole mouse brain, by introducing these small immuno-probes in live animals.



Sup. Figure 7. Penetration of anti-calbidin scFv into fixed HEK cells or live cells.

Immunofluorescence immunocytochemistry on transiently transfected cells. HEK cells were transfected with a plasmid encoding Flag-tagged human calbindin. **a**, Chemically fixed cells were labeled overnight with Alexa594 anti-calbindin L109/57 scFv in the absence of detergent. **b**, Chemically fixed cells were labeled overnight with Alexa594 anti-calbindin L109/57 scFv with 0.1% Triton-X. **c**, Live cells were labeled with Alexa594 anti-calbindin L109/57 scFv. The arrow indicates the cell that has intracellular scFv labeling. Arrowheads indicate puncta labeling in some cells.



Sup. Figure 8. Penetration of anti-calbindin scFv into fixed COS-1 cells.

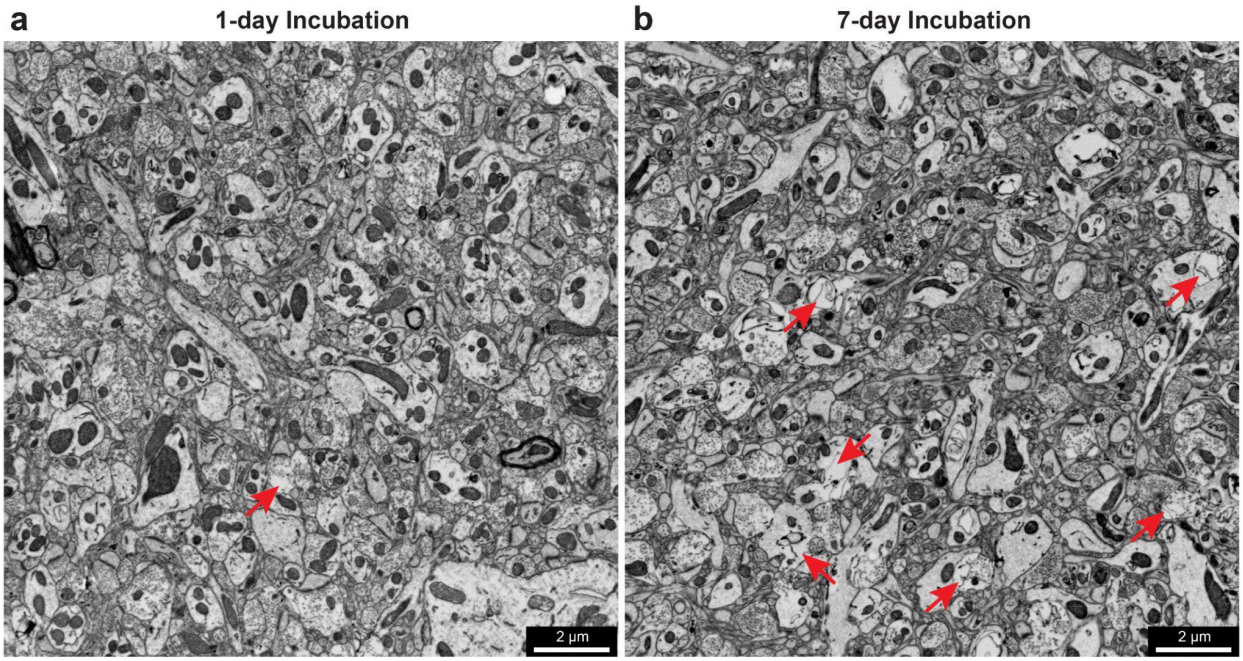
Immunofluorescence immunocytochemistry on transiently transfected cells. COS-1 cells were transfected with a plasmid encoding Flag-tagged human calbindin. Cells in panels A and B were labeled for 1 hour after fixation and prior to detergent permeabilization with (**A**) Alexa594 anti-calbindin L109/57 scFv or (**B**) anti-calbindin mouse mAb L109/39 (scFv and mAb labeling in red). After permeabilization, cells were labeled with rabbit anti-Flag (green) to detect calbindin, and Hoechst nuclear dye (blue). For cells in panels C and D all immunolabeling was performed after fixation and detergent permeabilization with (**C**) Alexa594 anti-calbindin L109/57 scFv or (**D**) anti-calbindin mouse mAb L109/39 (scFv and mAb labeling in red). Cells were simultaneously labeled with rabbit anti-Flag (green), and Hoechst (blue). Cells in all panels were imaged at the same exposure.

3. The animals were perfused with the fixative (4% paraformaldehyde + 0.1% glutaraldehyde). The low concentration of glutaraldehyde (0.1%) may not be sufficient to adequately preserve the lipids in the cell membrane. However, for detergent-free immunofluorescence labeling, scFv probes or nanobody probes were incubated for 3 days (50 µm) or 7 days (120 µm). This could potentially affect the ultrastructural morphology.

We agree this is a reasonable concern that the use of 0.1% glutaraldehyde does not sufficiently preserve lipids in the cell membrane, which may cause the ultrastructure to deteriorate when tissue samples are incubated with immuno-probes for prolonged periods like

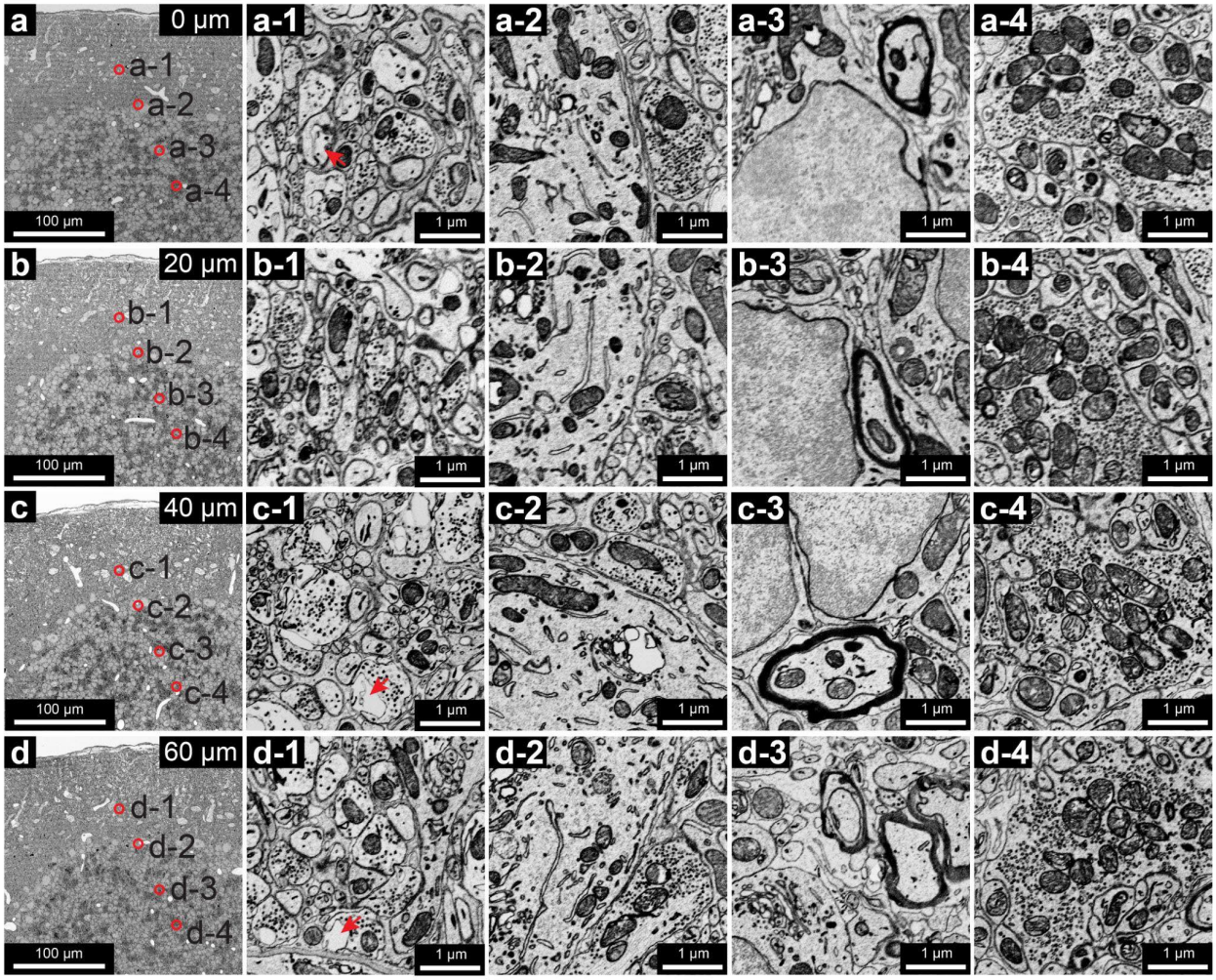
three or seven days. We were aware of this potential problem. The reasons we used 0.1% glutaraldehyde instead of a higher concentration was: first, glutaraldehyde is a harsher fixative which may modify epitopes on target proteins (Fischer et al. 2008), preventing immuno-probe labeling; Second, glutaraldehyde has higher autofluorescence than formaldehyde (Fischer et al. 2008), which causes high background in fluorescence microscopy. Because we only used 0.1% glutaraldehyde, we always postfixed the perfused brain for many hours (overnight). To prevent reversal of the formaldehyde fixation (Fischer et al. 2008) the brain samples were then sliced in ice-cold fixative (4% formaldehyde + 0.1% glutaraldehyde) and stored in the same fixative at 4 °C. The only exception to this protocol was our work with the neuropeptide NPY, which we, as others, have found to be difficult to label if the fixation is too extensive. In this case, we stored the slices in PBS at 4 °C. We also performed all the incubations, including the washing steps, at 4 °C to prevent ultrastructural degradation. In the manuscript, in Supplementary Figure 21, we examined the ultrastructure of a 2 mm, 2 mm, 120-µm thick cerebellum tissue sample incubated with scFv probes for seven days after light fixation (described above). As shown in the figure, ultrastructure at four locations across the cerebellar cortex layers including regions that are near the center of the block, is preserved well. After careful examination of the images during the revision process, we have noticed some abnormalities. In the superficial layer (the molecular layer) of the cerebellar cortex, which is mainly composed of neuronal processes and close to the surface of the block, we did observe some artifacts (new arrows in Supplementary Figure 21). We are unsure whether these artifacts are explained by mechanical or chemical or thermal factors that are different at the surface vs. the interior of the block. We have also modified the manuscript (line 217) to make readers aware of this issue. If reviewers are interested in examining the ultrastructure directly, we encourage reviewers to visit the Neuroglancer link of our vCLEM dataset at Neuroglancer LINK.

In addition, in a new set of experiments we performed for the revision that we will discuss in detail below in point 4, we showed that instead of 3-day or 7-day incubations, the anti-calbindin scFv can penetrate to the center of a 300-µm vibratome section with incubation of only one day. We found fewer tissue artifacts in the ultrastructure of 1-day incubated samples than the 7-day samples (see an example in new Supplementary Figure 6, arrows indicating artifacts). We have modified the text to make this point clearer (line 166). So, we conclude that, at least for some scFvs, 1-day incubations are sufficient.



Sup. Figure 6. Ultrastructure comparison between samples incubated for one day or seven days.

Ultrastructure of locations close to the surfaces of 300-µm cerebral cortex sections immunolabeled for one day (**a**) or seven days (**b**). Arrows indicate artifacts.



Sup. Figure 21. Well-preserved ultrasctructure from the surface (a) to the middle (d) of the 120-µm section.

Panel **1**-**4** in **a**-**d** show the ultrastructure at the locations labeled by the red circles in the right panels. Arrows indicate the artifacts potentially caused by prolonged incubation with scFvs for immunolabeling.

4. This study lack of novelty. The use of scFv has been well-established over the years, making it a solid foundation for the volume CLEM study. However, the choice of detergent-free immunofluorescence labeling raises questions about its suitability. Considering alternative approaches, such as utilizing low concentrations of saponin, might offer a more effective option for preserving membrane morphology during immuno-EM experiments.

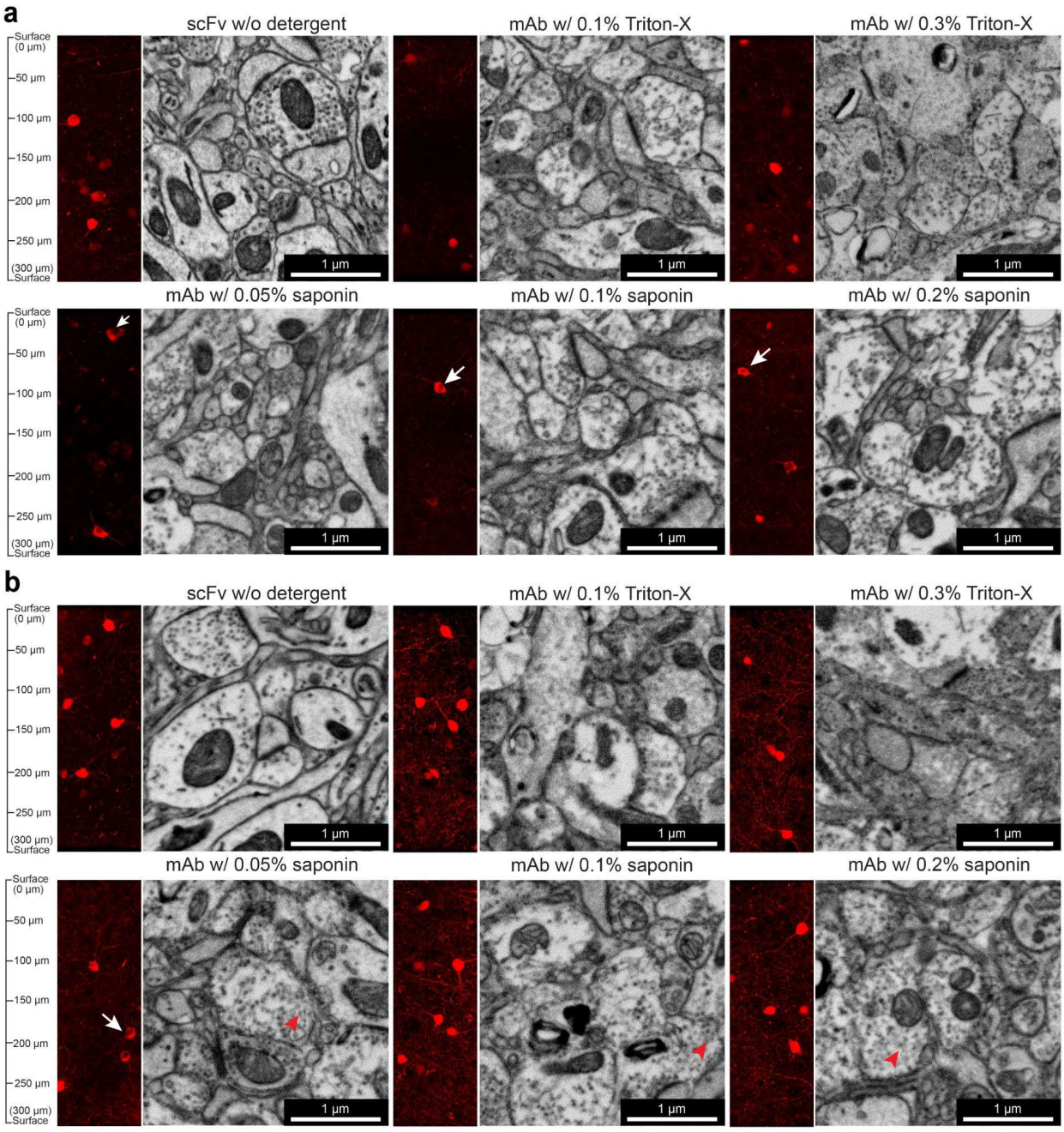
We agree with the reviewer that the use of scFvs is well-established (Bird et al. 1988; Huston et al. 1988; Monnier, Vigouroux, and Tassew 2013; Ahmad et al. 2012). The use of scFvs as immuno-probes for CLEM has been raised in a number of papers in discussion (de Beer and Giepmans 2020; Franek et al. 2024) but, to the best of our knowledge, this is the first actual demonstration of scFvs in volumetric CLEM.

We agree with the reviewer that when performing volumetric CLEM, alternative immunolabeling approaches other than those that employ scFvs should be considered, such as

fluorescently tagged primary IgG antibodies with saponin permeabilization. Therefore, in new experiments we compared detergent-free immunolabeling with scFv and Triton-X or saponin-enabled immunolabeling with a dye-directly conjugated monoclonal antibody (mAb) at various detergent concentrations (0.1% and 0.3% Triton-X; 0.05%, 0.1%, and 0.2% saponin) and with two different incubation times (1 day and 7 days). The experiments were performed on 300-µm cerebral cortex tissue blocks with an anti-calbindin L109/57 scFv and a dye-directly conjugated anti-calbindin L109/57 mAb. The epitope binding site of the mAb and the scFv were the same. As shown in new Supplementary Figure 5 a, after 1 day of incubation, only the scFv and the mAb with 0.3% Triton-X penetrated to the middle (i.e., 150 µm) of the section. The other experimental conditions showed various degrees of penetration: 0.1% Triton-X, ~50 µm; 0.05% saponin, ~30 µm; 0.1% saponin, ~80 µm; 0.2% saponin, ~100 µm. In all cases with saponin permeabilization, there was a lack of labeling in the cell nuclei (indicated by arrows). When we examined the ultrastructure of these labeled samples, the samples treated with detergent-free scFv labeling showed the best quality. The sample treated with 0.05% saponin showed good-quality EM ultrastructure. All the other samples showed compromised EM ultrastructure, the severity of which increased with the increase of detergent concentration. The membrane breaks in these samples would make automatic segmentation for connectomics challenging, as stated above in our answer to point 1. Although the sample treated with 0.05% saponin for one day showed no obvious ultrastructural artifacts, the mAb penetration was far shallower than the scFv (~30 µm vs. 150 µm) making volumetric CLEM on the samples larger than the penetration depth difficult.

As shown in new Supplementary Figure 5 b, after 7 days of incubation, scFvs without detergent and mAb with various concentrations of Triton-X or saponin can penetrate the middle of the 300-µm. However, we still observed in the case of 0.05% saponin a lack of labeling in the cell nuclei (indicated by arrows). Again, when examining the ultrastructure of these labeled samples, the sample treated with detergent-free scFv labeling showed the best quality and is similar to the one-day sample (which we have also mentioned in our answer to reviewer's point 3). All the other samples showed compromised EM ultrastructure, which was much worse when compared with the 1-day samples. Even the 0.05% saponin now showed membrane breaks. We also noticed after 7-day saponin incubation a new artifact: the vesicle-filled axonal profiles in samples treated with saponin for seven days showed a granular texture (indicated by arrowheads in Supplementary Figure 5 b). We think the protein-coagulating function of saponin, as the reviewer stated previously, may be the cause. These granules could pose challenges when synaptic vesicles need to be automatically detected and analyzed (as we did in the later part of this paper) for connectomic studies.

In addition, as we have mentioned in our answer to point 1, scFvs can penetrate 1-mm tissue blocks while saponin at 0.05% concentration only allows mAbs to penetrate into 250 µm after a seven-day incubation (Figure 1 g; new Supplementary Figure 4). These new results suggest that if a researcher wants to do a small-scale volumetric CLEM on a smaller tissue sample (such as several µm to 50-µm), directly dye-conjugated primary antibodies with a low concentration (0.05%) of saponin with a shorter incubation (one day) may be an option. However, should a researcher need to conduct large-scale volumetric CLEM on larger tissue samples (~1 mm in thickness), using scFvs for detergent-free immunolabeling is more advantageous. Large-scale volumetric CLEM is especially important for connectomics because a smaller volume is very likely to have fragmented cells/processes that prevent the mapping of the neural circuits. We have modified the text to make these points clearer (line 150; line 162).



Sup. Figure 5. Tissue penetration depth comparison of scFvs in the absence of detergent and fluorophore-conjugated mAbs with the treatments of various concentrations of detergents.

300-µm cerebral cortex sections were immunolabeled for one day (**a**) or seven days (**b**) with a calbindin-specific scFv conjugated with 5-TAMRA in the absence of detergent or with the scFv's parental mAb conjugated with FL550 in the presence of 0.1%, 0.3% Triton-X, or 0.05%, 0,1%, 0.2% saponin. Arrows indicate unlabeled cell nuclei. Arrowheads indicate granular textures associated with the treatment of saponin.

5. The choice of 0.3% Triton X-100 to demonstrate detergent issues in EM seems excessive. Most EM studies recommend not exceeding 0.1% Triton X-100, making it unnecessary to use a higher concentration for this purpose.

We agree with the reviewer that choosing 0.3% Triton X-100 is excessive to demonstrate the detergent's issue on EM ultrastructure. We have changed Figure 1 h and i so the comparison is with a sample treated with 0.05% saponin.

Reviewer #2 (Remarks to the Author):

This is a technology development manuscript describing the generation and implementation of an assortment of single chain antibody-based probes (scFvs) against different brain proteins to label tissue for correlative fluorescence/volumetric electron microscopy. The key advance is the nature of the labeling probes, which can diffuse deep into fixed brain tissue and penetrate cells without the need for detergent permeabilization. The authors do nice side-by-side comparisons with whole IgG antibodies to highlight this. Detergent-free labeling thus allows processing for ultrastructural analysis by EM, with excellent membrane preservation. Furthermore, the scFv labeling reagents can be easily labeled with different fluorescent dyes allowing the authors to visualize numerous (here they show 6) different labels in the same sample using spectral unmixing confocal microscopy. Serial section EM images of the same samples were then reconstructed and aligned with the fluorescence images to achieve correlated fluorescence/ultrastructure. Overall the data were compelling, with many beautiful examples of correlated fluorescence localization with 3D ultrastructure, nicely demonstrating the power of the technique. While the manuscript primarily focuses on tool development and offers little in the way of novel biological insight, I feel the potential future impact of the technique (i.e. ability to assign neural identities to volumetric connectomics EM datasets, ultrastructural localization of channels, receptors, etc.) will have broad appeal. There are several specific points that deserve attention:

-Nowhere in the manuscript do the authors validate their labeling reagents in a knockout background. In many cases the labeling pattern is distinct, consistent with previously published work and the localization of the signal makes sense with the correlated ultrastructure (i.e. vGLUT labels presynaptic terminals), but in some cases it is more ambiguous. For example, in Fig. S2a,e the authors argue that the CB and PV scFvs label more of the target proteins in the cell nucleus, is this real signal or are these probes picking up something non-specific in the nucleus that the IgG does not?

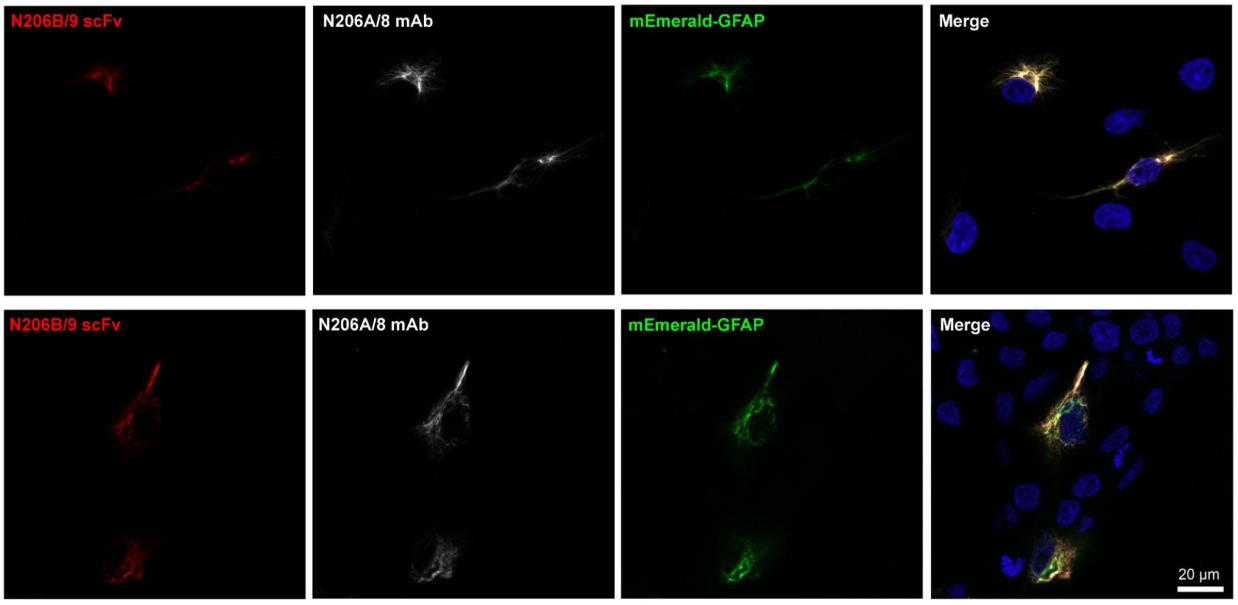
Concerning validation, we agree with the reviewer that the most crucial concern for immuno-probes or any similar affinity probes is whether they label or detect the actual target they are supposed to bind to. There are many cases when antibodies working in ELISA or Western blot settings fail to label their targets or have off-target labeling that creates abnormal background signals in immunohistochemistry (IHC).The parental (aka. progenitor) monoclonal antibodies (mAbs) from the UC Davis/NIH NeuroMab facility, whose sequences were used to generate the eight scFvs in this study, have undergone a strict validation process. In all but one case (the anti-NPY mAb), the mAbs have passed by at least three of the following: immunofluorescence on transfected COS-1 cells, Western blots on homogenized rat and mouse brains, IHC on rat and mouse brain sections, and IHC on mouse sections in a knockout background. These were accomplished in co-author James Trimmer's lab (for more details, see (Gong, Murray, and Trimmer 2016)). The validation tests of the eight mAbs used in the paper are now shown in new Supplementary Table 4). Although limited by the availability of KO brain samples, the three that we were able to test of them (N206Bb/9, GFAP R416WT; K28/43, PSD-95; K14/16, Kv 1.2) have passed the test of IHC on WT versus KO mouse brain sections, in that all detectable labeling observed in WT sections was eliminated in KO sections (all three also passed on WT/KO comparison by immunoblot) in a knockout background. While we want to test all the mAbs in a knockout background, but we hope the reviewer understands that it is

challenging to gather KO animals brain samples for all seven endogenous targets because some may be lethal mutations.

We also validated the scFvs in each case via IHC on rat and mouse brain sections and by immunofluorescence immunocytochemistry on transiently transfected COS-1 cells (also summarized in Supplementary Table 4; we also providde representative images in new Supplementary Figure 9, 10, 11 for the validation of the N206b/9, anti-GFAP R416WT scFv. Details of the methods of the validation tests for the other scFvs in this paper (and other scFvs) can be found in (Mitchell et al. 2023; Gong, Murray, and Trimmer 2016). We have modified the text to make these points clearer (line 139;line 143).

Sup. Table 4. Information on the validation of the scFvs and their parental mAbs.

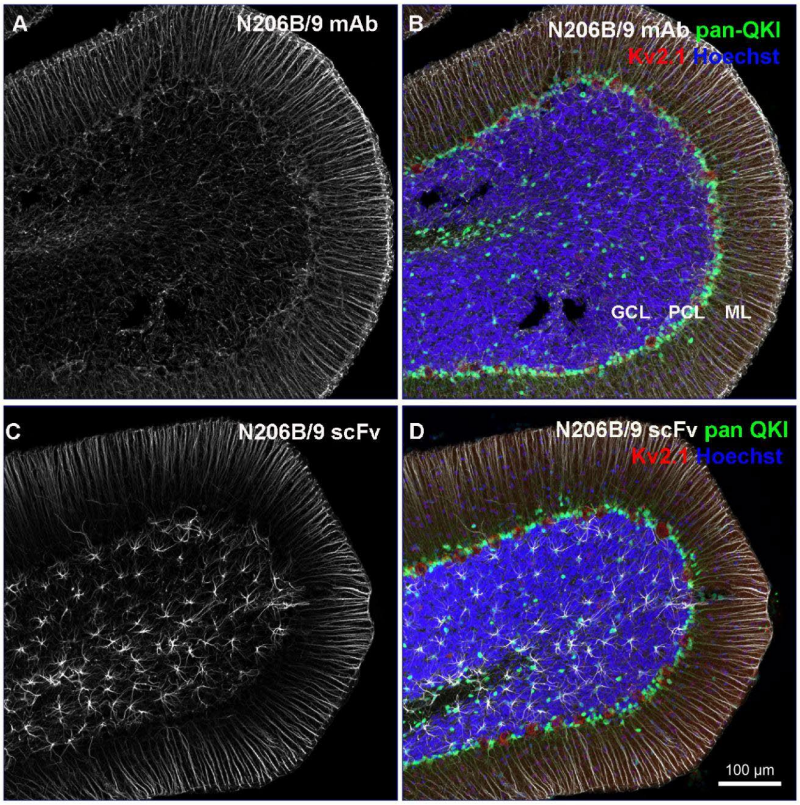
Target	Clone No.	mAb validation				scFv validation
		COS-IF	Brain IB	Brain IHC	KO Brain IHC	Method
GFP	N86/38	Pass	NA	NA	NA	COS-IF
Calbindin	L109/57	Pass	Pass	Pass	ND	Brain IHC and COS-IF
GFAP R416WT	N206B/9	Pass	Pass	Pass	Pass	Brain IHC and COS-IF
VGluT1	N28/9	Pass	Pass	Pass	ND	Brain IHC and COS-IF
PSD-95	K28/43	Pass	Pass	Pass	Pass	Brain IHC and COS-IF
Kv 1.2	K14/16	Pass	Pass	Pass	Pass	Brain IHC and COS-IF
Parvalbumin	L114/81	Pass	Pass	Pass	ND	Brain IHC and COS-IF
NPY	L115/13	Pass	Fail	Pass	ND	COS-IF



Sup. Figure 9. Validation of anti-GFAP scFv with Immunofluorescence immunocytochemistry.

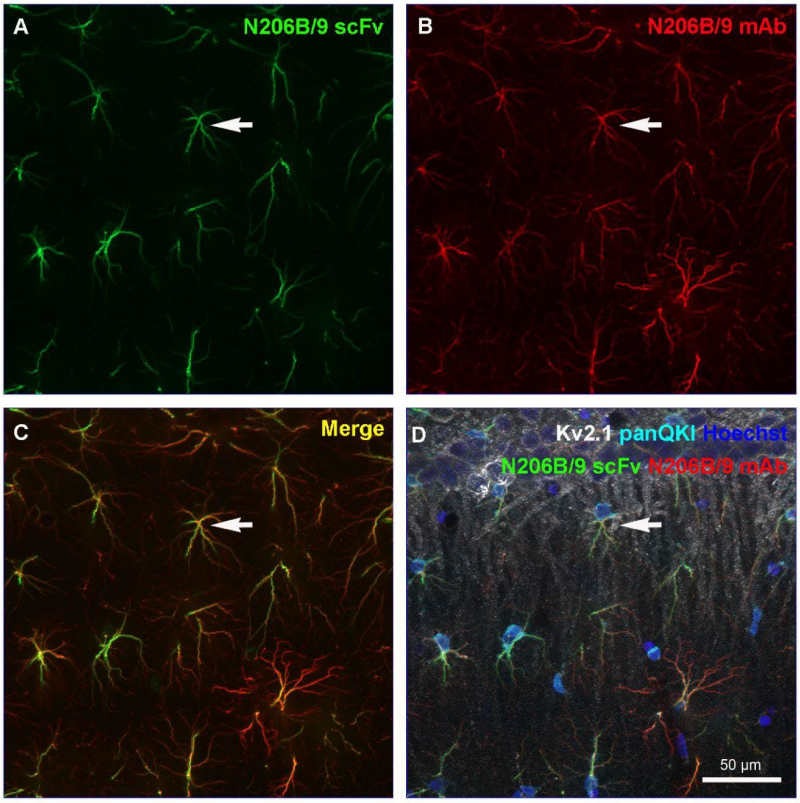
Immunofluorescence immunocytochemistry on transiently transfected cells. COS-1 cells (top row) and HEK293T cells (bottom row). Cells were transfected with a plasmid mEmerald-tagged human GFAP

(green) and double immunolabeled with the 5-TAMRA-labeled anti-GFAP N206B/9 scFv (red) and the anti-GFAP N206A/8 mouse IgG1 mAb (white). Hoechst nuclear labeling is shown in blue.



Sup. Figure 10. Validation of anti-GFAP scFv with Immunofluorescence immunohistochemistry (cerebellum).

GFAP scFv and original monoclonal antibody from which it was derived display the same tissue labeling pattern of a sagittal section through the rat cerebellum. **A**) Glial cells throughout the cerebellar granule cell layer (GCL) and prominent Bergmann glial process in molecular layer (ML) are labeled with hybridoma derived monoclonal antibody N206B/9. **B**) merged image includes labeling with a polyclonal rabbit antibody (KC) against the neuronal potassium channel Kv2.1, monoclonal antibody targeting glial specific RNA binding protein QKI (N147/6) and nuclear specific Hoechst labeling. **C**) An adjacent section labeled with N206B/9 derived scFv shows the same pattern of labeling. **D**) merged image with the same additional labeling as B.

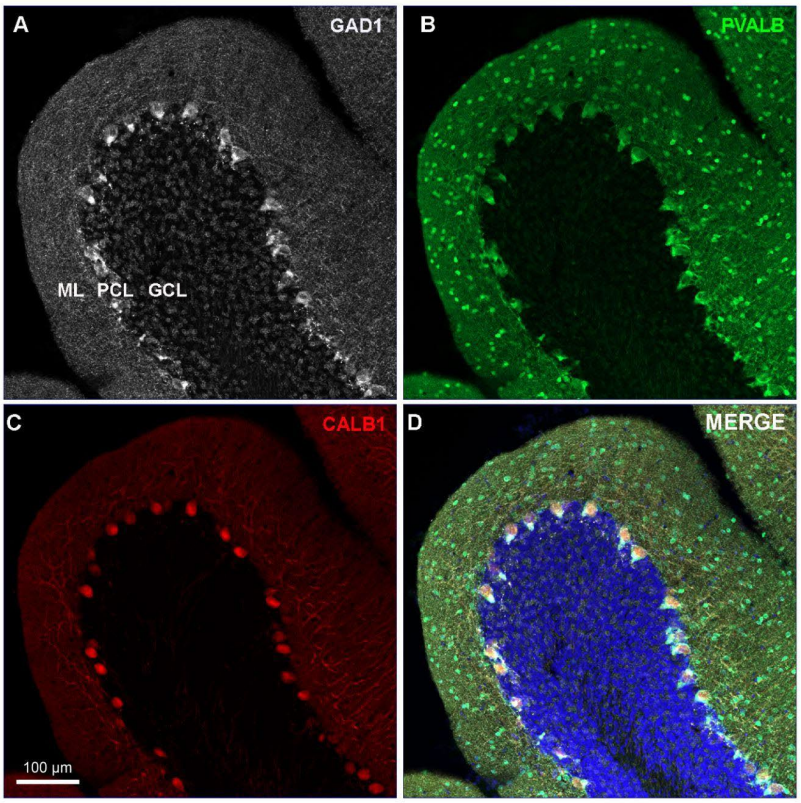


Sup. Figure 11. Validation of anti-GFAP scFv with Immunofluorescence immunohistochemistry (hippocampus).

Validation of scFv labeling pattern against hybridoma-generated monoclonal antibody N206B/9 from which it was derived. Multiplex immunofluorescent labeling of a sagittal section through rat hippocampal region CA1. **A**) 5-TAMRA conjugated N206B/9 derived scFv, **B**) Hybridoma derived monoclonal antibody N206B/9 indirectly labeled with Alexa fluor 647 conjugated goat anti-mouse IgG1 secondary antibody, **C**) merged images from A and B illustrating co-labeled astroglial cells (e.g arrowheads). **D**) Same multiplex image shown in C with additional neuronal specific potassium channel Kv2.1, glial specific pan-QKI RNA binding protein, and DNA marker Hoechst 33342 labeling.

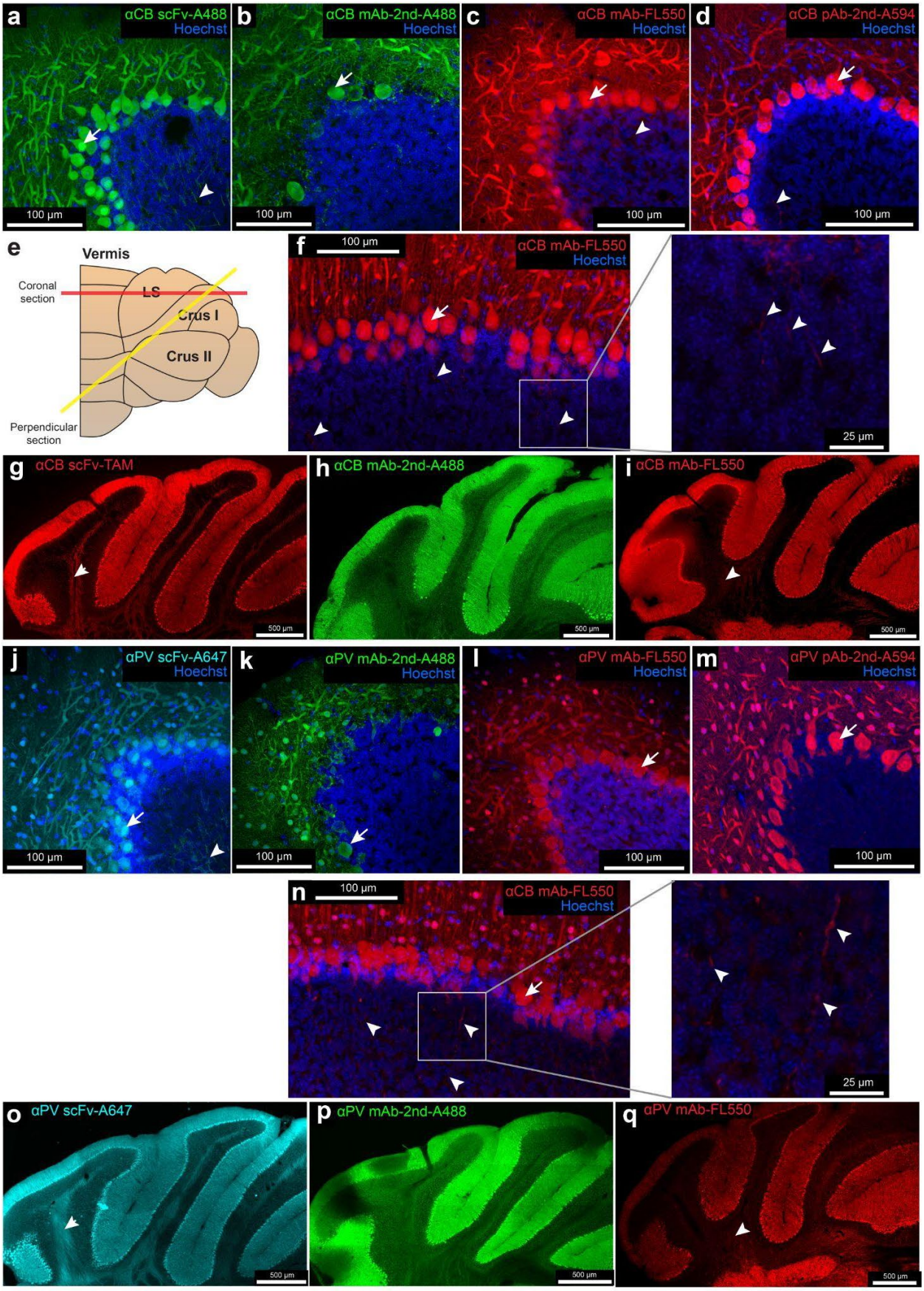
The second issue raised is concern over the ambiguous signals detected by the anti-calbindin and anti-parvalbumin scFvs in the cell nuclei of Purkinje cells. Clearly, in the original Supplementary Figure 2, the mAbs for calbindin and parvalbumin did not detect signals in the cell nuclei as the scFvs did. In the revised paper we now include the validation IHC images of the anti-calbindin and anti-parvalbumin mAbs from experiments done in James Trimmer's lab (new Supplementary Figure 14). These images showed the normal expected labeling, that included signal in some cell nuclei of Purkinje cells in the lateral hemisphere of the rat cerebellum. In the original manuscript, we mentioned previous studies reporting the detection of calbindin and parvalbumin in cell nuclei of Purkinje cells (Celio 1990; Brandenburg et al. 2021; German et al. 1997; Schmidt et al. 2007). We believe calbindin and parvalbumin are present in the cell nuclei. We therefore suspect that the reason our mAb labeling didn't detect signals in the cell nuclei is insufficient antibody incubation time.

To address this problem, in Triton-X-treated samples, we first did immunolabeling with the same anti-calbindin and anti-parvalbumin mAbs directly conjugated with the red fluorophore FL-550 in distinction to using secondary antibodies as we did previously. The directly conjugated mAbs exclude the requirement for secondary antibodies allowing for only one round of incubation. We extended the incubation time to seven days (versus 2 days of incubation with primary antibodies previously). The results showed that both mAbs can detect signals in the cell nuclei of most (>~90%) Purkinje cells (new Supplementary Figure 15 c, l). This staining is similar to the scFv labeling except that scFvs were detected in all Purkinje cells. The second approach we attempted was immunolabeling with commercial polyclonal antibodies (pAbs) against calbindin and parvalbumin. These antibodies are not directly conjugated with fluorophores, so we utilized $(Fab)_2$ as fluorescently tagged secondaries, which are smaller than conventional secondaries and supposedly can diffuse in tissue better. Our results showed that, again, both calbindin and parvalbumin could be detected by the pAbs in the cell nuclei in most but not all Purkinje cells (new Supplementary Figure 15 d and m). The third approach we used, was to slice the section in a different orientation to cut through the nuclei of most Purkinje cells in order to gain direct access to the nuclei in the vibratome section (see new Supplementary Figure 15 e). We immunolabeled with the same anti-calbindin or anti-parvalbumin mAbs directly conjugated with the fluorophore FL-550 with a seven-day incubation. This time, we observed labeling in nearly all Purkinje cells (Supplementary Figure 15 f and n). These results indicate that the signals detected in the cell nuclei by scFvs are real signals. The reason it is relatively harder for full-length antibodies (mAbs or pAb) to detect these signals can be attributed to their relatively weaker penetration ability, even in the presence of Triton-X. This can be addressed in ways like extending incubation time or slicing sections to expose the internal epitopes in cell nuclei better. We have modified the text to make these points clearer (line 148).



Sup. Figure 14. Validation of anti-calbindin and anti-parvalbumin scFvs with Immunofluorescence immunohistochemistry.

Labeling pattern of original mAbs used to generate scFvs against Parvalbumin and Calbindin in rat cerebellum. Sagittal section through cerebellum labeled with monoclonal antibodies L127/8 (**A**, GAD1), L114/8 R (**B**, PARV), and L109/57 (**C**, CALB1). The merged image (**D**) shows the colocalized pattern of labeling within Purkinje cell layer (PCL). ML, molecular layer, GCL, granule cell layer.


a
αCB scFv-A488
Hoechst
100 µm
b
αCB mAb-2nd-A488
Hoechst
100 µm
c
αCB mAb-FL550
Hoechst
100 µm
d
αCB pAb-2nd-A594
Hoechst
100 µm
e
Vermis
Coronal section
LS
Crus I
Crus II
Perpendicular section
f
100 µm
αCB mAb-FL550
Hoechst
25 µm
g
αCB scFv-TAM
500 µm
h
αCB mAb-2nd-A488
500 µm
i
αCB mAb-FL550
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j
αPV scFv-A647
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m
αPV pAb-2nd-A594
Hoechst
100 µm
n
100 µm
αCB mAb-FL550
Hoechst
25 µm
o
αPV scFv-A647
500 µm
p
αPV mAb-2nd-A488
500 µm
q
αPV mAb-FL550
500 µm

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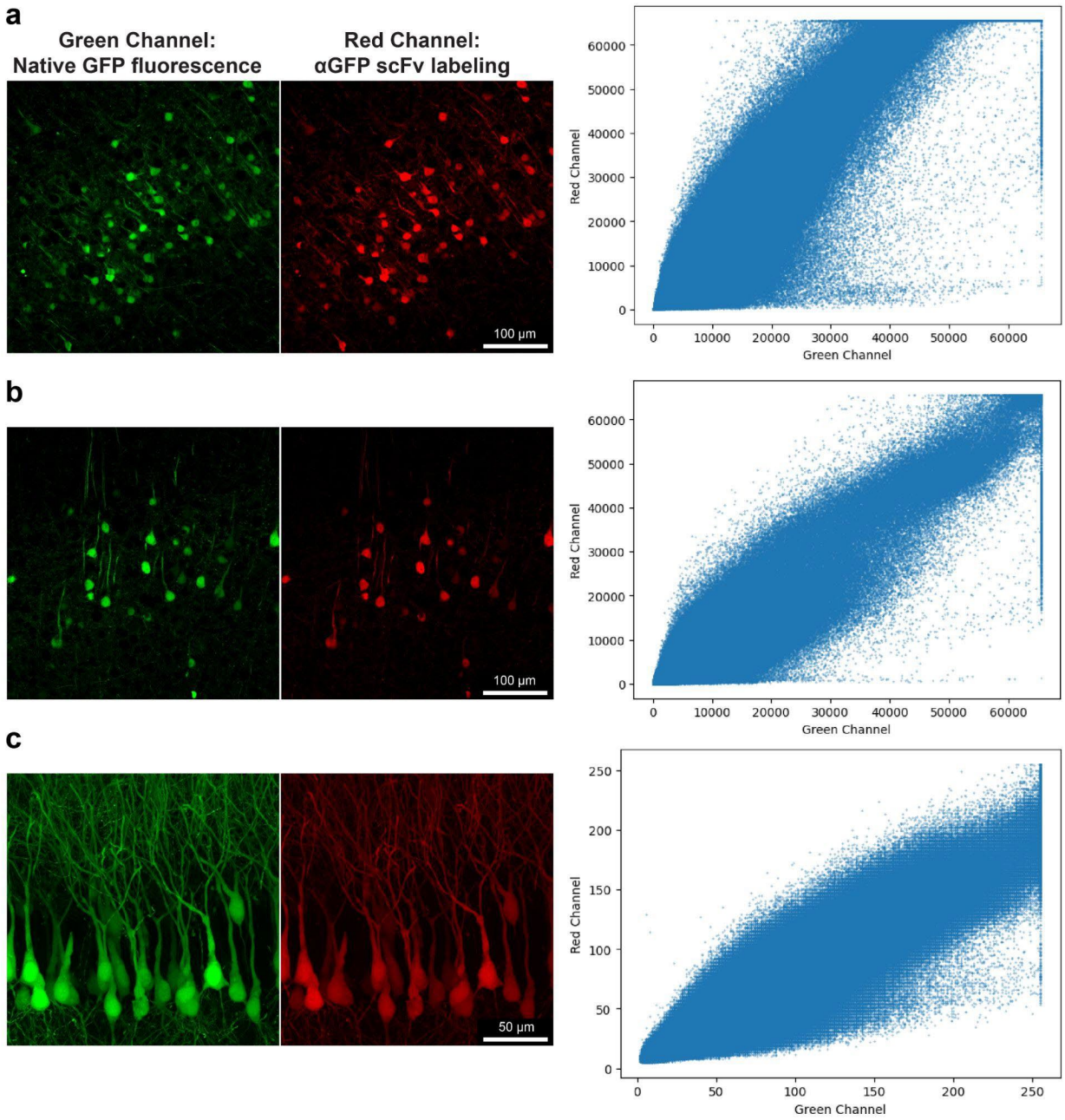
Sup. Figure 15. Validation of immunofluorescence by scFv probes and their parental mAbs (part 3).

Cerebellum Crus 1 sections were immunolabeled with a calbindin-specific scFv (**a**), or its parental mAb and secondary antibody conjugated with Alexa Fluor 488 (**b**), the mAb conjugated with FL550 (**c**), or a commercial calbindin-specific pAb and secondary $(Fab)_2$ conjugated with Alexa Fluor 594 (**d**). **e**, Schematics showing the cutting orientation that is parallel to the lobule of Crus 1, which intersects perpendicular to the planer Purkinje cells in Crus 1. **f**, Sections cut in this orientation immunolabeled with the mAb conjugated with FL550. The boxed inset is shown enlarged in the adjacent panel. Whole-section images of cerebellum Crus 1 sections immunolabeled with a calbindin-specific scFv (**g**), or its parental mAb and secondary antibody conjugated with Alexa Fluor 488 (**h**), or the mAb conjugated with FL550 (**i**). Arrows indicate labeled cell nuclei of Purkinje cells. Arrowheads indicate the labeled axons.

Cerebellum Crus 1 sections were immunolabeled with a parvalbumin-specific scFv (**j**), or its parental mAb and secondary antibody conjugated with Alexa Fluor 488 (**k**), the mAb conjugated with FL550 (**l**), or a commercial parvalbumin-specific pAb and secondary $(Fab)_2$ conjugated with Alexa Fluor 594 (**m**). **f**, Sections cut in this orientation in **e** immunolabeled with the mAb conjugated with FL550. The boxed inset is shown enlarged in the adjacent panel. Whole-section images of cerebellum Crus 1 sections immunolabeled with a parvalbumin-specific scFv (**o**), or its parental mAb and secondary antibody conjugated with Alexa Fluor 488 (**p**), or the mAb conjugated with FL550 (**q**). Arrows indicate labeled cell nuclei of Purkinje cells. Arrowheads indicate the labeled axons.

-*While many of the images are quite striking, overall the manuscript lacked any sort of quantitative analysis. Just as one example, in Fig. 1, showing a simple pixel correlation scatter plot comparing the YFP and GFP-scFv signal would give readers a better idea of how evenly the scFv is penetrating cells to label YFP.*

We thank the reviewer for highlighting the lack of quantitative analysis in comparing the specificity of scFvs and mAbs. The paper does contain other quantitative analyses (see Figure 7; Supplementary Figure 29; Supplementary Table 5, 6) but in response to the specific question raised, we have now created pixel correlation scatter plots for three images from two cortical and one hippocampal section from YFP-H mice, which were also immunolabeled with the anti-GFP scFv (new Supplementary Figure 2). Supplementary Figure 2 a is the raw image of Figure 1 b. As is shown in all three pixel correlating scatter plots, the signals from the scFv labeling (red) correlate with the native YFP fluorescence signal (green). There are pixels that only have values in the green channels, which correspond to the insufficiently labeled axons pointed out in Figure 2 a. There are very few pixels that only have values in the red (scFv) channel, which indicates that there is minimal off-target labeling of this anti-GFP scFv. This analysis gives us confidence in the specify of the scFv for green fluorescent protein. Doing this kind of double labeling is more problematic when comparing scFvs to mAbs that have the identical paratope as they compete for the same site. So, in these cases, as described in detail above, we had to be content with the comparative labeling of different tissue sections. We have modified the text to make this point clearer (line 128).



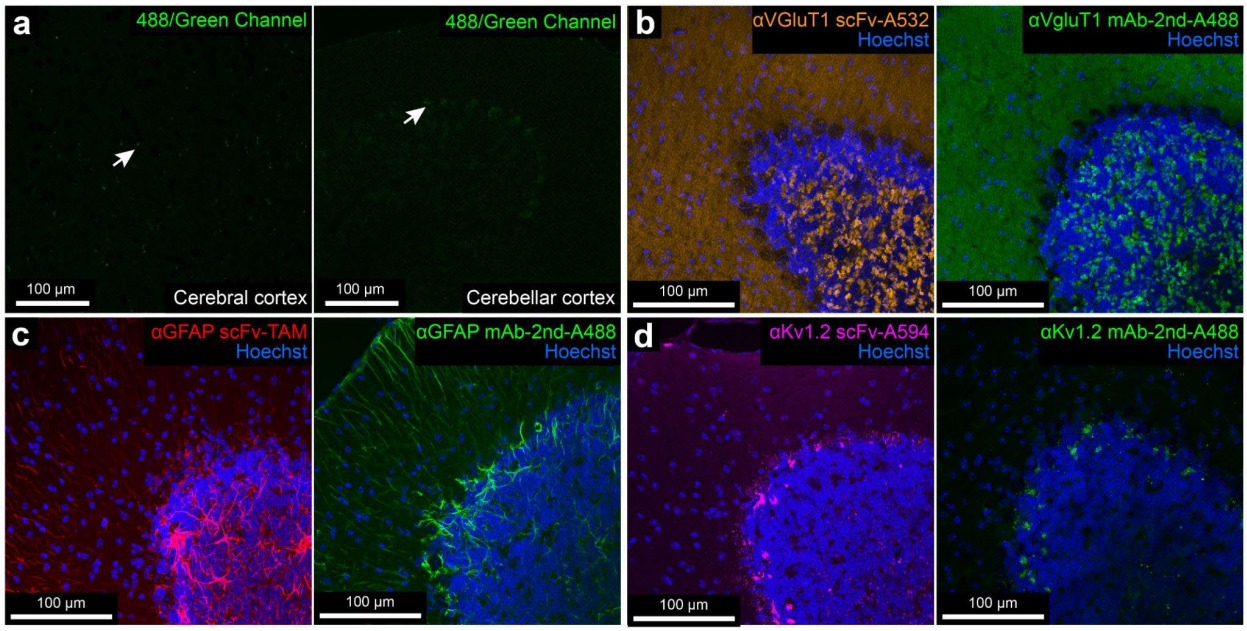
Sup. Figure 2. Pixel correlation scatter plots comparing the native YFP fluorescence signal and the red fluorescence from the labeling of the GFP- specific scFv.

Cerebral cortex samples (**a** and **b**) and hippocampus (**c**) from YFP-H mice were immunolabeled with a GFP-specific scFv conjugated with the red fluorophore 5-TAMRA. The images are raw data without any brightness/contrast adjustment. **a** is the raw image data of Figure 1 b.

-p. 9 ".....immunofluorescence patterns that were similar to or in some cases stronger than their parental mAbs in Crus 1 of the cerebellar cortex (Figure 2 a; Sup. Figure 2; Sup. Figure 3). In many cases the comparisons between mAb and scFv is not entirely fair since the mAb is labeled in the 488/green channel (in which brain tissue notoriously has more autofluorescence) and the scFv in the red channel e.g. NPY signal in Sup. 2b,d; PSD95 in Sup. 2f. Is the labeling

really that much cleaner or is the background signal in the green channel making the mAb appear worse than it is?

We apologize that the phrasing in our original manuscript may have caused a misunderstanding. In this sentence, "in some cases stronger than their parental mAbs in Crus 1 of the cerebellar cortex." only refers to the cases of calbindin and parvalbumin, as discussed in the previous point. In the other cases (GFAP, VGluT1, Kv 1.2, PSD-95, and NPY), we believe our results suggest that the labeling of scFvs and mAbs are comparably good in terms of both signal and background. We understand the legitimate concern that the tissue sections fixed with formaldehyde and glutaraldehyde may have a higher background in the 488/green channel. Glutaraldehyde especially has stronger autofluorescence (Fischer et al. 2008). However, we only used 0.1% glutaraldehyde in our preparation prior to osmium staining. After adequate washing with PBS, the brain tissue sections do not show strong autofluorescence in the 488/green channel (as now shown in new Supplementary Figure 12 a). There is some autofluorescence, mostly from lipofuscin granules in cell bodies (arrows in new Supplementary Figure 12 a) with broad excitation/emission spectra (Di Guardo 2015; Marmorstein et al. 2002). But this autofluorescence is found in all channels. We emphasize that we are not attempting to make a case that scFv labeling is cleaner than that obtained with mAb, as the results from both are very similar. Indeed we also performed new experiments with red fluorophore-conjugated mAbs for calbindin and parvalbumin, as discussed in our answer to the reviewer's point 1, and didn't find any difference in the background level (new Supplementary Figure 15). We have adjusted the phrasing in the manuscript (line 145) to avoid any further misunderstanding.



Sup. Figure 12. Validation of immunofluorescence by scFv probes and their parental mAbs (part 1).

a, Confocal images from unlabeled the cerebral cortex and cerebellar cortex of a wild-type mouse showed limited background in the 488/green channel. Arrows indicate background signals lipofuscin granule. **b-d**, Cerebellum Crus 1 sections were immunolabeled with scFvs targeting VGluT1, GFAP, and

Kv 1.2; or these scFvs' parental mAbs and secondary antibodies conjugated with Alexa Fluor 488.

-p. 9 ".....found that the anti-calbindin scFv penetrated to a depth of ~150 µm in a 300-µm tissue slice". So the probe labeled throughout the entire slice?

We apologize for the lack of clarity, yes, we meant that they labeled through the entire 300 µm slice (150 µm from each side). Given the recent availability of directly conjugated mAbs we have removed Figure 1 d and e to Supplementary Figure 3 b (we remade figures from raw images showing the penetration across the 300 µm thickness) and c, and added new Figure 1 f-i, Supplementary Figure 4 and 5 of the results of a comparable experiment of scFv labeling on 300-µm and 1-mm thick brain tissue sections in the absence of detergent. In Figure 1 and Supplementary Figure 4, scFvs are shown to label throughout a 1-mm thickness with a seven-day incubation; in Supplementary Figure 5, scFvs can label a 300-µm thickness sample with either a 1- or 3-day incubation. EM ultrastructure of all these samples was good. We have modified the text to make these points clearer (line 150).

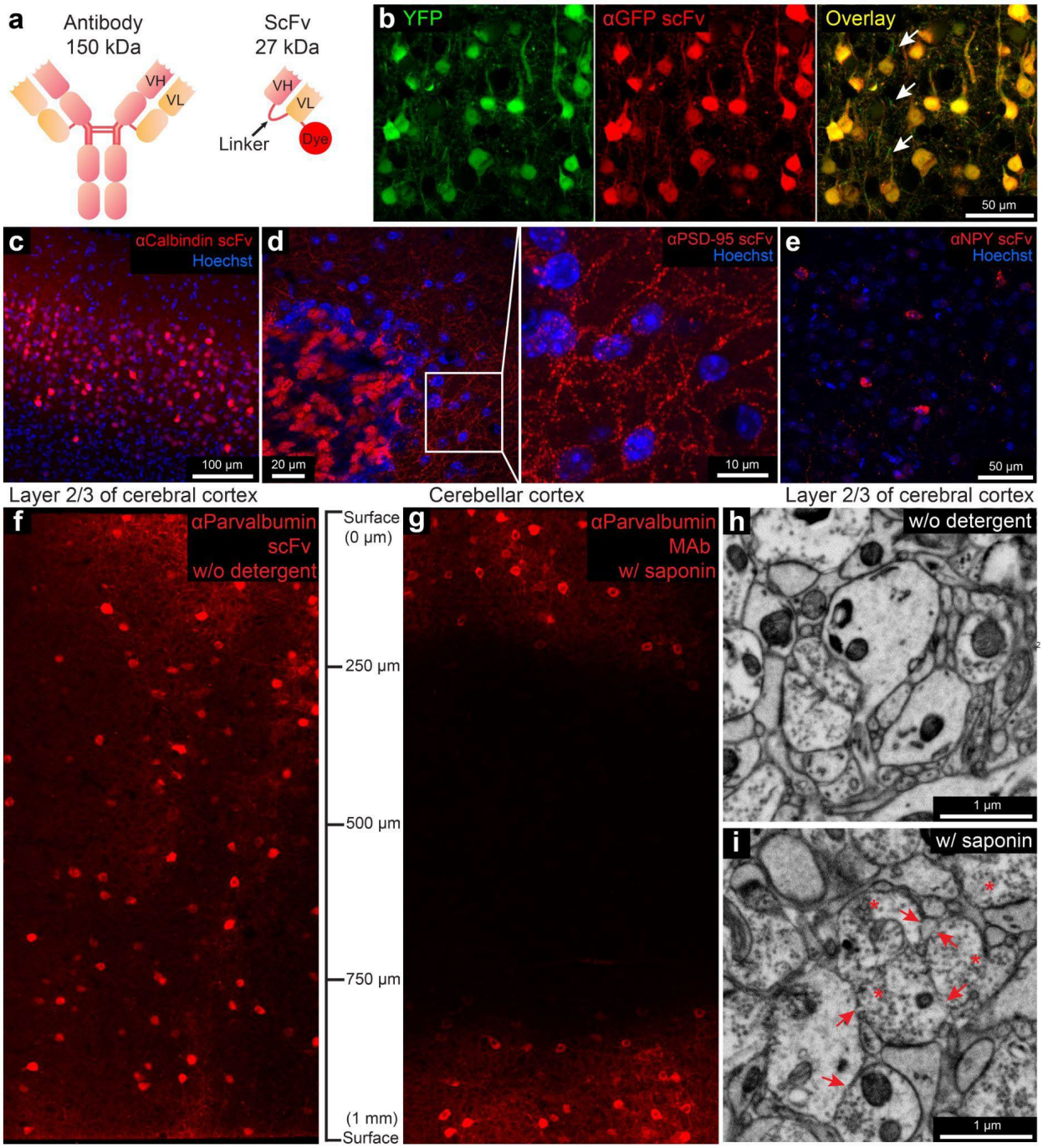
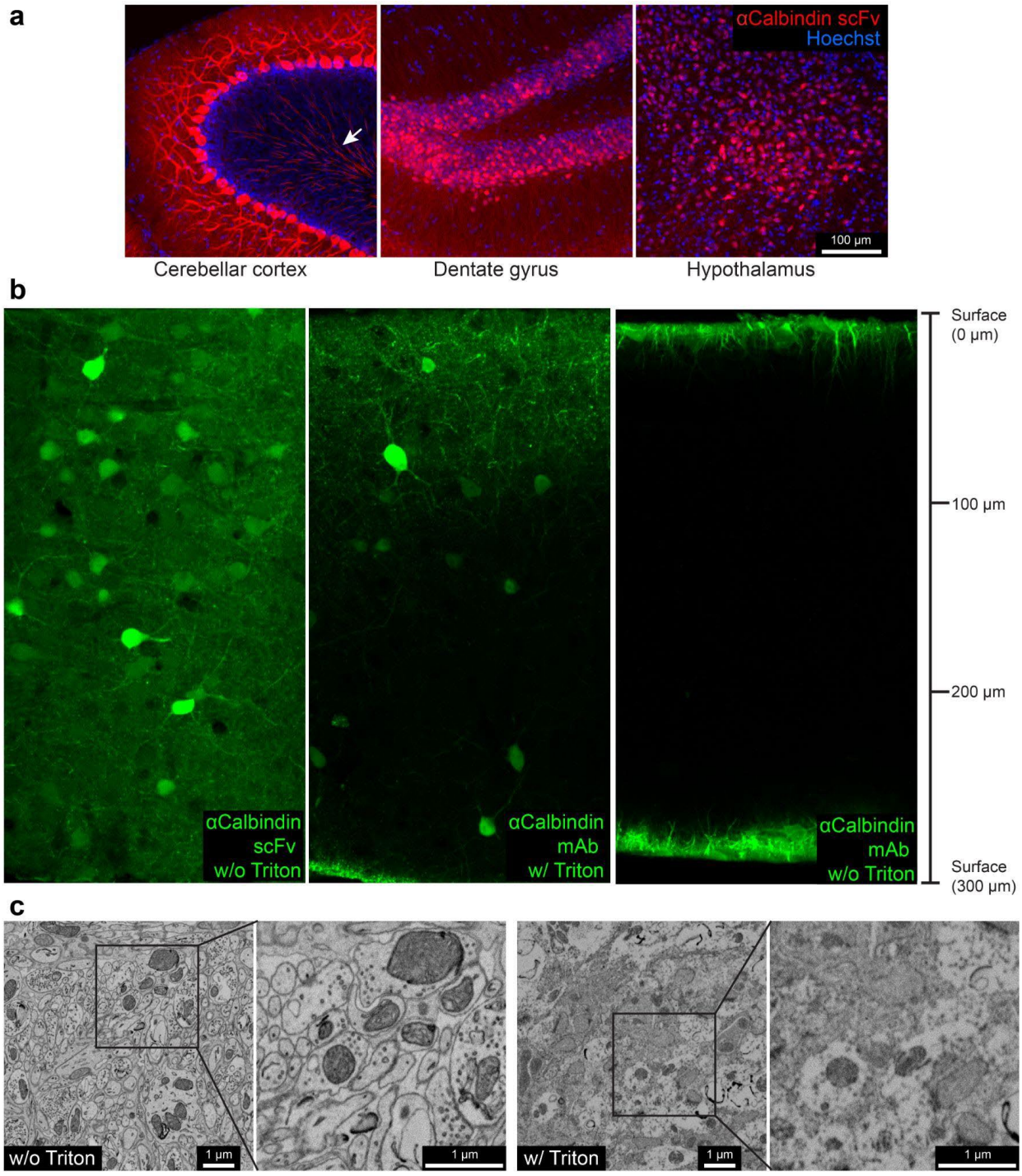


Figure 1. Fluorescent scFv probes label brain tissues without detergents to preserve electron microscopy ultrastructure.

a, Schematic representations of a full-length IgG antibody and an scFv probe with a conjugated fluorescent dye. **b**, Confocal images from the cerebral cortex of a YFP-H mouse labeled using a GFP-specific scFv probe conjugated with the red dye 5-TAMRA. Arrows show thinner neuronal processes, perhaps myelinated, that are not labeled by scFv. **c**, Layer ⅔ of the cerebral cortex labeled with a calbindin-specific scFv probe. **d**, Cerebllum cortex of Crus 1 labeled with the PSD-95 specific scFv. Right panel is the enlarged boxed inset from left. **e**, Cerebral cortex labeled with the NPY-specific scFv. **f** and **g**, Tissue penetration depth comparison of a parvalbumin-specific scFv without detergent and its parental

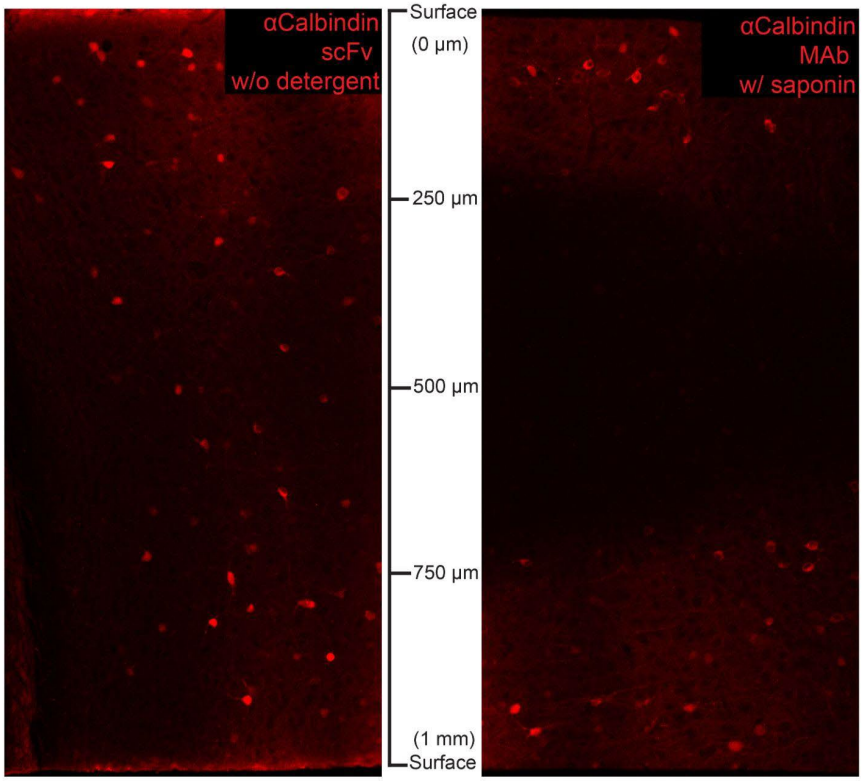
mAbs directly conjugated with fluorophores with 0.05% saponin on 1-mm cerebral cortex tissue sections with a 7-day incubation. **h** and **i**, Comparison of ultrastructure from samples incubated 7 days without detergent and with 0.05% saponin. Arrows indicate membrane breaks. Asterisks indicate abnormal appearing vesicle-filled axonal terminals.



Sup. Figure 3. Immunolabeling results of anti-calbidin scFv and tissue penetration comparison.

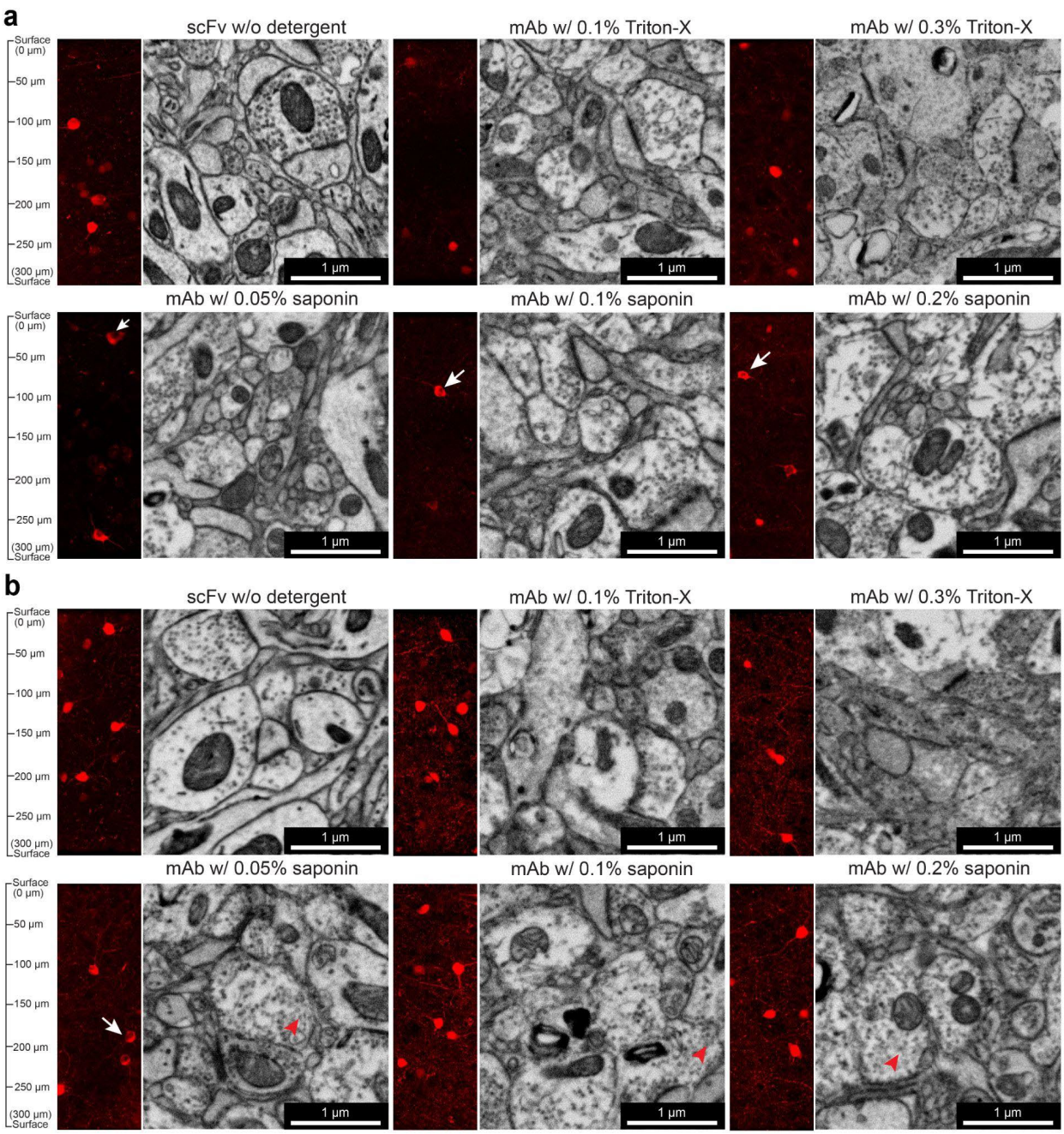
a, Additional brain regions labeled with a calbindin-specific scFv probe conjugated with 5-TAMRA. The arrow in the left panel shows myelinated Purkinje cell axons in the granule layer. **b**, Tissue penetration

depth comparison of scFvs, mAbs (plus secondary antibodies), and the role of detergents in improving the depth of labeling. **c**, Comparison of ultrastructure with and without 0.5% Triton X-100 on scFv labeled samples. Boxed insets are shown at higher magnification in adjacent panels. of ~30 μm; the nanobody can penetrate into a depth of ~150 μm.



Sup. Figure 4. Penetration of anti-calbidin scFv into 1-mm tissue sample.

Tissue penetration depth comparison of a calbindin-specific scFv without detergent and its parental mAbs directly conjugated with fluorophores with 0.05% saponin on 1-mm cerebral cortex tissue sections with a 7-day incubation.



Sup. Figure 5. Tissue penetration depth comparison of scFvs in the absence of detergent and fluorophore-conjugated mAbs with the treatments of various concentrations of detergents.

300-µm cerebral cortex sections were immunolabeled for one day (**a**) or seven days (**b**) with a calbindin-specific scFv conjugated with 5-TAMRA in the absence of detergent or with the scFv's parental mAb conjugated with FL550 in the presence of 0.1%, 0.3% Triton-X, or 0.05%, 0,1%, 0.2% saponin. Arrows indicate unlabeled cell nuclei. Arrowheads indicate granular textures associated with the treatment of saponin.

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REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have developed a method employing fluorescent single-chain variable fragments (scFvs) for conducting multiplexed detergent-free immunolabeling and volumetric-correlated-light-and-electron-microscopy (vCLEM) on the same samples. In this manuscript, they detail the development of eight fluorescent scFvs targeting specific markers crucial for brain studies. Through experimentation, six fluorescent probes were successfully visualized in the cerebellum using confocal microscopy with spectral unmixing, followed by vEM analysis of the identical sample. The outcomes reveal an exceptional blend of ultrastructure alongside multiple fluorescence channels, offering valuable insights into cellular composition and organization.

This approach facilitated the documentation of a previously poorly characterized cell type and the precise subcellular localization. Leveraging scFvs derived from existing monoclonal antibodies opens up the possibility of generating numerous such probes, which could significantly enhance molecular overlays for connectomic investigations.

This study represents a significant advancement in the field of vCLEM and holds great promise for researchers seeking to unravel intricate neuronal networks. The revised version of the manuscript effectively addresses key questions and concerns. I don't have further comments.

Reviewer #2 (Remarks to the Author):

The revised manuscript thoroughly addresses all of the concerns raised in my initial review. I think the manuscript will have broad appeal and I appreciate the effort put in to address the referees' comments. I strongly recommend publication in Nature Communications.

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