nature portfolio

Peer Review File

Single-molecule methods for characterizing receptor dimers reveal metastable opioid receptor homodimers that induce functional modulation

Corresponding Author: Dr Peng Zhou

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
The manuscript by Peng Zhou et al titled: “Single-molecule detection of transient dimerization of opioid receptors 1: Homodimer’s effect on signaling and internalization” reports results from studies using single-molecule imaging to derive homodimer-monomer dissociation equilibrium and rate constants. The authors report the involvement of 9-26 amino acid C-terminal domains in opioid receptor homodimerization and find that membrane permeable C-terminal peptides that mimic these domains can block receptor homodimerization. Moreover, the study reports finding differences in activation of downstream G-proteins following agonist treatment by KOR and DOR homodimers in comparison with the receptor monomers; this is not observed between MOR homodimers and monomers.

The results with MOR studies contradict findings from other studies using single molecule imaging: Moller et al (PMID: 32541966) reported that MOR homodimers form only upon treatment with DAMGO (negligible under un-activated conditions). Others (PMID: 29769636, PMID: 33686301, PMID: 34657173) reported negligible homodimers under physiological concentrations. The similarities and differences between the published studies using single molecule imaging and the current studies need to be rigorously addressed. The authors argue that the present study develops theories to evaluate koff from the distribution of single-molecule colocalization durations, and another to estimates KD (kon can be calculated as koff/KD). However, these theories need to be tested experimentally with stringency in order to determine the ranges at which koff, KD, kon and homodimer lifetimes remain constant. Also, in this study, the authors report the involvement of C-terminal region and MOR TM1 in homo/heteromer regions. This has been previously reported using other approaches which also show that peptides that mimic dimer motifs can block receptor dimer formation. As is, these findings do not significantly advance our current knowledge of the dynamics of receptor dimerization particularly with native receptors in their endogenous setup.

To help the authors improve the manuscript several recommendations are included below:

General Comments:
1. The results section sometimes refers to data that is in the second paper on heterodimers – this is inappropriate. They should remove any mention of the second paper from the current manuscript.
2. Figure legends are very cryptic making it difficult to understand the different figure panels.
3. Authors should not assume that only those who are carrying out single molecule detection studies are reading their manuscript. Thus, it would be helpful if the terms in the different equations in the manuscript (including figures) and supplemental Materials are defined and those that can be experimentally obtained stated. This would be very helpful for those not working in this area of research particularly new students.
4. In the results section it would be helpful to specifically mention which results are based on theoretical and on experimental calculations.
5. There are portions in the results section that are better fit for the discussion section e.g. Lines 258- 266 starting from” In contrast … at low membrane densities.”
Lines 295-303, lines 353-359 starting from “Accordingly…”
6. The figures and supplemental figures should be arranged in the order they appear in the results.

Specific comments

1. As the authors pointed out in the introduction the existence of homo- and heterodimers of opioid receptors particularly at physiological expression levels remains controversial because they could be artifacts due to the expression of very high OR concentrations in the cells. However, in this study authors use electroporation -a technique known for high expression of proteins.
a. No mention is made on the Methods section of how much cDNA was used for electroporation and how were receptor levels determined to be in the physiological range and not overexpressed at the cell surface at the time the experiments were carried out.
b. Do the homomer-monomer dissociation equilibrium and rate constants change with amounts of cDNA of opioid receptors being electroporated into the cells?
c. Also, authors use total spot number densities in the range of 0.5-1.5 spots/μm2.
How are the results affected if the range if the number of spots is the same (0.5-1.5) but the μm2 is < or > than 1?

2. The authors used a monomeric TM protein CD47, after linking the SNAPf-tag and mGFP-tag to its N- and C-termini respectively, to evaluate the labeling efficiency of SNAPf-tag to the extracellular domain of opioid receptors that was difficult to evaluate due to homodimer formation. This approach assumes that the SNAPf-tag binds to the extracellular domain of opioid receptors with a similar efficiency as to the extracellular domain of CD47. A more appropriate strategy would be to exchange the extracellular domain of CD47 for that of each individual opioid receptor.

3. Supplementary Fig 1E (legend and in Methods lines 885-887). Calculation of SNAPf-tag labeling efficiency is very cryptic. The figure provides and equation but the terms of the equation like Fo, exp, x, Co, yo are not defined.

4. Fig.1 C shows co-localization index with opioid receptor mutants with deletions of the opioid receptors N-terminal extracellular domains (KOR N1-53, MOR N1-51 and DOR N1-35). How were these mutants generated? Methods (line 828-829) mention OR point mutants, but these are not point mutations. Also, were these mutants labeled with the SNAPf-tag? Was the labeling efficiency comparable to that of the wild-type receptors? What was their cell surface expression levels and functional activity compared to wild-type receptors?

5. To evaluate the homodimer lifetimes, authors examined colocalization event of spots
with different colors within a threshold distance R = 200 nm since their theory in Supplementary Note 1 suggests that the selection of R does not affect the measured koff value, although it could affect its SEM. The authors should show this at least for 1 receptor by choosing at different R values for their analysis (< and >200 nm).

6. Lines 168-169. Not clear what is meant by “This result holds despite the fact that the generally used colocalization threshold distance is ≈200 nm, which is much greater than the molecular scale of 3~10 nm“. This would have to be supported by using data for different co-localization thresholds as suggested in 5 above.

7. Supplemental Fig. 1F.
Description of this panel missing from results and/or Material and Methods section.
What does % molecules in the y-axis mean?

8. Supplemental Fig. 3c says “Also see f”
There is no f panel in the figure or in the figure legend.

9. Lines 246- 252 starting from” KD was first determined … like other statistical parameters).” Should be moved to methods

10. Fig 2D.
The fig does not have solid curves. It has dashed lines and solid circles.
Fig legend is not clear. What mathematical functions were calculated from KD value? Since the authors use physiological expression levels found in the literature were the KD values obtained from the literature? How were the values in the y-axis obtained

11. Fig 3B shows co-localization index with KOR 351-364, 359-380, 365-380, 365-371, 372-380, MOR 358-398, 358-382, 371-382, 383-398, and DOR 339-372, 357-372, 357-364, 365-372. How were these mutants generated? Methods (line 828-829) mention OR point mutants, but these are not point mutations.

What was their cell surface expression levels and functional activity compared to wild-type receptors – this is critical since many deletion and point mutations affect maturation of the receptors leading to a loss of significant decrease in cell surface expression.

12. Lines 342-344
Not clear. Since data fits single exponential function there is only one t component. The t2 component in the figure corresponds to that of the t2 for wild type receptors shown in Fig 1D obtained by the sum of two exponential functions.

13. Fig 3D shows co-localization index with point mutations of KOR, MOR and DOR.
What was their cell surface expression levels and functional activity compared to wild-type receptors?

14. Line 390.
Change K-, M-, D-, to MOR, DOR and KOR for consistency.

Reviewer #2

(Remarks to the Author)
A combination of new theory combined with advanced single-molecule experiments complemented with data analysis developed by Zhou et al. enabled quantification of koff and KD for monomer-dimer equilibrium for the plasma membrane-associated opioid receptors (ORs). Beyond ORs, this dimerization kinetics approach could be applied to other plasma membrane molecules, and it is thus a significant advance in the field. The authors identified that residues close to c-terminal cytoplasmic domains of ORs are responsible for specific homodimerization interactions while trans-membrane domain of MOR (TM1MOR) showed non-specific interactions; as such, the Authors suggest that TM domains may be involved in both homo and heterodimerization. Developed peptides (based on the residues close to c terminus) specifically reduced OR homodimerization and may be valuable tools for the field as they show unique effects on signaling for the three canonical ORs. The results are generally rigorous and well interpreted; however, few questions should be addressed before the manuscript is ready for publication in Nature Communications.

Suppl Fig 1c-f shows CD47 was used to assess labeling efficiency. Why was this molecule chosen when LDL receptor was used later in the manuscript as a monomeric model molecule (and KD was provided)? Is the accessibility of tag/labeling efficiency associated with extracellular loop in different receptors an issue?

The Authors should explain why pair cross-correlation functon(PCCF) 0-100 nm was used for calculation of colocalization index given the decay rate of the function and 50 nm bin.

Was colocalization threshold distance of 200 nm in part effective for calculations due to generally low molecular densities (one or fewer fluorescently labeled molecules in the diffraction limited spot)?

Were molecule paths largely random?

SEM for MOR KD was ~3 higher than that for KOR and DOR. Could this be associated with the bigger heterogeneity in MOR organization between cells?

Supplementary Table 1 should include number of independent measurements.

DeltaN-OR mutants do not fit well in the figure 1c, especially as they are discussed later in the manuscript. Perhaps include with Fig. 3b.

In several places secondary binding site (TM domain) is hinted but not explained; perhaps revise so the flow is not interrupted (e.g. include this part later: Line 333 “which might be the TM domains, as previously suggested for ORs26, 49, 50, 52, 53 and other class A GPCRs.21, 39, 40, 41 However, as shown in Supplementary Fig. 4a; the aa identities of TM domains among the three ORs are quite high, and thus the homo-interaction between TM domains might be less specific. We will revisit the TM interactions for homodimerization later.”)
This part is even more challenging at current location:
Line 445 “The reason for this higher tendency to form homodimers even in the presence of FAM-Dpep-TAT is unknown, but we propose that the agonist-induced conformational changes enhanced the interaction in the TM domains, because DOR homodimerization also involves TM-domain interactions (see Fig. 5, which will be discussed in
449 the next subsection).” Moving this to discussion could be beneficial.

The Authors should discuss why was FAM-TET peptide more efficient in preventing homodimerization (significant effect at 3 uM vs 6 uM for Xpep-GFP).

Rationale for addition of pyrenebutyrate to cells should be included (line 393) (presumably to increase peptide translocation).

While MOR-KOR heteromers are not likely, did the Authors test the effect of TM1MOR on KOR-KOR homodimerization (or on another GPCR)? The question is how (un)specific is the effect of TM1MOR peptide.

Is it possible that other TM domains or extracellular loop regions that were not tested (e.g., TM4) facilitate interactions for homomers (albeit the effect would be lower)? Perhaps include a sentence regarding this in the discussion.

The Authors used low expression levels (where oligomers could be largely neglected). Based on their conclusions regarding both C terminal domains and TM domains affecting OR organization and their simulation data (Fig S3), it may be useful to discuss if oligomers could be present (in addition to monomers and dimers) on the cell membranes in physiological conditions. There is a little note on this in the companion paper, something similar would be helpful here.

Minor comments:
Line 376 Fig 4a,b: typo for figure referencing?

Few parts in the results are more appropriate in the discussion:
Lines 222-227; Lines 295-303; Lines 431-436; Lines 556-568

“I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts."

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
In this manuscript Zhou and co-workers utilize a single molecule fluorescence based approach to provide a novel inspection of opioid receptor dimerization. For about 50 years the concept of opioid receptor oligomerization has been proposed, initially based of pharmacological data, then biochemical and fluorescent and bioluminescent experimental data. This early work however mostly relied on recombinant cell systems that overexpress the opioid receptors. Recent studies using more sophisticated techniques have not provided a clear answer whether GPCRs, or opioid receptor, form dimers. Zhou et al show that opioid receptors form temporary dimers at a rate that his higher than random encounters. The duration and occurence is mathematical and increase with receptor expression. The authors, identify regions in each OR that contribute to the dimerization. The authors created interfering Xpeps to break up dimers, which did not impact internalization but did have unique effects on agonist-induced signal transduction. The authors have carried out a tremendous amount of work with multiple controls in some cases using distinct controls for the same question.
I have a small number of questions and suggestions that if addressed can assist other readers in understanding the interpretation of the work.

The authors use a chimeric Gqi protein to divert the OR signal transduction to calcium mobilization, what was their reasoning for this? Was it to better align the speed and short duration of dimer formation with a fast signaling endpoint or was it done for easy fluorescent microscopic analysis. In either case, it would be helpful if the authors could carry out or at least address if similar results can be expected for cAMP inhibition.

On page 6, line 124, the authors state that their SNAPf-OR was labelled with two SNAP ligands at 60 and 61% effeciency. Perhaps I am understanding this incorrectly, but I read this as one type of SNAPf-OR simulataneously being labeled with the 549 and CF660R, which can maximally lead to all receptors 100% being labeled, making 60+61% not possible.

Page 22, line 588. This Result.. may be confused as refering to the results in this manuscript, by a careless reader. in line 589 please elaborate on the however, see14, such that the reader does not have to dig up the paper and hope to understand what the authors want the reader to come away with.

Reviewer #5

(Remarks to the Author)
In the present work Zhou et al. investigated opioid receptors (ORs) homodimerization using single-molecule imaging techniques at low physiological concentrations. This study confirmed that μ-, κ-, and δ-ORs transiently form homodimers, revealing that their C-terminal cytoplasmic domains drive specific homodimerization while transmembrane domains contribute less specifically. Additionally, the authors provide accurate estimates of kinetic and thermodynamic dimerization constants. The authors produced an important amount of data that are informative when compared to traditional methods (e.g., BRET, FRET) that use higher protein densities. Considering the pharmacological relevance of the ORs, the findings might provide insight into the modulation of analgesic response through ORs dimerization.

The authors submitted another paper (namely part 2) that uses the same approach to study the heterodimerization of ORs. Considering that the topic of the two papers is the same and even the methodology is identical, I suggest merging the two works into one that could highlight the main findings for the ORs homo- and heterodimers, reporting all the technical information in the supplementary information. The current manuscripts are lengthy and include numerous technical details that, while relevant to specialists, could be streamlined by merging the works. Key suggestions include:
1. Focus: Emphasize C-terminal interactions in homodimers, N-terminal interactions in heterodimers, and expand on signaling and internalization pathways (see comments below).
2. Data Presentation: Include thermodynamic and kinetic constants in tables; detailed TM interaction results could be moved to supplementary information.
3. Broader Impact: Combining these studies will yield a robust foundation for future investigations into opioid receptor and GPCR dimerization that will be of interest for the Nat Commun readership once the following points will be addressed.

- Cell line environment: Given that experiments were performed in CHO-K1 cells lacking endogenous OR expression, it would be beneficial if the authors discussed how CHO-K1's plasma membrane composition might influence GPCR dimerization, potentially differing from native environments in homo- and heterodimer formations. Effects of lipids composition and cholesterol concentration are known on GPCRs dimerization (10.1016/j.sbi.2011.09.007) and they should be discussed;

- Impact of fluorescent dyes on dimerization: Fluorescent tags or dyes used in experiments may affect ORs dimerization and binding characteristics, potentially altering receptor behaviour (e.g., OR tilting with respect to the membrane plain and protomer-protomer contacts). Discussing how dye selection and labeling strategies were controlled or assessed for minimal interference would add rigor, particularly since GPCRs are sensitive to changes in structure and membrane environment;

- Binding mode of OR dimers: No information is provided on whether the OR dimer binding mode is symmetric or asymmetric, nor on specific binding interfaces between protomers. Additional experiments, such as structural modeling or crosslinking assays, could clarify the binding orientation and interface characteristics of OR dimers. This data would help to elucidate the structural basis for dimer stability and its functional implications especially for drug discovery, adding depth to the study;

- Effect of ORs dimerization on cell signaling: The paper provides additional evidence of OR dimer formation, which is valuable for the field. However, the concept of OR dimerization is not novel in itself and has been demonstrated in previous studies. As such, to meet Nature Communications standards, it may be beneficial to frame these findings within a larger narrative, such as detailing how dimerization influences OR signaling and receptor trafficking. Additional experiments on the effect of ORs dimers on cell signaling and selective activation of specific pathways (biased signaling) would significantly advance our understanding of OR biology;

- Functional Insights into OR dimerization: Expanding the discussion of OR interactions with G proteins and beta-arrestins for both monomeric and dimeric forms would be highly beneficial. Could the authors conduct biased signaling studies under varying receptor concentrations to link dimerization ratios with signaling changes?

- Oligomerization Investigation: Although the focus of the works is on dimers, OR oligomerization also influences GPCR signaling. Could the experimental data provide some insight into C-terminal and transmembrane contacts driving oligomerization?

- Receptor concentrations for KD values: It would be valuable to ensure that receptor concentrations used in the experiments are suitable for accurate determination of dissociation constants (KD). Verifying that concentrations fall within physiological ranges and optimizing them to avoid artifacts from underexpression will ensure the reliability of dimerization affinity measurements, making the findings more applicable to in vivo conditions;

Further points for paper 1:

- Supplementary Information: The derivation in Supplementary Note 1 could benefit from some clarification. Equations lack explicit calculations, particularly in reaching equation 4, where the reference (12) is ambiguous. Additionally, certain boundary condition derivations, especially the Bessel function identity used, would benefit from detailed explanation for the general readership. In detail, the authors refer to reference 12 but no chapter is suggested. Is it chapter 3.2.1 for the "First boundary value problem with domain $0 < r < R$"? If so, please cite it properly. Authors should specify which point of the book in reference 12 should be followed and report explicit transformations to obtain equation 4. Moreover, one condition reported during the demonstration is that $1 = 2\Sigma m\, J0(y\mu n) / [\mu n J1(\mu n)]$ is taken from $xv = 2\Sigma m\, Jv(y\mu m) / [\mu m Jv+1(\mu m)]$, which is valid only for $v>0$ (eq. 10.23.21 in NIST as reported by the authors). However, they use it for $v=0$, is that correct? And why the authors have a counter by variable m if it does not appear in the formula?
Finally, I could not find any property of Bessel functions for which $J0(\mu n) - J'0(\mu n)\mu n\varepsilon/R = J1(\mu n)\mu n\varepsilon/R$. I found two different ways to write J1 as a function of J0, but none of them meets the identity reported in the manuscript ($d/dx[xmJm(x)] = xmJm-1(x)$ and $J'v(x) = 1/2[Jv-1(x) - Jv+1(z)]$).
The authors should clarify these and all the other steps in the equations derivation to allow understanding the mathematics for a reader not expert in the field and favouring the reproduction of the data;

- Dimer Disruption by C-Terminal (C-ter) Peptides: The use of C-terminal peptides as a tool to disrupt dimerization is an indirect method, as these peptides may interfere with receptor dimerization even if the dimers are formed primarily through TM domain interactions. This limitation should be acknowledged, as it does not conclusively demonstrate that C-ter interactions alone govern OR dimerization. Discussing this as a methodological limitation and suggesting alternative experimental approaches (e.g., mutational analysis targeting TM regions) could strengthen the study's conclusions;

- TM1's Inhibitory Role: The conclusion that TM1 inhibits homodimerization might be clarified by including negative controls to confirm TM interactions (e.g., using other TM peptides);

- Comparative Temperature Analysis: The authors refer to a previous KOR homodimer study obtained at a different temperature. The authors should perform an additional experiment on KOR dimerization at 20°C to provide a direct comparison with the previous study and discuss the differences;

- Literature Citations: line 334 on page 13, and line 87 on page 4 of paper 2, consider citing chemokine receptor dimerization studies (doi: 10.1038/ni1027; doi: 10.1016/j.molcel.2018.02.034), including a recent work on CCR5 and CXCR4 (doi: 10.1038/s41467-023-42082-z).
Additionally, on line 367 at page 14 the authors' statement "Furthermore, if we could develop such homodimerization blockers, they could become extremely useful tools to dissect the functions of OR monomers and homodimers, and might inform future developments of more effective analgesic treatments with fewer side effects and less tolerance development." should be motivated and supported by references;

Figures: For Figure 2B, please clarify the data on the right side by adding columns.

Reviewer #6

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Version 1:

Reviewer comments:

Reviewer #2

(Remarks to the Author)
The Authors have thoroughly addressed all of my concerns, and the manuscript is significantly improved. The work by Zhou et al. incorporates substantial new insights into opioid receptor organization along with notable methodological advancements; it is ready for publication in Nature Communications.

Reviewer #3

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4

(Remarks to the Author)
The authors performed additional experiments, ran more controls and expanded discussions to adequately address my questions as well as those provided by other reviewers.

Reviewer #5

(Remarks to the Author)
- The hetero- and homodimerization of opioid receptors is well-studied. The main novelty of these two papers lies in the use of low concentrations that effectively mimic physiological conditions, and in the precise determination of thermodynamic and kinetic constants.
The abundance of technical details makes it easy for the reader to lose sight of the main messages. I remain convinced that the authors should reorganize their material into one strong, clear paper focused on the thermodynamics and kinetics of OR dimerization, and a second, more technical paper suitable for a specialized journal, such as Nature Protocols.

- Due to time and resource constraints, the authors communicated that they cannot investigate the effect of G proteins on OR dimerization (point 3 in the rebuttal letter)

- We acknowledge the authors' efforts in addressing the other points, including conducting additional experiments.

Reviewer #6

(Remarks to the Author)
I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #7

(Remarks to the Author)
The two manuscripts by Zhou et al. include an extensive amount of well-designed and well-performed experiments with state-of-the-art techniques to analyze the dynamics of interacting single fluorescent molecules in mammalian transfected cells. These studies clearly demonstrate that μ, κ, and δ opioid receptors (MOR, KOR, and DOR, respectively) undergo

repeated transient homodimerization (first manuscript) and heteromerization (second manuscript) with functional significance. The authors develop a very sound experimental and theoretical framework which this reviewer believes can represent a seminal work in the field of GPCR oligomerization. The studies include elegant experiments with the use of membrane-permeable synthetic peptides to disclose the differential involvement of specific C-terminal, transmembrane (TM) and N-terminal domains of the different ORs forming homomers or heteromers. In the second manuscript they also include in vivo experiments that provides evidence for the rationale of considering the heteromers as therapeutic targets. More specifically, to counteract the tolerance of opioid-induced analgesia by disrupting the MOR-DOR heteromers with the intracranial administration of a heteromer-disrupting peptide. A key result of these studies is that the degree of oligomerization depends on the membrane expression levels of the receptors, and they demonstrate their significant functional presence within a putative low physiological expression range.

I have two main general comments that apply to both manuscripts that I believe deserve special consideration. These comments do not intend to add more experimental burden, although I would hope the authors will address them in future experiments.

1-The authors repeatedly state that the experiments are performed at “physiological expression levels”. More specifically they state that 1 and 10 copies/μm2 correspond to low and high physiological membrane expression levels, respectively. This reviewer is not aware of any study that provides a clear answer to what are the normal membrane expression levels of any GPCR in neurons. None of the studies referenced to support this statement (refs. 26, 29 and 33, from the first manuscript) or from the references of those studies provide experimental evidence for what those physiological levels are. This is a real key question, since the authors clearly demonstrate that the amounts of homodimers and heterodimers (and heterotrimers, in the second manuscript) that exist at any time depend considerably on the real physiological expression. Based on many published correlational functional in vitro, in situ and in vivo studies on GPCR heteromers, and based on the well-known lack of uniformity of the neuronal plasma membrane and on many studies that demonstrate that GPCRs tend to concentrate in specific signaling areas of the plasma membrane (such as the concentration of MORs in primary cilia mentioned in the second manuscript), this reviewer believes that physiological levels are more often probably higher than 10 copies/μm2. This would imply that GPCR oligomers have a more significant functional role, including higher order than dimers, as implied in the second manuscript. At least, those that contribute to the most significant signaling effects at the cellular level. I do sympathize very much with the authors’ theoretical discussion about the pulse-like signals (in the second manuscript), which implies that summation, aggregation of brief signals are what is needed to promote a detectable cellular signal, which promotes the functional role of GPCR oligomers. In fact, in the first manuscript the authors can only obtain a significant detectable Gi-mediated signal with preparations with higher, then probably more physiological, receptor expression. In summary, I do not believe that the authors can state (as in the legend to Fig. 2 in the first manuscript), that “the expression level employed in this study is basically well within the physiological range”. I would state the following: that there are no clear results in the literature about which are the physiological expression levels of opioid receptors, but that there is reasonable doubt that those membrane levels are lower than 1 copy/μm2, which is what has been used in previous studies of single-molecule tracking where oligomers were rarely detected; that the present study shows unequivocally that opioid receptor oligomers (homo and heterodimers) represent a significant proportion of functional entities at levels between 1-10 copies/μm2; and that it is very possible that those levels are higher in areas of the plasma membrane with more signaling significance, which would imply an even higher functional significance of GPCR oligomers.

2-The authors are probably not aware of a recent study by De Oliveira et al (Pharmacol Res, 2022, 182:106322) that reports MOR dimers and heteromers of MOR with galanin Gal1 receptors, and that analyzes in detail their TM oligomeric interfaces by using several techniques also including single-molecule imaging with TIRF microscopy. Instead of a single-molecule tracking analysis, they analyze the number of steps after photobleaching of single or co-localized fluorescent spots, which is a well-validated technique to study the stoichiometry of protein complexes, and that the authors of the present manuscripts have used in previous studies (Nagata et al., Proc Natl Acad Sci USA, 2013, 110:5034-5039). By using all possible 7 TM-peptides of the MOR, De Oliveira et al. found that TM5 and TM6 are involved in MOR dimerization, which is well in agreement with the crystallographic results of Manglik et al. (Nature, 2012, 485:321-326), and they obtained the same results by using the 7 TM-peptides of the MOR with bimolecular fluorescence complementation. This is an additional technique than the authors of the present manuscripts have also used and validated for the study of oligomeric interfaces in what I consider a seminal paper in the field (Kasai et al., J Cell Biol, 2011, 192:463-480). With both techniques and by also using the TM-peptides of the Gal1R, De Oliveira et al. also provided evidence for a tetrameric quaternary structure of the MOR-Gal1R heteromer (with two dimers of each receptor) and a significant involvement of TMs in the heteromeric interface.
In the first manuscript, Zhou et al. find conclusive evidence for a role of the intracellular C-terminal domains in MOR, DOR and KOR homodimerization, which was not evaluated in the study by De Oliveira et al. Then, by using the TM1-peptide of the MOR they find a weaker involvement of TM1 of MOR in MOR homodimerization, which was not observed in the study of De Oliveira et al., and conclude that TMs have a less specific, weaker and secondary role. The authors should moderate this conclusion and discuss their results in relation to those obtained by De Oliveira et al. According to those results, the use of TM5- and TM6-peptides of the MOR should have also disclosed a significant role of the corresponding TMs in MOR homodimerization. Therefore, most probably their conclusion only applies to TM1. It is unfortunate that the authors were not aware of the study by De Oliveira et al. and did not analyze the effect of TM5- and TM6 peptides. As I mentioned above, I understand that there are so many more additional experiments that the authors could add to their extensive study, but I would encourage the authors to run these experiments in the future. Nevertheless, they should moderate their general interpretation about the lesser role of TMs in oligomerization. What the study of De Oliveira et al, and other studies that use TM-peptides to interfere with the interfaces of GPCR oligomers, show is that the contribution of each interface is not additive, that the interference with any of the different interfaces leads to a near maximal disruption. And that is, in fact, what the authors show in the second manuscript, where TM1 and TM4 play a similar significant role than the N-terminal domains in MOR-DOR heteromerization, which they discuss as an apparent cooperative allosteric effect.

An additional very compelling study that demonstrates the significant role of TMs in GPCR heteromerization was recently published study by the same research group of De Oliveira et al. This study uses TM-peptides with different lengths and amino acid substitutions to disclose the specific residues involved in a TM5-TM6 interface of the cannabinoid CB1 receptor-serotonin 5-HT2A receptor heteromer, which leads to the prediction of small molecules that can specifically interfere with this heteromerization (Matsoukas et al., J Med Chem, 2025, 68, 261-269). This study represents the proof of concept of the possibility of using small molecules, instead of non-brain-penetrating peptides, to interfere with specific GPCRs heteromerization. I suggest the authors add this possibility to expand their suggestions about translational implications, such as those suggested at the end of the Discussion in the second manuscript about MOR-DOR heteromers and the tolerance to the analgesic effects of opioids.
Finally, the existence of electrostatic interactions between basic amino acids (particularly adjacent arginine residues) and acidic amino acid residues (particularly adjacent glutamic or aspartic or phosphorylated residues) in intracellular domains mediating receptor oligomerization has been previously stressed and it should be acknowledged. Particularly important has been the work of the late Amina Woods (see for instance, Navarro et al., J Biol Chem, 2010, 285:27346-27359).

REVIEWER COMMENTS

Dear Reviewers,

Thank you very much for critically reading our manuscripts and providing constructive and important comments, suggestions, and advice.

The revisions in the manuscripts are shown in the following ways.

The revised parts of the main text are shown in dark-red fonts. Following the advice of reviewers, some sentences have been moved, which are shown in blue fonts. MS2 has been comprehensively revised for readability as recommended by Reviewer 1, and these revisions are shown in green fonts.

New data added to the figures in the revised manuscripts are indicated by surrounding the data by magenta rectangles, and their captions are shown in dark-red fonts.

Figure panels that existed in the original manuscripts that were moved to new figures are indicated by surrounding the data (panels) by blue rectangles, and their captions are shown in blue fonts.

Thank you very much again for serving as reviewers for our manuscripts.

Sincerely,

Peng and Aki, representing all co-authors

Reviewer #1 (Remarks to the Author):

The manuscript by Peng Zhou et al titled: "Single-molecule detection of transient dimerization of opioid receptors 1: Homodimer's effect on signaling and internalization" reports results from studies using single-molecule imaging to derive homodimer-monomer dissociation equilibrium and rate constants. The authors report the involvement of 9-26 amino acid C-terminal domains in opioid receptor homodimerization and find that membrane permeable C-terminal peptides that mimic these domains can block receptor homodimerization. Moreover, the study reports finding differences in activation of downstream G-proteins following agonist treatment by KOR and DOR homodimers in comparison with the receptor monomers; this is not observed between MOR homodimers and monomers.

The results with MOR studies contradict findings from other studies using single molecule imaging: Moller et al (PMID: 32541966) reported that MOR homodimers form only upon treatment with DAMGO (negligible under un-activated conditions). Others (PMID: 29769636, PMID: 33686301, PMID: 34657173) reported negligible homodimers under physiological concentrations. (A) The

similarities and differences between the published studies using single molecule imaging and the current studies need to be rigorously addressed. The authors argue that the present study develops theories to evaluate koff from the distribution of single-molecule colocalization durations, and another to estimates KD (kon can be calculated as koff/KD). (B) However, these theories need to be tested experimentally with stringency in order to determine the ranges at which koff, KD, kon and homodimer lifetimes remain constant. (C) Also, in this study, the authors report the involvement of C-terminal region and MOR TM1 in homo/heteromer regions. This has been previously reported using other approaches which also show that peptides that mimic dimer motifs can block receptor dimer formation. As is, these findings do not significantly advance our current knowledge of the dynamics of receptor dimerization particularly with native receptors in their endogenous setup.

To help the authors improve the manuscript several recommendations are included below:

Thank you very much for your critical reading of our manuscript and many constructive comments and valuable suggestions.

Before moving to the specific points raised by Reviewer 1, since some of the above points are not repeated below, let us address them in the following. The three major points raised by Reviewer 1 here are indicated by (A), (B), and (C) above.

(A) The similarities and differences between the published (OR) studies using single molecule imaging and the current studies need to be rigorously addressed.

Thank you very much for raising this issue. In fact, these previous discrepant results were cited in the Introduction in the original manuscript and are also cited in the revised manuscript (**p. 4, lines 69-80**), and as described there, they gave us an incentive to initiate this study.

More specifically, for the revision of this manuscript, we performed further experiments to compare our results with previous quantitative studies and provided possible explanations for the apparent discrepancies. Please note that there have only been two studies that obtained all three thermodynamic and kinetic parameters. Please see **Table 1 and p. 24, lines 662-679** in the revised manuscript.

Reviewer 5 raised a similar issue in point 7 with some more details, so please also see our responses there. As pointed out by Reviewer 5, just trying to find the existence of dimers without controlling or examining the expression levels (concentrations in the plasma membrane, such as the number density of OR molecules/μm^2) can lead to artifacts, inducing confusion in the literature, as indicated by Reviewer 1. This issue is now mainly addressed in the **section, "(8) Proper molecular concentrations (number densities in the PM) for dimer detection and K_D determination" in Supplementary Note 2** in the revised manuscript.

(B) However, these theories need to be tested experimentally with stringency in order to determine the ranges at which koff, KD, kon and homodimer lifetimes remain constant.

We appreciate your raising this important issue. For this, we performed further experiments and provided explanations. Please see **Table 1**, and related text on **p. 10, lines 251-255, and p. 11, lines 272-286** in the revised manuscript.

(C) Also, in this study, the authors report the involvement of C-terminal region and MOR TM1 in homo/heteromer regions. This has been previously reported using other approaches which also show that peptides that mimic dimer motifs can block receptor dimer formation.

We think Reviewer 1 is referring to the paper by Cvejic and Devi (1997) about the C-terminal region for DOR homodimer formation and the report by He et al. (2011) about MOR-TM1 for DOR-MOR heterodimerization. However, Cvejic and Devi did not perform blocking experiments using the amino acid sequence for DOR homodimerization (one would need to make the peptide membrane-permeable, as we did in this work). For MOR-TM1, in the present research (in these two manuscripts collectively), we showed that MOR-TM1 cannot be used as a specific blocker because it binds to both DOR and MOR, blocking MOR-MOR, DOR-DOR, and DOR-MOR dimerizations. Therefore, even though these are only small portions of our research results, we believe that they are useful and important additions to the literature.

General Comments:

1. The results section sometimes refers to data that is in the second paper on heterodimers – this is inappropriate. They should remove any mention of the second paper from the current manuscript.

Following the advice by Reviewer 1, we deleted almost all of these references to paper 2 in the Results, except for two cases. The remaining ones are the following.

p.11 lines 292-294 “In the following part of this report and in the companion paper, we use the colocalization index when showing the direct experimental data is preferable, whereas we show the KD values when providing the fundamental constants is desirable.”

p.18 lines 500-501 “The absence of a $TM1^{MOR}$ effect on KOR homodimerization is consistent with the lack of MOR-KOR heterodimerization.”

2. Figure legends are very cryptic making it difficult to understand the different figure panels.

We appreciate this comment by Reviewer 1. We comprehensively revised the figure legends. For details, please see our responses to your specific comments.

3. Authors should not assume that only those who are carrying out single molecule detection studies are reading their manuscript. Thus, it would be helpful if the terms in the different equations in the manuscript (including figures) and supplemental Materials are defined and those that can be

experimentally obtained stated. This would be very helpful for those not working in this area of research particularly new students.

4. In the results section it would be helpful to specifically mention which results are based on theoretical and on experimental calculations.

We are grateful to you for making these points. We tried to write the paper so it will be readily understandable for scientists working in the fields of GPCRs and membrane biology, as well as useful for scientists who are interested in single-molecule imaging of molecules associated with the plasma membrane (PM). However, obviously, the efforts to make it generally readable and clear were not sufficient. So, in this revision, we made greater efforts to clarify the meanings of the equations and the definitions of the quantities that appear in the equations, and to make it easily discernable whether the data are obtained by experiments, calculations, or simulations.

5. There are portions in the results section that are better fit for the discussion section e.g. Lines 258- 266 starting from" In contrast … at low membrane densities."
Lines 295-303, lines 353-359 starting from "Accordingly…"

Thank you very much for raising this point.

Lines 258 – 266: This part is now included in the part of testing the developed method. For this, we performed another set of experiments to obtain the K_D value for FPR(D71A) homodimers. Please see **p. 11, lines 272-286**.

Lines 295-303: We moved this part to the Discussion. Reviewer 1 states in the second paragraph of the overall statement, "Moller et al (PMID: 32541966) reported that MOR homodimers form only upon treatment with DAMGO (negligible under un-activated conditions). Others (PMID: 29769636, PMID: 33686301, PMID: 34657173) reported negligible homodimers under physiological concentrations. The similarities and differences between the published studies using single molecule imaging and the current studies need to be rigorously addressed." This part (Lines 295-303) of our discussion in the Results section in the original manuscript was indeed addressing this comment, and we slightly expanded this part after moving it to the Discussion (**p. 24, lines 662-679**) in the revised manuscript.

Lines 353-359: We would like to keep this part at the original location. If we move this part to Discussion, we need to repeat our explanation of the basic results, which will become quite lengthy, and we hope to avoid this.

6. The figures and supplemental figures should be arranged in the order they appear in the results.

Sorry for our mistakes. These have now been fixed in the revised manuscript.

Specific comments

1. As the authors pointed out in the introduction the existence of homo- and heterodimers of opioid receptors particularly at physiological expression levels remains controversial because they could be artifacts due to the expression of very high OR concentrations in the cells. However, in this study authors use electroporation -a technique known for high expression of proteins.

Thank you very much for pointing out the necessity for further clarifying the following points a and b.

a. No mention is made on the Methods section of how much cDNA was used for electroporation and how were receptor levels determined to be in the physiological range and not overexpressed at the cell surface at the time the experiments were carried out.

b. Do the homomer-monomer dissociation equilibrium and rate constants change with amounts of cDNA of opioid receptors being electroporated into the cells?

Let us address both a and b together. Thank you very much for your concerns. For transfection, 200 ng plasmid cDNA in 0.1 mL transfection buffer was added to 5×10^6 cells. This is now described in the Methods (**p. 44, lines 987-992**).

However, the key point in our experimental design is that we specifically *measured the OR expression levels in each cell*, by counting the number of fluorescent spots, and observed the cells with expression levels of 1.0 ± 0.5 fluorescent spots/μm^2 (sum of the spots in two colors; for homodimer observations; see **p. 6, lines 141-143**). We hope that Reviewer 1 agrees that this is a more direct method to determine the expression levels and thus is better than simply using the same amount of cDNA for transfection and failing to measure the exact expression levels.

As described in the **caption to Fig. 2c** in both the original and revised manuscripts (further clarification on **p. 12, lines 325-328**), the OR molecular expression level is approximately 1.8 molecules/μm^2 based on the labeling efficiencies of OR molecules (66% and 61% for SNAP-Surface 549 and SNAP-CF660; **p. 6 line 135-136; Supplementary Fig. 1f**) and the OR protomer fractions in homodimers (10-20% as stated on **p. 12, line 321 and the caption to Fig. 2c**). The physiological expression levels of ORs have been described as being between 0.3 and 10 molecules/μm^2 according to previous reports (Asher et al., 2021; Gentzsch et al., 2020; Meral et al., 2018), which are cited in both the original and revised manuscripts; however, OR expression levels might vary more considerably in particular nerve circuits and under various pathological and pharmacological conditions (DiCello et al., 2020; Erbs et al., 2015; Erbs et al., 2014; Pierre et al., 2019). Therefore, the expression levels employed in this study are basically well within the physiological range. This is now described in the **caption to Fig. 2c.**

Under the conditions of transfection and fluorescence labeling described in the previous paragraphs, in general, the populations of cells exhibiting expression levels of 1.0 ± 0.5 fluorescent

spots/μm^2, and those with greater or lower expression than this level were each about one-third of the cells found in the dish (lower expression here includes the cells that do not show any fluorescence labeling even under single-molecule imaging conditions). This has been clarified further in the revised manuscript (**p. 47, lines 1051-1054 in Methods**).

Another key point in our experimental design is, in addition to using the physiological OR expression levels, to evaluate K_D rather than just detecting homodimers or obtaining the dimer/monomer ratio under the given expression levels. K_D is a fundamental constant, by which we can calculate dimer fractions at various expression levels (which might vary between 0.3 and 10 molecules/μm^2; i.e., by a factor of >30, among different cell types, as described in the previous paragraph); i.e., in principle, K_D and rate constants should not be affected by the OR density on the cell surface. However, as pointed out by Reviewer 1, their *estimates* might be affected by expression levels. For K_D, we tested this by simulation and found that the systematic errors of the evaluation by our method will be within 8% for K_D values between 1 and 15 when the expression levels between 0.3 and 2 spots/μm^2 are employed for K_D evaluation. This point has now been added to the **to Supplementary Fig. 3d**.

The k_{off} value will not be affected by the expression levels, as long as they are within the range where the occurrences of incidental homo-oligomers greater than dimers are limited. Such occurrences have been evaluated, as shown by our new Monte Carlo simulation result (**Supplementary Fig. 2f**). These points have now been further clarified in the **caption to Supplementary Fig. 2f and the section "(6) Experimental justification of using a threshold distance *R* of 200 nm" in Supplementary Note 1.**

c. Also, authors use total spot number densities in the range of 0.5-1.5 spots/μm2.
How are the results affected if the range if the number of spots is the same (0.5-1.5) but the μm2 is < or > than 1?

We are sorry, but we are not sure about the question raised here. These numbers represent protein copy numbers per square micron, and thus the number "1" does not have any specific meaning. To avoid confusion, in the revised manuscript, we indicate this expression range as 1.0 ± 0.5 μm^2 (and use the simplified expression of ≈1) **throughout the report**.

2. The authors used a monomeric TM protein CD47, after linking the SNAPf-tag and mGFP-tag to its N- and C-termini respectively, to evaluate the labeling efficiency of SNAPf-tag to the extracellular domain of opioid receptors that was difficult to evaluate due to homodimer formation. This approach assumes that the SNAPf-tag binds to the extracellular domain of opioid receptors with a similar efficiency as to the extracellular domain of CD47. A more appropriate strategy would be to exchange the extracellular domain of CD47 for that of each individual opioid receptor.

We are grateful for this suggestion. We assume that in your comment, the first and second "SNAPf-tag"s that appear in your statement above represent the SNAPf-tag protein and the third "SNAPf-tag" represents a SNAP-ligand conjugated with a fluorescent probe. Since the fluorescently-labeled SNAP ligand covalently binds to the SNAPf-tag protein, the SNAPf-tag protein and thus the protein of interest (POI) can be fluorescently labeled. Accordingly, the concern raised by Reviewer 1 will be that the conformation of the SNAPf-tag protein, and thus its labeling efficiency, might be affected by the domain of the POI to which it is bound.

To address this issue together with Reviewer 2's point 1 (why CD47 and not TM^{LDLR}?), we examined the following additional four cases (2-5 shown below; the description of molecule 1 [SNAPf-CD47-mGFP] was in the original manuscript, and is reproduced in the revised manuscript). Please see **Supplementary Fig. 1e** for schematic figures. Your recommendation is represented by proteins 3-5.

1) SNAPf-CD47-mGFP

2) SNAPf-TM^{LDLR}-mSG

3) SNAPf-(N-terminal domain of MOR)-TM^{LDLR}-mSG

4) SNAPf-MOR-mSG used at a density of 0.1 fluorescent spots/μm^2

5) SNAPf-(MOR C-terminal deletion mutant)-mSG used at a density of 0.1 fluorescent spots/μm^2

Here, mSG = monomeric StayGold (Ando et al., 2024), which was employed for the newly tested molecules 2-5 because it is more readily detectable than mGFP due to its brightness and much slower photobleaching; N-terminal domain of MOR = amino acids 1-70 of MOR; MOR C-terminal deletion mutant = Δ358-382, monomeric mutant developed in this study.

The labeling efficiencies are plotted as a function of the SNAP-dye concentrations (**Supplementary Fig. 1g**). The SNAPf-tag proteins in these five proteins exhibited very similar values, indicating that the labeling efficiencies of SNAP-Surface 549 and SNAP-CF660R for binding to the SNAPf-tag protein are generally not affected much by the POI domains. We suspect that the somewhat long 21-amino acid linker we employed for conjugating the SNAPf-tag protein to the POIs, SGGGSGGx3, might have helped to provide similar labeling efficiencies for the different POIs. Specifically, the labeling efficiencies were estimated to be 0.6631 ± 0.0107 for SNAP-Surface 549 and 0.6148 ± 0.0077 for SNAP-CF660R (mean ± SEM of five proteins) at a SNAP-dye concentration of 300 nM. These results are now described in **Supplementary Fig. 1e-g and their captions** in the revised manuscript.

3. Supplementary Fig 1E (legend and in Methods lines 885-887). Calculation of SNAPf-tag labeling efficiency is very cryptic. The figure provides and equation but the terms of the equation like Fo, exp, x, Co, yo are not defined.

We are sorry that this part was poorly described. The explanations are now greatly improved, and as described in our responses to Reviewer 1's point 2 and Reviewer 2's point 1, the improved and expanded explanations are provided in **Supplementary Fig. 1d-f and their captions** in the revised manuscript.

4. Fig.1 C shows co-localization index with opioid receptor mutants with deletions of the opioid receptors N-terminal extracellular domains (KOR ΔN1-53, MOR ΔN1-51 and DOR ΔN1-35). How were these mutants generated? Methods (line 828-829) mention OR point mutants, but these are not point mutations. Also, were these mutants labeled with the SNAPf-tag? Was the labeling efficiency comparable to that of the wild-type receptors? What was their cell surface expression levels and functional activity compared to wild-type receptors?

Please note that, following the recommendation by Reviewer 2 (point 7), we moved all colocalization index data of N-terminal deletion mutants to **Fig. 3b**.

We apologize for our oversight. The deletion and point mutants of opioid receptors (ORs) were generated using a Q5® Site-Directed Mutagenesis Kit (NEB, Cat #E0554). We have incorporated this information on **p. 44 lines 976-978 in the Methods**, along with the complete plasmid sequences in **Supplementary Data 1** in the revised manuscript. All mutants are tagged with the SNAPf-tag protein at the N-terminus, and based on the data shown in **Supplementary Fig. 1g**, we speculate that the labeling efficiencies of all the mutants are very similar to each other and to the wild-type ORs. These points are now described on **p. 6, lines 136-138**.

As described in our response to point 1, we always selected the cells expressing 1 ± 0.5 spots/μm^2. (total spot number for two colors), including the investigations using various mutants.

While a systematic analysis of the functional consequences in mutants with impaired homodimerization capacity is a compelling avenue for future investigation (which is mentioned in the **Discussion, p. 27, lines 742-744** in the revised manuscript), as Reviewer 1 suggested, the current study focuses on revealing how these mutations alter the dimerization dynamics and equilibrium. Therefore, we consider the in-depth functional characterization of these mutants more appropriate as the basis for a distinct future study.

5. To evaluate the homodimer lifetimes, authors examined colocalization event of spots with different colors within a threshold distance R = 200 nm since their theory in Supplementary Note 1 suggests that the selection of R does not affect the measured koff value, although it could affect its SEM. The authors should show this at least for 1 receptor by choosing at different R values for their analysis (< and >200 nm).

Following this excellent recommendation, we performed an experimental examination for the use of R = 200 nm (examined in the range from 150 nm to 300 nm), and the result and its explanation

are given in **"(6) Experimental justification of using a threshold distance *R* of 200 nm" in Supplementary Note 1**.

6. Lines 168-169. Not clear what is meant by "This result holds despite the fact that the generally used colocalization threshold distance is ≈200 nm, which is much greater than the molecular scale of 3~10 nm". This would have to be supported by using data for different co-localization thresholds as suggested in 5 above.

Thank you very much for pointing this out. Our statement indicated by Reviewer 1 was indeed unclear. It has been revised as "Specifically, although the colocalization event of two fluorescent spots is defined by a threshold distance *R*, which is 200 nm in the present study and thus generally much greater than the molecular scale (such as 3-10 nm), we can evaluate the k_{off} and τ_2 values of dimers (associated molecules) by obtaining the duration distribution of the colocalization defined by a threshold distance (such as 200 nm) much greater than the molecular scale." This is now stated on **pp. 7-8, lines 184-194**.

7. Supplemental Fig. 1F.
Description of this panel missing from results and/or Material and Methods section.
What does % molecules in the y-axis mean?

Thank you very much for pointing this out. This figure was cited in the Methods section in the original manuscript. The figure panels are histograms and they are now shown in **Supplementary Fig. 2g** in the revised manuscript. The y-axis label was changed to "% SNAPf-MOR Trajectories" and the x-axis is doubly labeled as "Trajectory length in time" and "Trajectory length in frames". Further explanation is provided in the **caption to Supplementary Fig. 2g**.

8. Supplemental Fig. 3c says "Also see f"
There is no f panel in the figure or in the figure legend.

Sorry for this oversight. When we removed this figure panel f in our preparation of the original manuscript, we failed to remove this sentence. This sentence has been deleted.

9. Lines 246- 252 starting from" KD was … like other statistical parameters)." Should be moved to methods

As recommended by Reviewer 1, this sentence was moved to the **Methods** (**pp. 50-51 lines 1165-1177**) and **Software and statistical analysis** (**p. 55, lines 1311-1323**).

10. Fig 2D.
The fig does not have solid curves. It has dashed lines and solid circles.

Fig legend is not clear. What mathematical functions were calculated from KD value? Since the authors use physiological expression levels found in the literature were the KD values obtained from the literature? How were the values in the y-axis obtained

This figure is now shown as **Fig. 2c** in the revised manuscript. Thank you very much for pointing this out. The graphs contained both solid and dashed parts of the function (curves), but the curves were drawn quite faintly, and therefore the solid curves appeared like dashed lines. We fixed this problem. The equation and its derivation are now shown **at the end of Supplementary Note 2, "(7) Molecular fraction of a membrane protein existing as dimers, calculated from K_D and the total number of molecules (protomers)."**

The matter of fact is that it has been difficult to obtain K_D values for **any membrane proteins** in membranes. To our knowledge, there are only two publications that reported all three thermodynamic and kinetic parameters (K_D, k_{off}, and k_{on}; both happen to be for GPCRs). Our group was the first to develop a method to evaluate all three constants: we obtained them for the internalization-deficient mutant of formyl peptide receptor (FPR(D71A)) (Kasai et al., 2011). After this, another paper appeared reporting these three values for MOR at 20˚C (Gentzsch et al., 2020). They are now listed in **Table 1** (new table produced for the revised manuscript) together with our new data obtained in this work, and are compared.

The lack of quantitative knowledge based on these fundamental constants is hampering the studies of molecular interactions and dimerizations of membrane molecules, which we hope to change. In the present research, we developed a theory and method to rigorously evaluate k_{off} by performing quite simple single molecule imaging (previous methods were somewhat intuitive). Furthermore, we developed a new easier and, in principle, more sensitive method to evaluate K_D (due to the use of a correlation method). Our previous method was too cumbersome to be applied broadly.

To make sure that these crucial points of this manuscript are clear, we further explained these points on **pp. 4-5, lines 90-110 in the Introduction and p. 23, lines 614 -628 at the beginning of the Discussion** In the revised manuscript.

11. Fig 3B shows co-localization index with KOR Δ351-364, Δ359-380, Δ365-380, Δ365-371, Δ372-380, MORΔ358-398, Δ358-382, Δ371-382, Δ383-398, and DORΔ339-372, Δ357-372, Δ357-364, Δ365-372. How were these mutants generated? Methods (line 828-829) mention OR point mutants, but these are not point mutations.

What was their cell surface expression levels and functional activity compared to wild-type receptors – this is critical since many deletion and point mutations affect maturation of the receptors leading to a loss of significant decrease in cell surface expression.

Thank you very much for pointing out these issues. We addressed all of these issues in our responses to point 4. Please refer to our responses there.

12. Lines 342-344

Not clear. Since data fits single exponential function there is only one t component. The t2 component in the figure corresponds to that of the t2 for wild type receptors shown in Fig 1D obtained by the sum of two exponential functions.

Thank you very much for pointing this out. To satisfy the length limit for an article in Nature Communications, perhaps we oversimplified the explanation.

These are about the results shown in **Fig. 3c**. There exists only one decay component for the deletion mutant, KOR(Δ365-380), MOR(Δ358-382), or DOR(Δ357-372). However, their time constants (τ's) are longer than the incidental colocalization lifetimes (τ_{inci}'s), as evaluated by the rotated overlays of the magenta and green images: 58 vs. 45 ms for the KOR mutant, 54 vs. 47 ms for the MOR mutant, and 54 vs. 45 ms for the DOR mutant. Based on these results, we speculate that these slightly longer decay time constants for these deletion mutants (τ's) compared to τ_{inci}'s are due to the existence of weak homodimerizations whose lifetimes are quite close to τ_{inci}'s and/or to the existence of very small fractions of homodimers. The lifetimes of such components could not be separated from the τ_{inci} component, but they would make the decay time constant (τ's) longer than τ_{inci}'s. The part mentioned by Reviewer 1 has been revised on **pp. 13-14, lines 358-367** in the revised manuscript.

13. Fig 3D shows co-localization index with point mutations of KOR, MOR and DOR.
What was their cell surface expression levels and functional activity compared to wild-type receptors?

Thank you very much for pointing out these issues. We addressed all of these issues in our responses to point 4. Please refer to our responses there.

14. Line 390.

Change K-, M-, D-, to MOR, DOR and KOR for consistency.

Thank you very much for pointing this out. In the original manuscript, we defined the "peptides with the same amino-acid sequences as those of the deleted parts of the mutants, conjugated to the C-terminus of mGFP" as "mGFP-Kpep, Mpep, and Dpep, collectively called mGFP-Xpeps" (**p. 15**). However, as indicated by Reviewer 1, Kpep, Mpep, and Dpep were not defined. In the revised manuscript, we explicitly defined them (**p. 15, lines 395-398; p. 15, lines 420-423**).

Reviewer #2 (Remarks to the Author):

A combination of new theory combined with advanced single-molecule experiments complemented with data analysis developed by Zhou et al. enabled quantification of koff and KD for monomer-dimer equilibrium for the plasma membrane-associated opioid receptors (ORs). Beyond ORs, this dimerization kinetics approach could be applied to other plasma membrane molecules, and it is thus a significant advance in the field. The authors identified that residues close to c-terminal cytoplasmic domains of ORs are responsible for specific homodimerization interactions while trans-membrane domain of MOR (TM1MOR) showed non-specific interactions; as such, the Authors suggest that TM domains may be involved in both homo and heterodimerization. Developed peptides (based on the residues close to c terminus) specifically reduced OR homodimerization and may be valuable tools for the field as they show unique effects on signaling for the three canonical ORs. The results are generally rigorous and well interpreted; however, few questions should be addressed before the manuscript is ready for publication in Nature Communications.

Thank you very much for your understanding of the purposes, results, and biological significances of the present work and for considering its impact on broader fields in membrane biology. Thank you very much also for giving a nice summary of this work and your kind words.

For the convenience of addressing the points, we have numbered the following specific points raised by Reviewer 2.

(1) Suppl Fig 1c-f shows CD47 was used to assess labeling efficiency. Why was this molecule chosen when LDL receptor was used later in the manuscript as a monomeric model molecule (and KD was provided)? Is the accessibility of tag/labeling efficiency associated with extracellular loop in different receptors an issue?

Thank you very much for raising this point. It simply reflects our study history. In our previous work (Tsunoyama et al., 2018), we employed CD47 as a monomeric, non-raftophilic molecule, and we initially used CD47 as a monomer control molecule in the present research. With the progress of this work, we started using the transmembrane domain of TM^{LDLR} as a monomeric non-raftophilic single-path transmembrane protein, as a control molecule of various TM domains of ORs. Following the comments by Reviewer 2, we also examined four other molecules under monomeric conditions, including TM^{LDLR}. Please see our response to Reviewer 1's Specific comment 2, where Reviewer 1 expressed related concerns. The molecule using TM^{LDLR} is molecule (2) there. For a quick review of this point, please see **Supplementary Fig. 1e and its caption.** The result is shown in **Supplementary Fig. 1g and its caption**.

(2) The Authors should explain why pair cross-correlation functon(PCCF) 0-100 nm was used for calculation of colocalization index given the decay rate of the function and 50 nm bin.

We appreciate your raising this issue. By broadening the range from 0-50 nm to 0-100 nm, we might lose the sensitivity for colocalization but will gain a better signal-to-noise ratio (lower SEM). While the K_D determination uses the entire PCCF, the colocalization index is simply determined by the ratio. Therefore, we decided to use the ratio after averaging over two bins (0-100 nm and 400-500 nm), which would also provide better signal-to-noise ratios. This is now described at the end of the **caption to Supplementary Fig. 2e**.

(3) Was colocalization threshold distance of 200 nm in part effective for calculations due to generally low molecular densities (one or fewer fluorescently labeled molecules in the diffraction limited spot)?

Thank you very much for raising this important point. This is now explicitly examined by simulation and the result is shown in **Supplementary Fig. 2f**, where the ratio of the number of incidental oligomers (those greater than dimers) vs. the number of true dimers is plotted as a function of the colocalization threshold distance (R). As indicated by Reviewer 2, with an increase of R, the ratio will increase, and with an increase of the expression levels (number density of fluorescent spots; see curves with different colors), the ratio becomes greater. At the density of ≈1 fluorescent spots/μm^2 used in this study, the ratio is ≈0.025 at R = 200 nm, which is a very small fraction unlikely to greatly affect the determined K_D. These points are now described in the **caption to Supplementary Fig. 2f**. The justification of using R = 200 nm is summarized on **p. 7, lines 174-183 in the Results, p. 48, lines 1091-1096 and the paragraphs that follow, and "(6) Experimental justification of using a threshold distance *R* of 200 nm" in Supplementary Note 1**. Please also refer to our response to point 5 raised by Reviewer 1.

(4) Were molecule paths largely random?

In the CHO-K1 cell plasma membrane, >95% OR trajectories were classified into simple Brownian by the criteria described in **Kusumi et al., 1993**. This is now described on **p. 6, lines 146-147.**

(5) SEM for MOR KD was ~3 higher than that for KOR and DOR. Could this be associated with the bigger heterogeneity in MOR organization between cells?

We would think it rather represents the statistical variations of the measured SEM values. As Reviewer 2 indicated, it is true that the SEM for the MOR K_D was greater than those of the KOR and DOR K_Ds. However, the ratio of the SEM vs. mean value for MOR is about the same as that for KOR, suggesting that the SEM for the MOR K_D would not be associated with greater heterogeneity in MOR organization among different cells, compared with the heterogeneity in KOR organization. Meanwhile, this ratio is about 3x smaller in the case of the DOR K_D. Therefore, we think that such SEM variations represent the statistical variations of the measured SEM values. This level of SEM

variations was difficult to reduce because the number of cells we examined was ≈20 (for the precise numbers of cells, see **Supplementary Data 2**), which is already quite hard to do, considering the time and efforts for experiments and analysis. This point is now included in **Table 1** In the revised manuscript.

(6) Supplementary Table 1 should include number of independent measurements.

Thank you very much for pointing out our oversight. We have added the number of independent measurements in **all tables, including Supplementary Table 1**.

(7) DeltaN-OR mutants do not fit well in the figure 1c, especially as they are discussed later in the manuscript. Perhaps include with Fig. 3b.

We agree. We have now placed the ΔN mutant data in **Fig. 3b**.

(8) In several places secondary binding site (TM domain) is hinted but not explained; perhaps revise so the flow is not interrupted (e.g. include this part later: Line 333 "which might be the TM domains, as previously suggested for ORs26, 49, 50, 52, 53 and other class A GPCRs.21, 39, 40, 41 However, as shown in Supplementary Fig. 4a; the aa identities of TM domains among the three ORs are quite high, and thus the homo-interaction between TM domains might be less specific. We will revisit the TM interactions for homodimerization later.")
This part is even more challenging at current location:
Line 445 "The reason for this higher tendency to form homodimers even in the presence of FAM-Dpep-TAT is unknown, but we propose that the agonist-induced conformational changes enhanced the interaction in the TM domains, because DOR homodimerization also involves TM-domain interactions (see Fig. 5, which will be discussed in 449 the next subsection)." Moving this to discussion could be beneficial.

We are grateful for this suggestion. These statements were indeed out of place and destroyed the flow. Parts of these were moved to **p. 18, line 481 in the subsection of "MOR's transmembrane domain 1 ($TM1^{MOR}$) is involved in MOR homodimerization with less specificity."** In the Discussion, the involvement of TM domains has already been discussed quite extensively and therefore no further changes were made (**p. 26, lines 703-715**).

(9) The Authors should discuss why was FAM-TET peptide more efficient in preventing homodimerization (significant effect at 3 uM vs 6 uM for Xpep-GFP).

This is indeed a good point, which we refrained from discussing because we were afraid that, if we did, it might sound too speculative. We think mGFP-Xpeps are less effective for blocking homodimerization compared with Xpep-TATs, due to steric hindrance from the larger mGFP

bound to Xpeps. Following the recommendation by Reviewer 2, this is now mentioned on **p. 16, lines 431-433** in the revised manuscript.

(10) Rationale for addition of pyrenebutyrate to cells should be included (line 393) (presumably to increase peptide translocation).

Thank you very much for raising this point. Reviewer 2 guessed correctly that the addition of pyrenebutyrate is to increase the efficiency of peptide translocation across the membrane, and it would be a good idea to mention this so that readers do not need to check the reference cited there. We added this point on **p. 16, lines 423-425**.

(11) While MOR-KOR heteromers are not likely, did the Authors test the effect of TM1MOR on KOR-KOR homodimerization (or on another GPCR)? The question is how (un)specific is the effect of TM1MOR peptide.

This is an interesting idea, and therefore, we examined the influences of $TM1^{MOR}$ and TM^{LDLR} (negative control) on KOR homodimerization, and found no significant effect (**Fig. 5e**). This is now described on **p. 18, lines 501-509**.

(12) Is it possible that other TM domains or extracellular loop regions that were not tested (e.g., TM4) facilitate interactions for homomers (albeit the effect would be lower)? Perhaps include a sentence regarding this in the discussion.

Thank you very much for raising this point. We included discussions about this point on **p. 26, lines 709-718** in the Discussion.

(13) The Authors used low expression levels (where oligomers could be largely neglected). Based on their conclusions regarding both C terminal domains and TM domains affecting OR organization and their simulation data (Fig S3), it may be useful to discuss if oligomers could be present (in addition to monomers and dimers) on the cell membranes in physiological conditions. There is a little note on this in the companion paper, something similar would be helpful here.

Thank you very much for raising an interesting issue. We added a paragraph discussing this issue in the Discussion (**p. 26, lines 716-719**). Furthermore, inspired by this point raised by Reviewer 2 as well as point 7 raised by Reviewer 5, we calculated the densities of hetero-trimers (DOR homodimers + MOR monomer and MOR homodimers + DOR monomer in the membrane containing MOR and DOR) based on the K_Ds for MOR homodimerization and DOR-MOR heterodimerization (companion paper, **Fig. 8a**).

Minor comments:

(1) Line 376 Fig 4a,b: typo for figure referencing?

Fixed (**p. 15, lines 404-407**).

(2) Few parts in the results are more appropriate in the discussion:
Lines 222-227; Lines 295-303; Lines 431-436; Lines 556-568

Thank you very much for your recommendations.

Lines 222-227 and Lines 295-303: Moved to Discussion with slight expansion (**pp. 24, lines 662-670**).

Lines 431-436: Moved to Discussion with slight expansion (**p. 25, lines 680-687**).

Lines 556-568: Moved to Discussion with slight expansion (**p. 27, lines 742-756**).

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Reviewer #4 (Remarks to the Author):

In this manuscript Zhou and co-workers utilize a single molecule fluorescence based approach to provide a novel inspection of opioid receptor dimerization. For about 50 years the concept of opioid receptor oligomerization has been proposed, initially based of pharmacological data, then biochemical and fluorescent and bioluminescent experimental data. This early work however mostly relied on recombinant cell systems that overexpress the opioid receptors. Recent studies using more sophisticated techniques have not provided a clear answer whether GPCRs, or opioid receptor, form dimers. Zhou et al show that opioid receptors form temporary dimers at a rate that his higher than random encounters. The duration and occurence is mathematical and increase with receptor expression. The authors, identify regions in each OR that contribute to the dimerization. The authors created interfering Xpeps to break up dimers, which did not impact internalization but did have unique effects on agonist-induced signal transduction. The authors have carried out a tremendous amount of work with multiple controls in some cases using distinct controls for the same question.
I have a small number of questions and suggestions that if addressed can assist other readers in understanding the interpretation of the work.

Thank you very much for your careful and critical reading of the manuscript and providing constructive comments. Thank you very much also for your kind words.

For the convenience of addressing the points, we numbered the following specific points raised by Reviewer 4.

(1) The authors use a chimeric Gqi protein to divert the OR signal transduction to calcium mobilization, what was their reasoning for this? Was it to better align the speed and short duration of dimer formation with a fast signaling endpoint or was it done for easy fluorescent microscopic analysis. In either case, it would be helpful if the authors could carry out or at least address if similar results can be expected for cAMP inhibition.

We thank Reviewer 4 for raising this important issue. Observing Gi activation is quite tricky. Unlike the stimulatory effect by Gs-coupled receptors, the decrease in the cAMP level from its steady-state level is subtle and difficult to quantify with high sensitivity. Even before stimulation, GPCRs generally induce low levels of constitutive basic signals, and in the case of Gi-coupled GPCRs, upon stimulation, the constitutive signals occurring at low levels are further decreased. This is difficult to detect and therefore two methods have traditionally been used. (1) Using cells overexpressing Gi-coupled GPCRs, first the adenylyl cyclase is artificially activated by forskolin to increase the cytoplasmic cAMP levels and then, following the stimulation of overexpressed GPCRs, the reduction of cAMP levels is observed. (2) Using cells expressing Gqi5 together with physiological expression levels of Gi-coupled GPCRs (in our case, 1 ± 0.5 fluorescent spots/μm^2, the same as

those used for single-molecule imaging), the increase in the cytoplasmic Ca^{2+} level is observed. Namely, by using the chimeric G protein, Gqi5, the OR signal transduction is diverted from Gi-mediated cAMP inhibition to calcium mobilization. The signal is greatly amplified in the IP_3/Ca^{2+} pathway, and the OR expression levels can be kept in the physiological range.

Both methods have their own artifactual problems. In (1), cAMP levels can be determined, but the analyses are done under non-physiological conditions, using forskolin to stimulate the adenylyl cyclase and overexpressing GPCRs. In (2), Gi-coupled GPCR activation is, with the use of Gqi5, artificially linked to Ca^{2+} signaling, which is normally downstream of Gq GPCRs. However, the Gi-coupled GPCR expression levels are kept in the physiological range and there is no need to artificially activate the adenylyl cyclase.

Therefore, in either case, we will need to use somewhat artificial conditions for assaying the Gi-coupled GPCR downstream signals. We chose method 2, because we wanted to use cells expressing ORs at levels similar to those used for single-molecule imaging of OR homodimerization. These points are now further clarified in the **caption to Supplementary Fig. 1a**.

As recommended by Reviewer 4, we compared the results of (1; cAMP) and (2; Ca^{2+}) for a few cases in the revised manuscript.

First, we asked Prof. Asuka Inoue of Kyoto University, a leading specialist in GPCR signaling, to join in this project. His group routinely performs cAMP assays for GPCRs under various conditions, basically using method (1) we described above (Inoue et al., 2012; Janetzko et al., 2022; Kawakami et al., 2022; Ono et al., 2023; Tatsumi et al., 2024). For the current study, they employed one of the cAMP assay methods using GloSensor (Promega, but with modified protocols) (Kawakami et al., 2022; Tatsumi et al., 2024). Together with the Inoue group, we found the following (**Supplementary Fig. 1a-c**).

(1) The DAMGO (an MOR agonist) dose dependence of the cytoplasmic cAMP response in MOR-Halo7 (which was used in experiments reported in the heterodimerization paper) expressing HEK cells is very similar to that observed by the Ca^{2+} response via Gqi5 in MOR-Halo7 expressing CHO-K1 cells. The DAMGO dose dependences of the cAMP response are very similar between non-tagged MOR and MOR-Halo7 (**Supplementary Fig. 1a-c**), which is consistent with the lack of an effect of Halo conjugation to MOR observed by Ca^{2+} responses (**Supplementary Fig. 1d**).

(2) The SNC80 (a DOR agonist) dose dependence of the cAMP response in SNAPf-DOR expressing HEK cells is very similar to that observed by the Ca^{2+} response in SMAPf-DOR expressing CHO-K1 cells. The SNC80 dose dependences of the cAMP response are very similar between non-tagged DOR and SNAPf-DOR (**Supplementary Fig. 1a-c**), which is consistent with the lack of an effect of SNAPf conjugation to DOR observed by Ca^{2+} responses (**Supplementary Fig. 1d**).

Furthermore, we performed cAMP assays for two crucial cases. One examined the effect of the peptide blocker for DOR-DOR homodimers on the agonist-induced signaling response (Supplementary Fig. 7b-d in paper 1) and the other measured the effect of the peptide blocker for

DOR-MOR heterodimers (Dpep(20-42)DM) on the agonist-induced signaling response (Supplementary Fig. 8a-c in paper 2). In all examinations, we found excellent agreements between the cAMP assays and Ca^{2+} assays.

(2) On page 6, line 124, the authors state that their SNAPf-OR was labelled with two SNAP ligands at 60 and 61% effeciency. Perhaps I am understanding this incorrectly, but I read this as one type of SNAPf-OR simulataneously being labeled with the 549 and CF660R, which can maximally lead to all receptors 100% being labeled, making 60+61% not possible.

We apologize for the confusion. Since the fluorescent spot numbers for green and magenta spots are equalized, for the mixed labeling, the labeling fraction for each dye was approximately half of the labeling efficiency determined as shown in **Supplementary Fig. 1d-f**. Since the method of evaluating the labeling efficiency was improved by the advice from Reviewers 1 and 2, the fluorescence labeling efficiencies under our normal observation conditions (labeling at 300 nM) determined this way were 0.6631 ± 0.0107 for SNAP-Surface 549 and 0.6148 ± 0.0077 for SNAP-CF660R (mean ± SEM of five proteins). Therefore, for the entire molecular population, the labeling efficiencies will be about 33% and 31%, respectively. This point has now been clarified at the **end of the caption to Supplementary Fig. 1f. and p. 6, line 134-138** in the revised manuscript.

(3) Page 22, line 588. This Result.. may be confused as refering to the results in this manuscript, by a careless reader. in line 589 please elaborate on the however, see14, such that the reader does not have to dig up the paper and hope to understand what the authors want the reader to come away with.

Thank you very much for pointing this out. This part has been rewritten (**p. 24, lines 643-647**).

Reviewer #5 (Remarks to the Author):

In the present work Zhou et al. investigated opioid receptors (ORs) homodimerization using single-molecule imaging techniques at low physiological concentrations. This study confirmed that μ-, κ-, and δ-ORs transiently form homodimers, revealing that their C-terminal cytoplasmic domains drive specific homodimerization while transmembrane domains contribute less specifically. Additionally, the authors provide accurate estimates of kinetic and thermodynamic dimerization constants. The authors produced an important amount of data that are informative when compared to traditional methods (e.g., BRET, FRET) that use higher protein densities. Considering the pharmacological relevance of the ORs, the findings might provide insight into the modulation of analgesic response through ORs dimerization.

Thank you very much for your careful and critical reading of our manuscripts and giving us comprehensive reviews and important advice.

For the convenience of addressing the points, we numbered the following specific points raised by Reviewer 5.

(1) The authors submitted another paper (namely part 2) that uses the same approach to study the heterodimerization of ORs. Considering that the topic of the two papers is the same and even the methodology is identical, I suggest merging the two works into one that could highlight the main findings for the ORs homo- and heterodimers, reporting all the technical information in the supplementary information. The current manuscripts are lengthy and include numerous technical details that, while relevant to specialists, could be streamlined by merging the works. Key suggestions include:
1. Focus: Emphasize C-terminal interactions in homodimers, N-terminal interactions in heterodimers, and expand on signaling and internalization pathways (see comments below).
2. Data Presentation: Include thermodynamic and kinetic constants in tables; detailed TM interaction results could be moved to supplementary information
3. Broader Impact: Combining these studies will yield a robust foundation for future investigations into opioid receptor and GPCR dimerization that will be of interest for the Nat Commun readership once the following points will be addressed.

After intensive discussions among co-authors, we decided to request that we keep the format of two separate manuscripts. The main reasons are the following. (1) As serious papers dealing with fundamentally important scientific questions, we think it is proper to provide fundamentally important raw data in the main figures. Some data can be moved to Supplementary information parts, but the main results should stay in the main figures. (2) Due to the limited word length for each paper, if we combine these two papers, we will need to oversimplify our explanations of our

results, critical findings, and their meanings. This is what we really want to avoid. For example, the importance of obtaining and using critical quantitative data (thermodynamic and kinetic constants K_D, k_{off}, and k_{on} for receptors) is difficult to convey to scientists who study membrane molecules at the levels of tissues and animals. (3) The methods developed for evaluating K_D, k_{off}, and k_{on} described in the homodimer paper (paper 1) are very powerful and should become critical tools for broad research areas of molecular interactions in the membrane in general. Therefore, we would not like to have this important method development part totally buried in very large amounts of other data when the two manuscripts are combined. This point is further explained in the following paragraphs.

Previously, it has been difficult to evaluate these values and, despite their critical importance in any quantitative studies of plasma membranes, there have scarcely been any studies obtaining these three constants, except for only two cases in the literature. We were the first to develop (and publish) such a method, providing these three constants for formyl peptide receptor (Kasai et al., 2011)**.** However, the method requires lengthy experiments and was less sensitive compared with the method developed here (also, the previous method to obtain k_{off} lacked mathematical rigor, which has been corrected by the theory described in paper 1), and so, it has not been used much. We believe quantitative evaluations of molecular interactions in the membrane, using K_D, k_{off}, and k_{on}, is one of the most crucial missing pieces in membrane biology, and advancing quantitative understanding of molecular interactions are one of the most critical and urgent issues for advancing membrane biology.

The descriptions of molecular interactions in the membrane have been very qualitative: for example, with regard to the formation of homodimers of ORs, people argue about the presence or absence of homodimers and heterodimers, which is somewhat useless without the evaluation of KD, because depending on the number densities of ORs in the membrane employed in individual studies and the sensitivities of the employed methods, researchers can obtain either answer (absence or presence) as pointed out by Reviewer 5. Therefore, quantitative understanding is a key issue now. We are hoping to change this situation, by the methods we developed in this study.

The first manuscript has the important aspect of explaining such a key technology as well as showing its usefulness and power by applying it to the detection of OR homodimers, which is an important scientific subject matter. We do not want to have this method hidden under the large data sets for OR dimerization from the broad readership. Based on Reviewer 5's comments, we now consider that these points were not clear in the original manuscript, and therefore, we revised paper 1 to clarify this point on **pp. 4-5, lines 90-105 in the Introduction and p. 23, lines 614 -628 at the beginning of the Discussion**. To further clarify this point, we retitled the first manuscript. It is now entitled, "**Single-molecule characterization of receptor dimerization: Metastable opioid receptor homodimers modulate signaling and internalization**".

With regard to the three key suggestions by Reviewer 5, we will address them in our responses to specific points. However, we would like to simply point out the following two issues.

(1) Recommended focuses are fine with us. However, please note that, for DK heterodimerization, the interaction sites are not the N- or C-terminal domains, but the EL3 domains of DOR and KOR, and TM1 and TM4 of DOR. Therefore, oversimplification should be avoided.

(2) As recommended by Reviewer 5, thermodynamic and kinetic constants have been moved to tables in the revised manuscript.

We respectfully argue against moving the TM data to the Supplementary information. Secondary binding domains supporting/enhancing dimerization are also important for understanding the nature of homo- and hetero-dimerization. Furthermore, in the literature, TM interactions are often considered to be most important for homo- and hetero-dimerizations (Dijkman et al., 2018; Ferré et al., 2014; Huang et al., 2015; Johnston et al., 2011; Manglik et al., 2012; Meral et al., 2018).

Reviewers 2 and 3 (Reviewer 3 is a junior reviewer) mentioned their interests in TM contributions for dimerization.

Our conclusions that $TM1^{MOR}$ interactions with MOR and DOR have supporting roles for enhancing both MOR-MOR and DOR-MOR dimerizations with lower specificity provide a crucial perspective not only in the field of opioid receptors, but for other GPCRs as well as many other transmembrane proteins. Therefore, we hope to keep the TM interaction part in the main figures and text.

(3) We agree with the "Broader impacts." However, we would like to emphasize the extremely important impact of the newly developed methods to evaluate the thermodynamic and kinetic constants for membrane molecules on any molecular interaction and binding studies of the plasma membrane and other cellular membrane systems. We would like to ensure that this point is not missed in the literature.

(2) Cell line environment: Given that experiments were performed in CHO-K1 cells lacking endogenous OR expression, it would be beneficial if the authors discussed how CHO-K1's plasma membrane composition might influence GPCR dimerization, potentially differing from native environments in homo- and heterodimer formations. Effects of lipids composition and cholesterol concentration are known on GPCRs dimerization (10.1016/j.sbi.2011.09.007) and they should be discussed;

Thank you very much for your important suggestion. In the **caption to Supplementary Fig. 7**, we added an explanation, stating that the PM composition, and particularly the amount of cholesterol, might influence GPCR dimerization (Dijkman et al., 2018; Harding et al., 2009; Oates and Watts, 2011). Furthermore, the MOR homodimer lifetimes were evaluated in the intact cell PM, cholesterol-depleted PM, cholesterol-repleted PM after depletion, and PM of GM3-depleted CHO-K1 cells (Yamashiro et al., 1995). No significant differences were found. These results are

described on p**. 19, lines 516-520** in the revised main text and the data are shown in **Supplementary Fig. 7**.

(3) Impact of fluorescent dyes on dimerization: Fluorescent tags or dyes used in experiments may affect ORs dimerization and binding characteristics, potentially altering receptor behaviour (e.g., OR tilting with respect to the membrane plain and protomer-protomer contacts). Discussing how dye selection and labeling strategies were controlled or assessed for minimal interference would add rigor, particularly since GPCRs are sensitive to changes in structure and membrane environment;

We appreciate your point here. To address the point raised by Reviewer 5, we varied the tags (SNAPf and Halo7), tag positions (N- and C-termini), and fluorescent probes (SNAP Surface 549, CF660, and Surface Fluor 650). We examined the DOR dimer duration histograms and dimer lifetimes using the three combinations, and found no significant changes. These results are now shown in **Supplementary Fig. 2h** and described on **p. 10, lines 248-250**.

(4) Binding mode of OR dimers: No information is provided on whether the OR dimer binding mode is symmetric or asymmetric, nor on specific binding interfaces between protomers. Additional experiments, such as structural modeling or crosslinking assays, could clarify the binding orientation and interface characteristics of OR dimers. This data would help to elucidate the structural basis for dimer stability and its functional implications especially for drug discovery, adding depth to the study;

Thank you very much for raising an interesting point. We examined this possibility by using an AI-based structure predictor, Chai Discovery. We were able to obtain a prediction only for DOR's C-terminal-domain homodimer structure. The result exhibited the clear 180˚-rotational symmetry of the C-terminal-domain dimer. This result is shown in **Supplementary Fig. 5**, and its limitations are described in its **caption and the Methods**.

(5) Effect of ORs dimerization on cell signaling: The paper provides additional evidence of OR dimer formation, which is valuable for the field. However, the concept of OR dimerization is not novel in itself and has been demonstrated in previous studies. As such, to meet Nature Communications standards, it may be beneficial to frame these findings within a larger narrative, such as detailing how dimerization influences OR signaling and receptor trafficking. Additional experiments on the effect of ORs dimers on cell signaling and selective activation of specific pathways (biased signaling) would significantly advance our understanding of OR biology;

(6) Functional Insights into OR dimerization: Expanding the discussion of OR interactions with G proteins and beta-arrestins for both monomeric and dimeric forms would be highly beneficial. Could the authors conduct biased signaling studies under varying receptor concentrations to link dimerization ratios with signaling changes?

We thank Reviewer 5 for these important suggestions. We agree with the statement by Reviewer 5, "the concept of OR dimerization is not novel in itself", but respectfully argue against the statement, "(the concept of OR dimerization) has been demonstrated in previous studies." The concept has been around for some time, but various studies concluded that ORs do not form homodimers or heterodimers (some stated that under physiological expression levels), despite the presence of quite clear biochemical data for DOR (Cvejic and Devi, 1997), while the homodimerization of MOR and KOR has been more controversial. Reviewer 4 agreed with this point.

By developing methods based on single-molecule imaging to measure key thermodynamic and kinetic constants, K_D, k_{off}, and k_{on}, we can now conclusively state that the OR monomer-dimer equilibrium exists. Furthermore, our results provided a new dynamic view in which all three ORs undergo rapid interconversions between monomers and homodimers within a few seconds, even at expression levels of about 1 copy/μm^2, from a clear and solid quantitative basis (described in the Results section in the original manuscript). These points have been further clarified on **p. 24, lines 655-661**.

We also agree that "frame these findings within a larger narrative, such as detailing how dimerization influences OR signaling and receptor trafficking" is important, and this point has been addressed in Fig. 6 in the original manuscript as well as in the revised manuscript. Fig. 6 shows the OR internalization and downstream signaling with/without the homodimer blocker peptides; i.e., in the presence and absence (much lower amounts) of homodimers. Therefore, in the original manuscript, we basically addressed your point "experiments on the effect of ORs dimers on cell signaling and selective activation of specific pathways (biased signaling) would significantly advance our understanding of OR biology." We respectfully argue that OR internalization would be a critical aspect of biased signaling, which could be better called "biased response", because OR internalization is coupled with OR signal downregulation and is one of the major downstream pathways after β-arrestin binds to OR. Considering the presence of a variety of possible β-arrestin downstream pathways, we chose to observe a phenomenon that is clearly related to OR functional regulation, and how it differs between OR monomers and dimers (with and without homodimer blocker peptides). To clarify these points, we comprehensively revised the related parts in the revised manuscript. Please see **p. 19, lines 523-530, p. 21, lines 584-5591, p. 22, lines 603-606, and p. 27, lines 742-751** in the revised manuscript.

Furthermore, I would like to draw your attention to the very high levels of quantification of OR internalization performed in this work (**Fig. 6a, b and Table 2**). Studies of OR internalization at this accurate quantification level are rarely seen in the literature. These points were probably not clear in the original manuscript, and so, we further clarified them in the revised manuscript (**p. 20, lines 548-553**).

(7) Oligomerization Investigation: Although the focus of the works is on dimers, OR

oligomerization also influences GPCR signaling. Could the experimental data provide some insight into C-terminal and transmembrane contacts driving oligomerization?

We appreciate your suggestion. Although the primary sites for inducing homodimers are the C-terminal domains, our results suggest the involvement of the TM domains for enhancing dimer affinity, but with less specificity. Therefore, although under our expression conditions we hardly detected homo-trimers, homo-tetramers, and greater homo-oligomers, in cells where the expression levels are higher, ORs might form homo-oligomers greater than homodimers. Reviewer 2 raised a similar issue. We added a paragraph discussing this issue in the Discussion (**p. 26, lines 720-725**).

The consideration for ORs' greater oligomers will become more important for hetero-oligomers, due to the presence of specific independent affinities for homodimerization in the C-terminal domains and for heterodimerization in the N-terminal domains. Although this point has not been directly raised by Reviewer 5, her/his comment, as well as point 13 raised by Reviewer 2, inspired us to examine this possibility using calculations (because we did not see significant numbers of hetero-trimers or greater hetero-oligomers under our expression conditions); i.e., we calculated the densities of hetero-trimers as well as homo- and hetero-dimers in the plasma membrane containing MOR and DOR, based on the K_Ds of MOR homodimerization and DOR-MOR heterodimerization (companion paper, **Fig. 8b**).

(7) Receptor concentrations for KD values: It would be valuable to ensure that receptor concentrations used in the experiments are suitable for accurate determination of dissociation constants (KD). Verifying that concentrations fall within physiological ranges and optimizing them to avoid artifacts from underexpression will ensure the reliability of dimerization affinity measurements, making the findings more applicable to in vivo conditions;

Thank you very much for raising these critically important issues here. Two separate but related issues have been raised. (1) Are our OR expression levels employed to determine K_D within the physiological range? (2) Since, when determining K_D, its determination accuracy would be higher if the expression levels are comparable to K_D, are our OR expression levels quite comparable to the determined K_D? Did we use the right expression levels in this sense? Are they compatible with the physiological expression levels?

For (1), In the literature, previous papers stated that the physiological expression levels of ORs are in the range of 0.3 – 10 copies/μm^2 (Asher et al., 2021; Gentzsch et al., 2020; Meral et al., 2018), whereas the expression level we employed was approximately 1.8 molecules/μm^2, as described in our response to Reviewer 1's points 1a, b, and the **caption to Fig. 2c**. Therefore, the expression level employed in this study is basically well within the physiological range.

For (2), Reviewer 5's statement "optimizing them (concentrations used for the experiments to determine K_D) to avoid artifacts from underexpression will ensure the reliability of dimerization affinity measurements" is very correct. We found that Reviewer 5's concern about "artifacts from

underexpression" is crucial in some of the previous studies. In the papers reporting that no MOR and KOR homodimers exist at normal physiological expression levels (Asher et al., 2021; Drakopoulos et al., 2020), the authors employed the lower end of the physiological expression levels of 0.1-0.3 copies/μm^2. Meanwhile, we employed somewhat higher number densities of ≈ 1.8 OR molecular copies/μm^2, which is still well within the physiological expression range, to obtain the K_Ds for ORs (we found that 10-20% of OR protomers exist as dimers in the fluorescence image). Therefore, the dimerization event number per unit time in our study could be greater by a factor of 30-300 compared with the previous studies. Accordingly, the discrepancy might simply be due to the use of OR number densities lower than the sensitivity limit (very few homodimers exist in an image) in previous studies that reported negative results. This is now described on **p. 24, lines 662-670**.

The obtained K_D values for ORs (6.0-16.6 copies/μm^2) were about 3-9 times greater than the expression levels we used (1.8 copies/μm^2), which suggests that the expression levels employed here will provide reasonably correct K_D values for OR homodimerization (see SEMs in **Table 1**).

Another crucial consideration about the relationship between the expression level and K_D provides the upper limit of the measurable K_D, using the method developed here. For dimers with larger K_Ds, the number density of molecules needs to be increased as discussed above, whereas it also must be maintained at levels that do not induce excessive incidental oligomers greater than dimers. The simulation results shown in **Supplementary Fig. 3d** in the revised (also original) manuscript show that a true K_D value of 15 (as well as 1 and 5) can be evaluated with systematic errors less than 5%, even in the range of expression levels between 0.5 and 1.5 spots/μm^2 (8% in the range from 0.3 and 2 spots/μm^2), while expression levels greater than 2-3 spots/μm^2 could induce greater systematic errors.

Following this excellent point raised by Reviewer 5, we summarized these important considerations to be made when the developed method is used for experimental evaluations of K_D for membrane molecules, in the **section, "(8) Proper molecular concentrations (number densities in the PM) for dimer detection and K_D determination" in Supplementary Note 2**.

Further points for paper 1:

(8) Supplementary Information: The derivation in Supplementary Note 1 could benefit from some clarification. Equations lack explicit calculations, particularly in reaching equation 4, where the reference (12) is ambiguous. Additionally, certain boundary condition derivations, especially the Bessel function identity used, would benefit from detailed explanation for the general readership. In detail, the authors refer to reference 12 but no chapter is suggested. Is it chapter 3.2.1 for the "First boundary value problem with domain $0 < r < R$"? If so, please cite it properly. Authors should specify which point of the book in reference 12 should be followed and report explicit transformations to obtain equation 4. Moreover, one condition reported during the demonstration is

that 1 = 2Σm J0(yμn) / [μnJ1(μn)] is taken from xv = 2Σm Jv(yμm) / [μmJv+1(μm)], which is valid only for v>0 (eq. 10.23.21 in NIST as reported by the authors). However, they use it for v=0, is that correct? And why the authors have a counter by variable m if it does not appear in the formula? Finally, I could not find any property of Bessel functions for which J0(μn) - J'0(μn)μnε/R = J1(μn)μnε/R. I found two different ways to write J1 as a function of J0, but none of them meets the identity reported in the manuscript (d/dx[xmJm(x)] = xmJm-1(x) and J'v(x) = 1/2[Jv-1(x) - Jv+1(z)]).
The authors should clarify these and all the other steps in the equations derivation to allow understanding the mathematics for a reader not expert in the field and favouring the reproduction of the data;

We thank Reviewer 5 for carefully verifying the calculation. We have revised Supplementary Note 1 to address this concern and make the derivation easier to follow for non-experts.

As for Reviewer 5's specific points:
(1) Eq. 4. Indeed, the solution to the diffusion equation is given in Section 3.2.1. of reference 12, as suggested by Reviewer 5. Eq. (4) is obtained by taking the expression from reference 12 and substituting g(t)=0 (the absorbing boundary condition) and the initial condition given in the supplements for f(t). We explicitly explain these steps in the revised version.
(2) v=0. We agree with Reviewer 5 that the equality used in the text (10.23.21 from NIST) has been rigorously proven only for v>0 and therefore cannot be invoked for the case v=0. We have numerically verified that the equality holds for v=0 as well, but we are not aware of a mathematical proof. In any case, the fact that S(0)=1 can be seen as a direct consequence of the normalization of the initial condition. Therefore, in the revised version, we avoided using the equality from NIST and simply used the normalization condition to argue that S(0)=1.
(3) Finally, in the last step in Eq. (8), μn is used as a zero value of the function $J_0(x)$, and therefore the zero order term in the expansion vanishes. We also used $J'_0(x)=-J_1(x)$. This equality can be found in Eq. 10.6.2 in the NIST book. We added a sentence to explain this step in the revised version.

(9) Dimer Disruption by C-Terminal (C-ter) Peptides: The use of C-terminal peptides as a tool to disrupt dimerization is an indirect method, as these peptides may interfere with receptor dimerization even if the dimers are formed primarily through TM domain interactions. This limitation should be acknowledged, as it does not conclusively demonstrate that C-ter interactions alone govern OR dimerization. Discussing this as a methodological limitation and suggesting alternative experimental approaches (e.g., mutational analysis targeting TM regions) could strengthen the study's conclusions;

We employed three experimental approaches to conclude that C-terminal domains (without sequence similarities) are responsible for ORs' homodimerization: (1) deletion mutants (Fig. 3b-c), (2) point mutants (Fig. 3d), and (3) two ways of adding the peptides with sequences found by

approaches (1) and (2) (Fig. 4). Using these three approaches, the methodological limitation of the use of the peptide can be covered. This point has been added on **p. 25, lines 691-695**.

Perhaps, Reviewer 5 is mainly addressing the problem that in our examination of OR monomer/homodimer functions (G-protein-downstream signaling observed by Ca^{2+} response via Gqi5 and OR internalization, probably via β-arrestin, collectively called biased response in our answer to point 5), we only used the homodimer blocker peptides and not deletion or point mutants to observe the signals and internalization of OR monomers. This issue was in fact addressed at the end of the Results in the original manuscript. Following the advice in Minor point 2 by Reviewer 2, we moved this part to the Discussion and slightly expanded our explanation. Please see **p. 27, lines 744-756**.

(10) TM1's Inhibitory Role: The conclusion that TM1 inhibits homodimerization might be clarified by including negative controls to confirm TM interactions (e.g., using other TM peptides);

We appreciate your suggestion. Indeed, we used TM^{LDLR} (transmembrane domain of LDL receptor) as a negative control for blocking DOR and MOR homodimerization (**Fig. 5e**) in the original manuscript. However, prompted by this comment, we included the results of blocking KOR dimerization by various peptides (both $TM1^{MOR}$ and TM^{LDLR} were negative) in **Fig. 5e**. Furthermore, inspired by your comment, we examined the interactions of TM^{LDLR} with MOR and DOR (negative controls), which indicated scarce interactions (**Fig. 5b**).

(11) Comparative Temperature Analysis: The authors refer to a previous KOR homodimer study obtained at a different temperature. The authors should perform an additional experiment on KOR dimerization at 20°C to provide a direct comparison with the previous study and discuss the differences;

As recommended by Reviewer 5, we examined KOR homodimerization at 20˚C, and the results are shown in **Table 1** in the revised manuscript and discussed in the **Discussion** (**pp. 24-25, lines 671-678**). This part was originally in the Results section, but following the recommendation in Reviewer 2's second Minor point, we moved the description to the Discussion.

Our K_D value is smaller than the previously reported value (Cechova et al., 2021) by a factor of ≈6. The reason for this difference is unknown. However, the SEM/mean ratio in our case is 20%, whereas it is 47% in the previous report, suggesting that our estimate might be more accurate.

(12) Literature Citations: line 334 on page 13, and line 87 on page 4 of paper 2, consider citing chemokine receptor dimerization studies (doi: 10.1038/ni1027; doi: 10.1016/j.molcel.2018.02.034), including a recent work on CCR5 and CXCR4 (doi: 10.1038/s41467-023-42082-z).

Thank you very much for your suggestion. We cited the first two papers in paper 1 and the third paper in paper 2 at the places you indicated.

Additionally, on line 367 at page 14 the authors' statement "Furthermore, if we could develop such homodimerization blockers, they could become extremely useful tools to dissect the functions of OR monomers and homodimers, and might inform future developments of more effective analgesic treatments with fewer side effects and less tolerance development." should be motivated and supported by references;

Thank you very much for your suggestion. We have provided references for this statement (**p. 15, lines 395-398**).

(13) Figures: For Figure 2B, please clarify the data on the right side by adding columns.

As recommended by Reviewer 5 in the general remark at the beginning of this review, we have moved these tabulated data in Figure 2b to Tables. The left part of Figure 2b was moved to Table 1, which was expanded with new results. The right part of Figure 2b has now been deleted because virtually the same data are shown in **Table 3**. We clarified that the part of the table you mentioned shows the "Predicted %OR protomers existing as dimers at five expression levels: 0.3, 1, 3, 10, and 30 (copies/μm^2)."

Reviewer #6 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

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REVIEWER COMMENTS

Reviewer #2 (Remarks to the Author):

The Authors have thoroughly addressed all of my concerns, and the manuscript is significantly improved. The work by Zhou et al. incorporates substantial new insights into opioid receptor organization along with notable methodological advancements; it is ready for publication in Nature Communications.

Thank you very much for your very kind and positive support.

Reviewer #3 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Thank you very much for your very kind and positive support.

Reviewer #4 (Remarks to the Author):

The authors performed additional experiments, ran more controls and expanded discussions to adequately address my questions as well as those provided by other reviewers.

Thank you very much for your very kind and positive support.

Reviewer #5 (Remarks to the Author):

Reviewer 5's comments for homo- and hetero-dimerization appear to have been accidentally switched between the two manuscripts in the decision letter from the editor. Therefore, we placed them in the way we think is correct. These parts are shown using green fonts.

- The hetero- and homodimerization of opioid receptors is well-studied. The main novelty of these two papers lies in the use of low concentrations that effectively mimic physiological conditions, and in the precise determination of thermodynamic and kinetic constants.
The abundance of technical details makes it easy for the reader to lose sight of the main messages. I remain convinced that the authors should reorganize their material into one strong, clear paper focused on the thermodynamics and kinetics of OR dimerization, and a second, more technical paper suitable for a specialized journal, such as Nature Protocols. (This paragraph is the same for both homo- and hetero-dimer manuscripts.)

Thank you very much for your kind words about our research results.

Thank you very much for your suggestion of changing the publication scheme. We authors seriously discussed the matter of the publication scheme again, and we respectfully prefer to retain the current two-manuscript format. We believe that the theory and methodology developed in this study and presented in the first paper are not mere protocols, but rather represent a fundamental advancement in single-molecule imaging research. We are sincerely grateful for your understanding and consideration.

- Regarding the symmetry of OR dimers (point 4 in the rebuttal letter), the authors did not provide a satisfactory answer. They presented potential interactions between the C-terminal cytoplasmic domains using an AI-based approach (Chai Discovery). However, the algorithm failed to provide a conclusive result, and the analysis was limited to a small intracellular portion at the C-terminal region, leaving the geometry of the dimers unresolved. The result is not even supported by more rigorous techniques, such as molecular dynamics simulations, mutagenesis or cross-linking experiments.

Thank you very much for your comments. The main focus of the present manuscript (and our findings) is that intracellular C-terminal domains significantly contribute to homodimer formation of three ORs. Furthermore, we found that the disorder scores of the C-terminal domains of three ORs are quite high. Therefore, we consider that our present results of intracellular C-terminal interactions would hardly be able to provide any better insights into the issue of the organization of TM domains in OR homodimers (such as symmetric or asymmetric binding mode) than our present knowledge. Meanwhile, encouraged by your suggestions, we addressed the symmetry issue of the intracellular C-terminal domains in DOR dimers.

In this second revised manuscript, as instructed by the Editor to follow the recommendations by Reviewer 7 on the issue raised by you, we moderated our statements on TM interactions (dark red fonts on **p. 26**). Furthermore, we have included a short discussion about the

symmetric/asymmetric binding models about OR dimerization (green fonts on **p. 26**). We hope this will be a step forward to address the important issue in the future.

- Points regarding the G protein interaction with opioid receptor dimers remain unaddressed. The authors reminded a sentence from the original manuscript proposing future studies on this topic. (The Reviewer 5's point in this paragraph is basically the same as that of the second point for the heterodimer manuscript.)

We thank Reviewer 5 for the suggestion. The effect of G proteins on OR dimerization will be a next important target of our single-molecule investigation.

- It is unclear why high cDNA levels result in low receptor expression (approximately 1 fluorescent spots/μm2). The authors should address this point in the manuscript.

Thank you very much for pointing this out. We made a mistake in the Methods section (Cell culture, transfection, and microscope observations). There, we wrote "For cDNA transfections for Ca^{2+} mobilization and cAMP assays with Gqi5 and cAMPinG1, approximately 2×10^5 cells were mixed with 1 μg OR cDNA plasmids in 100 μL transfection buffer." However, "1 μg OR cDNA plasmids" here was a mistake. The correct statement is "1 μg cDNA plasmids encoding Gqi5 and cAMPinG1." This part has been corrected (green fonts on **pp. 44-45**).

For OR expression for single-molecule imaging and Ca^{2+} assays using CHO-K1 cells, approximately 5×10^6 cells were mixed with 200 ng OR cDNA plasmids). Using this method, the major population of the cells exhibited 0.5~1.5 fluorescent spots/μm^2.

For establishing CHO-K1 cells used for cAMPinG1 cAMP assay, which stably expressed OR at levels 5-10 times higher than those used for single-molecule imaging, we transfected $2x10^5$ CHO-K1 cells with 1 μg OR cDNAs, and cloned the transfected cells (green fonts on **p. 55**).

- We acknowledge the authors' efforts in addressing the other points, including conducting additional experiments. (This paragraph is the same for both homo- and hetero-dimer manuscripts.)

Thank you very much for raising important points in the first review cycle for improving our manuscripts.

Reviewer #6 (Remarks to the Author):

I co-reviewed this manuscript with one of the reviewers who provided the listed reports. This is part of the Nature Communications initiative to facilitate training in peer review and to provide appropriate recognition for Early Career Researchers who co-review manuscripts.

Thank you very much for your very kind and positive support.

Reviewer #7 (Remarks to the Author):

The two manuscripts by Zhou et al. include an extensive amount of well-designed and well-performed experiments with state-of-the-art techniques to analyze the dynamics of interacting single fluorescent molecules in mammalian transfected cells. These studies clearly demonstrate that μ, κ, and δ opioid receptors (MOR, KOR, and DOR, respectively) undergo repeated transient homodimerization (first manuscript) and heteromerization (second manuscript) with functional significance. The authors develop a very sound experimental and theoretical framework which this reviewer believes can represent a seminal work in the field of GPCR oligomerization. The studies include elegant experiments with the use of membrane-permeable synthetic peptides to disclose the differential involvement of specific C-terminal, transmembrane (TM) and N-terminal domains of the different ORs forming homomers or heteromers. In the second manuscript they also include in vivo experiments that provides evidence for the rationale of considering the heteromers as therapeutic targets. More specifically, to counteract the tolerance of opioid-induced analgesia by disrupting the MOR-DOR heteromers with the intracranial administration of a heteromer-disrupting peptide. A key result of these studies is that the degree of oligomerization depends on the membrane expression levels of the receptors, and they demonstrate their significant functional presence within a putative low physiological expression range.

Thank you very much for your very kind words for our manuscripts. We are especially thankful for your providing us with in-depth explanations and literature reviews about the physiological expression levels of opioid receptors and TM interactions among various GPCRs.

I have two main general comments that apply to both manuscripts that I believe deserve special consideration. These comments do not intend to add more experimental burden, although I would hope the authors will address them in future experiments.

1-The authors repeatedly state that the experiments are performed at "physiological expression levels". More specifically they state that 1 and 10 copies/μm2 correspond to low and high physiological membrane expression levels, respectively. This reviewer is not aware of any study that provides a clear answer to what are the normal membrane expression levels of any GPCR in neurons. None of the studies referenced to support this statement (refs. 26, 29 and 33, from the first manuscript) or from the references of those studies provide experimental evidence for what those physiological levels are. This is a real key question, since the authors clearly demonstrate that the amounts of homodimers and heterodimers (and heterotrimers, in the second manuscript) that exist at any time depend considerably on the real physiological expression. Based on many published correlational functional in vitro, in situ and in vivo studies on GPCR heteromers, and based on the well-known lack of uniformity of the neuronal plasma membrane and on many studies that demonstrate that GPCRs tend to concentrate in specific signaling areas of the plasma membrane (such as the concentration of MORs in primary cilia mentioned in the second manuscript), this reviewer believes that physiological levels are more often probably higher than 10 copies/μm2. This would imply that GPCR oligomers have a more significant functional role, including higher order than dimers, as implied in the second manuscript. At least, those that contribute to the most significant signaling effects at the cellular level. I do sympathize very much with the authors' theoretical discussion about the pulse-like signals (in the second manuscript), which implies that summation, aggregation of brief signals are what is needed to promote a detectable cellular signal, which promotes the functional role of GPCR oligomers. In fact, in the first manuscript the authors can only obtain a significant detectable Gi-mediated signal with preparations with higher, then probably more physiological, receptor expression. In summary, I do not believe that the authors can state (as in the legend to Fig. 2 in the first manuscript), that "the expression level employed in this study is basically well within the physiological range". I would state the following: that there are no clear results in the literature about which are the physiological expression levels of opioid receptors, but that there is reasonable doubt that those membrane levels are lower than 1 copy/μm2, which is what has been used in previous studies of single-molecule tracking where oligomers were rarely detected; that the present study shows unequivocally that opioid receptor oligomers (homo and heterodimers) represent a significant proportion of functional entities at levels between 1-10 copies/μm2; and that it is very possible that those levels are higher in areas of the plasma membrane with more signaling significance, which would imply an even higher functional significance of GPCR oligomers.

Thank you very much for raising these important issues and thoroughly explain the key points when physiological expression levels are considered based on our present knowledge. We totally agree with your comments and revised the manuscripts following your recommendations.

For homodimer manuscript (paper 1), please see the parts shown in blue fonts on the following pages: **p. 3, 4, 5, 10, 12, 23, 24, 31-32** (Fig. 2c and its caption), **and p. 43** (Table 3).

For heterodimer manuscript (paper 2), please see the parts shown in blue fonts on the following pages: **p. 3, 4, 16, 17, 19, 21, 22, and 27**.

We very much liked your summary at the end of this paragraph, and adopted in the following way (**pp. 23-24**, in blue fonts; homodimer manuscript). "The results unequivocally demonstrated that the three classical ORs all form transient metastable homodimers and OR homodimers represent significant proportions of functional entities at local number densities over 0.3 copies/μm^2 at 37˚C, both before and after the agonist binding (Table 3 and Fig. 2c). It is very possible that local number densities are higher in the PM areas with more signaling significance, which would imply an even higher functional significance of KOR and DOR homodimers and oligomers." Thank you very much.

2-The authors are probably not aware of a recent study by De Oliveira et al (Pharmacol Res, 2022, 182:106322) that reports MOR dimers and heteromers of MOR with galanin Gal1 receptors, and that analyzes in detail their TM oligomeric interfaces by using several techniques also including single-molecule imaging with TIRF microscopy. Instead of a single-molecule tracking analysis, they analyze the number of steps after photobleaching of single or co-localized fluorescent spots, which is a well-validated technique to study the stoichiometry of protein complexes, and that the authors of the present manuscripts have used in previous studies (Nagata et al., Proc Natl Acad Sci USA, 2013, 110:5034-5039). By using all possible 7 TM-peptides of the MOR, De Oliveira et al. found that TM5 and TM6 are involved in MOR dimerization, which is well in agreement with the crystallographic results of Manglik et al. (Nature, 2012, 485:321-326), and they obtained the same results by using the 7 TM-peptides of the MOR with bimolecular fluorescence complementation. This is an additional technique than the authors of the present manuscripts have also used and validated for the study of oligomeric interfaces in what I consider a seminal paper in the field (Kasai et al., J Cell Biol, 2011, 192:463-480). With both techniques and by also using the TM-peptides of the Gal1R, De Oliveira et al. also provided evidence for a tetrameric quaternary structure of the MOR-Gal1R heteromer (with two dimers of each receptor) and a significant involvement of TMs in the heteromeric interface.

In the first manuscript, Zhou et al. find conclusive evidence for a role of the intracellular C-terminal domains in MOR, DOR and KOR homodimerization, which was not evaluated in the study by De Oliveira et al. Then, by using the TM1-peptide of the MOR they find a weaker involvement of TM1 of MOR in MOR homodimerization, which was not observed in the study of De Oliveira et al., and conclude that TMs have a less specific, weaker and secondary role. (A)

The authors should moderate this conclusion and discuss their results in relation to those obtained by De Oliveira et al. According to those results, the use of TM5- and TM6-peptides of the MOR should have also disclosed a significant role of the corresponding TMs in MOR homodimerization. Therefore, most probably their conclusion only applies to TM1. It is unfortunate that the authors were not aware of the study by De Oliveira et al. and did not analyze the effect of TM5- and TM6 peptides. As I mentioned above, I understand that there are so many more additional experiments that the authors could add to their extensive study, but I would encourage the authors to run these experiments in the future. Nevertheless, they should moderate their general interpretation about the lesser role of TMs in oligomerization. What the study of De Oliveira et al, and other studies that use TM-peptides to interfere with the interfaces of GPCR oligomers, show is that the contribution of each interface is not additive, that the interference with any of the different interfaces leads to a near maximal disruption. And that is, in fact, what the authors show in the second manuscript, where TM1 and TM4 play a similar significant role than the N-terminal domains in MOR-DOR heteromerization, which they discuss as an apparent cooperative allosteric effect.

An additional very compelling study that demonstrates the significant role of TMs in GPCR heteromerization was recently published study by the same research group of De Oliveira et al. This study uses TM-peptides with different lengths and amino acid substitutions to disclose the specific residues involved in a TM5-TM6 interface of the cannabinoid CB1 receptor-serotonin 5-HT2A receptor heteromer, which leads to the prediction of small molecules that can specifically interfere with this heteromerization (Matsoukas et al., J Med Chem, 2025, 68, 261-269). This study represents the proof of concept of the possibility of using small molecules, instead of non-brain-penetrating peptides, to interfere with specific GPCRs heteromerization. (B) I suggest the authors add this possibility to expand their suggestions about translational implications, such as those suggested at the end of the Discussion in the second manuscript about MOR-DOR heteromers and the tolerance to the analgesic effects of opioids.

(C) Finally, the existence of electrostatic interactions between basic amino acids (particularly adjacent arginine residues) and acidic amino acid residues (particularly adjacent glutamic or aspartic or phosphorylated residues) in intracellular domains mediating receptor oligomerization has been previously stressed and it should be acknowledged. Particularly important has been the work of the late Amina Woods (see for instance, Navarro et al., J Biol Chem, 2010, 285:27346-27359).

We appreciate your extensive explanations of TM interactions in the literature, which we missed previously.

Your three specific recommended revisions in your point 2 are marked as (A), (B), and (C) above, and we explain our revisions in the following.

(A) Following your recommendation, we moderated our conclusion about the TM interactions (less specific interaction between TM domains) and limited our discussion to MOR's TM1. Please see the parts highlighted in blue on the following pages.

> For homodimer manuscript (paper 1), please see the parts shown in dark red fonts on **p. 26**.
>
> For heterodimer manuscript (paper 2), please see the parts indicated by dark red fonts on the following pages: **p. 13, 25, and 26**

We now discuss our results in relation to those obtained by De Oliveira et al. on **p. 26** of the homodimer manuscript and also on **p. 26** of the heterodimer manuscript (shown in green fonts).

(B) This point is now discussed at the end of the heterodimer manuscript (dark red fonts on **pp. 28-29** in paper 2).

(C) This point has now been included on **p. 14** (dark red fonts) of the homodimer manuscript (paper 1).