This repository provides the source code and input files for extracting the density of a conformational probe, like engineered G proteins or nanobodies, at the plasma membrane. This work is based on the following manuscript: “Multimodal intrinsic activation of GPCRs in ultrastable plasma membrane nanodomains” by Kockelkoren et al.

Below, we provide installation guidelines and instructions for the use of the source code on an exemplary dataset.

Software dependencies and operating systems
This software is dependent on the use of Matlab (MathWorks Inc.) and requires input data processed by previously published algorithms by Kockelkoren and Lauritsen et al. in *Nature Chemical Biology* (2023). Importantly, the results produced by this code are dependent on the specific conformational probe and the fluorescent channels used to record it. Therefore, this code cannot directly be used for any dataset, but requires precise knowledge and calibration of the ‘zThresh’ value, as described in detail in the Methods section of the manuscript.
The source code can be operated on Windows, Mac and Linux using Matlab.

Versions the software has been tested on
The software has been tested on MATLAB versions: 2019b, 2022b and 2023a.

Any required-non-standard hardware
To run this code only Matlab (MathWorks Inc.) is required.

Instructions for installation
Matlab (MathWorks Inc.) can be downloaded from Mathworks on the following website and requires a MathWorks account: https://www.mathworks.com/help/install/install-products.html

Next to the base version of Matlab several toolboxes should be downloaded:
- Statistics and machine learning toolbox
- Curve fitting toolbox
- Parallel computing toolbox

Typical installation time on a ‘normal’ desktop computer is around 45 minutes. Hereafter, running the code on a typical desktop takes around 5 minutes.

Instructions to run data
1. To run the source code two input files are provided: “B1AR.mat” and “miniGStack.mat”.
2. Two codes are provided in this repository: “Extract_SensorDensity_final.m” and “PlottingDensity.m”. The first one is the main executable code and the latter is used for data plotting.
3. After opening the main source code in Matlab, the two input files should be added to the workspace (by dragging them into it).
4. Next, clicking the ‘Run’ button will result in a pop-up window that requires the manual selection of the most prominent peak (single mouse click and ‘Enter’ key) of the cytosolic intensity histogram. As described in the Methods, this peak

defines the average cytosolic intensity signal (Icyt) of the conformational probe, which is subsequently used for fitting.

5. The code will continue running and ultimately all output files are saved into ".mat" files in the chosen directory.
6. At the end of the run, the script will reveal the same image as in Figure 2b of the manuscript.

Outputs

There are 6 output files that are produced by the source code:

1. 'Fit.mat'
   Contains all fitting coefficients, the errors on the parameters and the adjusted R-squared values of the fit for every xy-pixel. The explanation of each coefficient can be found below. Here, 'dCoef' represents the Gaussian amplitude and is thus directly proportional to the density of the conformational probe.
2. 'FitType.mat'
   Contains for xy-pixel the fitting model that has been applied to it. Here the numbers 1 and 3 represent traces without a Gaussian contribution, while for traces categorized as 2 and 4 a Gaussian component has been identified. Number 5 is for unidentified traces that do not meet our classification criteria.
3. 'Icyt.mat'
   Contains the average intensity of the cytosolic signal of the conformational biosensor.
4. 'nom.mat'
   List of file names used as input.
5. 'results.mat'
   Contains a matrix called 'maxInt', which is the density of the conformational probe as reported in the manuscript. 'maxInt' is calculated from 'dCoef' after applying a 3x3 rolling-mean filter.
6. 'B1ARdata.mat'
   Contains all density information of the GPCR channel and the z-position of the plasma membrane.

As described in the Supplementary methods of the manuscript: "Multimodal intrinsic activation of GPCRs in ultrastable plasma membrane nanodomains" by Kockelkoren et al., we obtain the following parameters from the fitting procedure.

We used Eq. 1 to fit all axial intensity traces with 3 free parameters, i.e. A, B and D. A is the amplitude of the sigmoidal contribution, B is related to the width of the Gaussian and D is the amplitude of the Gaussian component. We use the Z position of the plasma membrane for parameter C, we fixed the offset F as the average intensity of the first 5 pixels of the trace, and we described E in terms of B.

$$I(z) = \frac{A}{1+e^{-B(z-C)}} + \frac{De^{-0.25E^2(z-C)^2}}{1+E^2(z-C)^2} + F \qquad \text{(Eq. 1)}$$

In the presence of intracellular vesicles, we adapted Eq. 1 and introduced a corrective term that fits the depletion created by the vesicle, as shown in Eq. 2 below. This introduces 3 additional free parameters that are constrained between Z_{PM} and the end

of the trace. G is the amplitude of the corrective term, H the centre position of the vesicle, I and J are related to the width and shape of the vesicle. The I term can be described in terms of B.

$$I(z) = \frac{A}{1+e^{-B(z-C)}} + \frac{De^{-0.25E^2(z-C)^2}}{1+E^2(z-C)^2} + Ge^{-\frac{(z-H)^2J}{2I^2J}} + F \qquad \text{(Eq. 2)}$$