Supplementary Figure 1

Optimized protocol to prepare calibration solution battery.

See Supplementary Methods for details.
Supplementary Figure 2

Half-gated NTC calibration for improved monitoring of [Ca\(^{2+}\)] fluctuations.

Normalised total count values (open circles) obtained by using the integrated count over the 5-10 ns post-pulse interval in the fluorescence decay, normalised against the count peak value (Fig. 5c). Red solid line, best-fit logistic function (as in Fig. 2b); reduced \(\chi^2\), 6.5278\(\times\)10\(^{-6}\); adjusted \(R^2\), 0.997.
Supplementary Methods

Calibration solution preparation (see also Supplementary Figure 1)

1. Pipette 3mL from HS into the high calcium Eppendorf tube H.
2. Add 6μL from 10mM stock OGB-1 solution F into H to make up 20μM concentration of OGB-1 in H. The concentration can be adjusted so that photon count rate is within 1-5% of the laser pulse repetition rate.
3. Pipette 1mL from LS into the low calcium Eppendorf tube L.
4. Add 2μL from F into L to make up 20μM concentration of OGB-1 in L. The concentration here should match that of H as decided at step 2 in order to maintain the same OGB-1 concentration throughout the measurements.
5. Use L as the mixing Eppendorf M, relabel it from L to M.
6. Extract 100μL from M into a new Eppendorf tube and labelled it N0 to indicate this is the nominally 0 mM calibration solution.
7. Extract 100μL from H and add into M then mix well. Solution will continue to mix whilst carrying out step 8.
8. Pipette 20μL from N0 and deposit it onto a clean sterile glass slide labelled S0. Quickly cover it with a coverslip and drop sufficient distilled water on top of the cover slip for water immersion objective. Take care to find the middle of the solution layer using fluorescent intensity. Then take a FLIM trace measurement as outlined in Procedure. This step will be simply stated as taking the measurement hereafter and name of the slides prepared are named as Sx where x is the nominal calibration values.
9. Extract 111.1μL from M into a new Eppendorf tube N1 to indicate this is the nominally 1 mM calibration solution. Extracts from M are placed into Eppendorf named as Nx where x is the nominal calibration values hereafter.
10. Extract 111.1μL from H and add into M then mix well.
11. Pipette 20μL from N1 and take measurement S1.
12. Extract 125μL from M into N2.
13. Extract 125μL from H and add into M then mix well.
14. Pipette 20μL from N2 and take measurement S2.
15. Extract 142.9μL from M into N3.
16. Extract 142.9μL from H and add into M then mix well.
17. Pipette 20μL from N3 and take measurement S3.
18. Extract 166.7μL from M into N4.
19. Extract 166.7µL from H and add into M then mix well.

20. Pipette 20µL from N4 and take measurement S4.

21. Extract 200µL from M into N5.

22. Extract 200µL from H and add into M then mix well.

23. Pipette 20µL from N5 and take measurement S5.

24. Extract 200µL from M into N5.

25. Extract 200µL from H and add into M then mix well.

26. Pipette 20µL from N6 and take measurement S6.

27. Extract 250µL from M into N6.

28. Extract 250µL from H and add into M then mix well.

29. Pipette 20µL from N6 and take measurement S6.


31. Extract 250µL from H and add into M then mix well.

32. Pipette 20µL from N7 and take measurement S7.

33. Extract 333.3µL from M into N7.

34. Extract 333.3µL from H and add into M then mix well.

35. Pipette 20µL from N7 and take measurement S7.

36. Extract 333.3µL from M into N7.

37. Extract 500µL from H and add into M then mix well.

38. Pipette 20µL from N8 and take measurement S8.


40. Pipette 20µL from N9 and take measurement S9.

41. Take H and relabel it N10.

42. Pipette 20µL from N10 and take measurement S10.

37. Analyse saved measurements using the NTC analysis in the procedure section (Fig. 2a) to obtain NTC values for each calibration solution.

38. Use smallest estimate of residue calcium (e.g. from all constituent chemicals and glass surfaces) as the nominal 0mM value, for example 1µM as nominal value.

39. Use WEBMAXC and key in respective constant parameters such as pH, temperature, [ATP], [Mg^{2+}] and [BAPTA] (10 mM + [OGB-1] used 20 µM in this example) as used during calibration procedure to estimate [Ca^{2+}].

40. Alter the [Ca^{2+}] from nominal values to estimated values from step 39.

41. Plot NTC value from step 37 vs estimated [Ca^{2+}] from step 40, and fit the logistic function $f(x)=A_2+(A_1-A_2)/(1+(x/x_0)^p)$ using Levenberg Marquardt algorithm (Fig. 2b). The Adjusted $R^2$ value is >0.99 and typical reduced $\chi^2$ is in the range of 10^{-5}. [Troubleshoot]

42. Invert the estimated logistic function to the form $f(x)=x_0([A_1-A_2]/(x-A_2))^{1/p}$ with fitted parameter values as the calibration function for calculating all future estimated [Ca^{2+}] using NTC, on images or traces.