### Fig. 1, Source Data 1. Comparison of available approaches for measuring membrane potential in cells.

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| --- | --- | --- | --- | --- | --- | --- |
|  | **Patch-clamp electro-**  **physiology** | **Single color fluorescence intensity imaging** | **Two component ratiometric fluorescent sensors (FRET-oxonol)** | **Ratiometric, electrochromic fluorescent sensors (ANEPPS)** | **GEVI-based FLIM approaches** | **FLIM with VoltageFluors (VF-FLIM, *this work*)** |
| **Absolute Vmem resolution (between cell comparisons)** | excellent | nonea | very poorb | poorc | very poord | good |
| **Quantification of Vmem changes on a given cell** | excellent | nonea | poorb | poorc | poord | excellent |
| **Compatibility with long time scales** | poore | poorf | goodg | goodg | good | good |
| **Temporal resolution** | sub-millisecond | ~1 msh | 2-500 msi | ~1 msh | secondsj | secondsj |
| **Minimal invasiveness, damage** | very poore | good | poork | good | good | good |
| **Throughput (cells/day)** | 10s | 1000s | 1000s | 1000s | 1000s | 1000s |
| **Spatial resolution** | Single value per electrodel | Subcellular | Subcellular | Subcellular | Single value per laser pathm | Single celln |

aMeasurements vary too much to be converted to absolute voltage or interpreted across populations of cells. This variability is attributable to numerous confounding factors, including dye loading, photobleaching, and sample movement 9.

bWhile in principle less variable than a single-color fluorescence intensity measurement, in practice, the signal depends strongly on the loading of two independent lipophilic indicators 13,80, which can vary substantially.

cANEPPS excitation ratios depend on a variety of non-voltage factors, in particular the membrane composition, leading to substantial artifacts in optical Vmem determinations 16,17.

dWith the GEVI CAESR in our hands, apparently poor protein trafficking produces large amounts of non-voltage-sensitive signal, which contaminates the FLIM recording and contributes to high cell to cell variability ([Fig. 1-supplement 6](#_Fig._1,_S5.)**,** [Methods](#_Resolution_of_VF-FLIM)).

ePatch-clamp electrophysiology requires physical contact with the cell of interest, which causes damage to the cell and, in whole cell configurations, washout of intracellular factors. Slight movement of the cell or sample generally result in loss of the patch.

fMovement of the cell and photobleaching of the dye both cause large changes to the signal over seconds to minutes.

gRatio-calibrated imaging approaches use a second signal (usually another color of fluorescence) to correct for differences in dye concentration or changes in the region of interest that contaminate single-color intensity signals. If the rate of photobleaching is the same for both components, photobleaching artifacts can also be avoided.

hLimited by photon count rates.

iLimited by probe movement in the membrane, which depends mostly on lipophilicity 12.

jPhoton counting based lifetime imaging, like epifluorescence intensity imaging, is limited by photon count rates. Large numbers of photons per pixel must be collected to fit TCSPC FLIM data, often using a line scanning confocal approach, leading to slower acquisition speeds than epifluorescence-based intensity imaging.

kToxicity from capacitive load of the sensor 12.

lThe spatial resolution of electrophysiology is compromised by space clamp error, preventing interpretation of Vmem in regions far from the electrode (e.g. many neuronal processes) 35,36.

mAs demonstrated by Cohen and co-workers 27; in our hands with CAESR, we also experienced significant improvements in voltage resolution by fitting a single curve per FLIM image instead of processing the images pixel-wise (see [Methods](#_Analysis_of_CAESR))

nIn this work, we calibrated VF-FLIM for Vmem measurements with single cell resolution. In principle, subcellular spatial resolution could be achieved with the VF-FLIM technique.