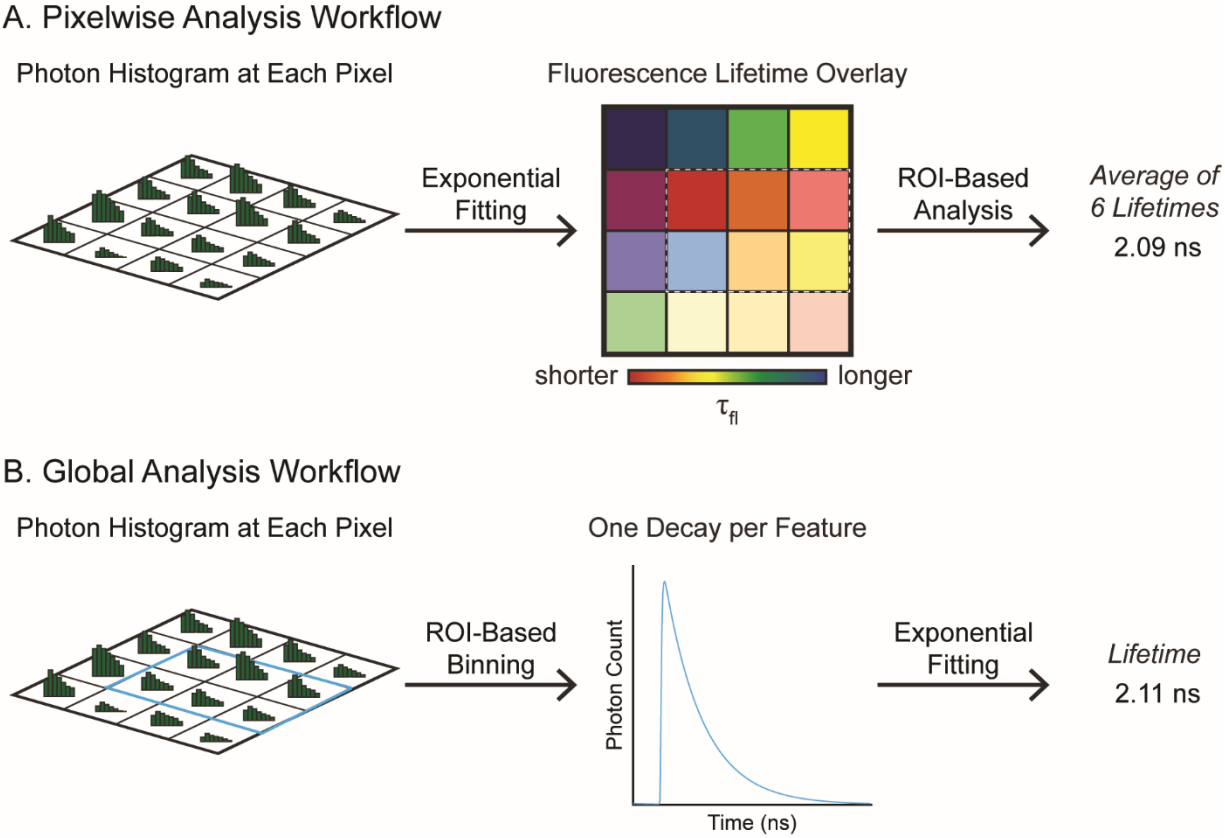
Technical Notes on Fitting VF-FLIM Data

Image Analysis: Selection of Global versus Pixelwise Analysis

Fluorescence lifetime analysis in the context of images can be broken down into two major classes: pixelwise and global (**Scheme 1**). In pixelwise analysis, the fluorescence decay constants τ are determined at each pixel and a heatmap of τ is generated. For summaries of results, the average of these τ values in the region of interest is often obtained. Global analysis involves consolidation of photon histograms for an entire region of interest (ROI) *before fitting*; decay constants are then determined per region of interest from the selected exponential model.



Scheme 1. Workflows for analysis of fluorescence decays.

(A) Analysis at each pixel involves fitting an exponential decay model to each position in the image. Often, images must be binned dramatically to obtain sufficient photons to perform each fit From this binned image, a region of interest (ROI, dotted line) is identified. The average across the pixels in the ROI is often used to represent the lifetime per ROI. (B) Global analysis involves combination of raw photon histograms from multiple pixels in the ROI (blue outline), followed by fitting of an exponential fit on the combined decay. These two approaches give similar results for large numbers of photons.

The selection of pixelwise versus global analysis methods depends largely on the application at hand, and there is no general rule. We have used both pixelwise and global analysis with

VoltageFluor FLIM data. Indeed, an advantage of the attached FLIM-FLAM code package is that it enables facile global and pixelwise analysis on the same dataset. Below, we outline some considerations in selecting an analysis mode.

On the one hand, in using VF-FLIM to map V_{mem} in a biological specimen, pixelwise analysis is often the most visually satisfying. Pixelwise analysis also provides an easier interface to discover unexpected relationships in the data, as the regions with interesting features do not need to be known a priori. However, care must be taken in the generation of images fit at each pixel, as many photons are required to accurately fit exponential decays. In many cases, acquiring enough photons to resolve the lifetime at each pixel limits the temporal resolution of the experiment. Most pixel by pixel fitting with VoltageFluors shows only the weighted average decay, as the individual decay constants and their amplitudes are considerably noisier.

If detailed information is desired about individual components of multiexponential fluorescence decays, global analysis is usually the best option. Sufficient photons to determine a fluorescence decay can be obtained much more easily on sensitive specimens, and often enough photons may be collected to resolve individual parameters of the decay model. Global analysis can also be used to extract information from carefully constructed regions of interest on the raw photon image, which may be useful for identifying fluorescence decays from fine structures such as neuronal processes.

Selection of an Exponential Decay Model

The simplest fluorescence decay model is a single exponential decay, reflecting a uniform population of molecules emitting with a decay constant τ. In practice, many fluorescence decays are not well described by a single fluorescence decay model. Multiple populations of emitters exist in most biological samples, resulting from heterogeneous probe environments and the fluorescence of endogenous chromophores. These multiple populations of emitters result in a fluorescence intensity I that decays over time t as a sum of exponential decays, each with amplitude a_i and decay constant τ_i (eqn. A1-1).

$$I(t) = \sum_{i=1}^{n} a_i e^{-t/\tau_i} \qquad \text{[A1-1]}$$

Selection of the most appropriate number of decay components is a statistically challenging problem. With VoltageFluors, a naïve expectation would be that the decays would show a single exponential form, arising from fluorophore uniformly localized to the interface between the plasma membrane and the extracellular space. However, in most cases, VF2.1.Cl in cells is best described by a biexponential fluorescence decay.

For fluorophores delivered to biological systems, a practical decay model is often selected by iteratively fitting additional terms and monitoring the concomitant decrease in the reduced chi squared. We demonstrated this approach when we reported a new suite of VoltageFluor dyes with FLIM characterization.[23] This model selection process is best performed on a standardized dataset. Ideally, the standards would be close to the system of interest (e.g. voltage-clamped HEK293T cells to develop standard fitting for non-voltage clamped HEK293T cells), but if that is not

possible, lifetime standards characterized by other researchers can be used.[24] The addition of terms must also be appropriate for the total number of photons acquired, as overfitting can produce artifacts in the results.

Selection of Other Fit Parameters

After the exponential model is selected, the most important additional parameter in the model is the shift in time between the instrument response function (IRF) and the fluorescence decay (sometimes called the color shift), as well as whether this parameter is fixed or allowed to vary during fitting. We follow the common convention of working with shift in units of bins in the photon histogram. In the data presented here, 1 bin is approximately 0.05 ns. A measured IRF at the same wavelength as the sample should have a shift very close to 0, but a measured IRF at a different wavelength may show a shift in time, especially on older detectors. In our experience, the best way to optimize the shift is to fit a monoexponential lifetime standard with the same emission spectrum as your fluorophore and then fix the shift to the shift obtained for that standard. Synthetic IRFs determined from the rising edge of the fluorescence decay are not implemented in FLIM-FLAM. Although they are common in commercial FLIM packages, we found a dramatic increase in the noise in VoltageFluor lifetimes determined from calculated IRFs, likely attributable to poor modeling of the short τ components present in certain VoltageFluor fluorescence decays.

The offset of the lifetime decay is the amount of non-time-resolved signal in the decay, arising most commonly from dark counts on the photon counting detector or from stray light in the room. If acquisitions were carefully performed in a cool, dark room, this parameter can generally be fixed to zero. When the lifetime of the fluorophore is much shorter than the period in between laser pulses, the offset can also be directly measured from the flat baseline of the TCSPC data.

The start and end time bin of the IRF should be selected to include the main IRF pulse but avoid other noise in the baseline. The start and end time of the decay should be a few bins in from either end of the signal region to avoid artifacts from the time to amplitude converter (TAC). Using the average of two measured IRFs in lieu of single, raw measured IRF can correct for variability in any individual IRF but is generally not required. The threshold in number of photons for fitting a decay depends on the number of parameters in your model; more photons are required to model more decay parameters. Whether enough photons have been counted is usually determined empirically based on the consistency of the result and the observation that collecting additional photons does not change the resulting lifetime. For VF2.1.Cl, we generally work with a minimum of 5000 photons total per decay trace, but the consistency of the lifetime obtained at a given number of photons should be checked for each new system, probe, and fit model.