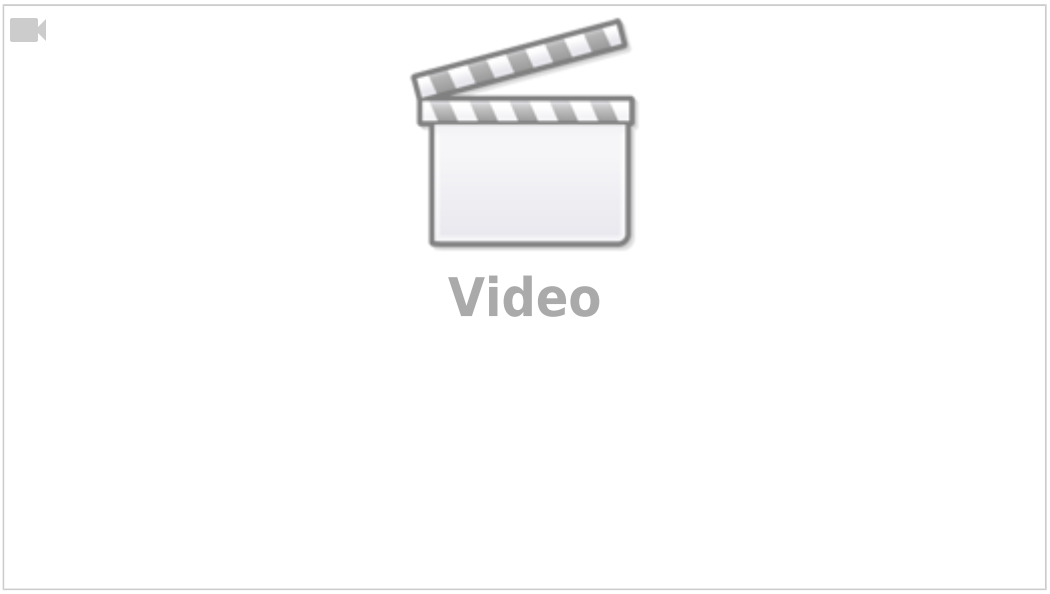
~~TOC~~

How to Measure the Instrument Response Function (IRF)

(For an explanation of the term Instrument Response Function see IRF)

The following video shows how to measure na IRF on confocal microscopes.



Low count rate during IRF measurements is important!

Make sure that the detection count rate is much lower than the count rate during a fluorescence decay measurement. Diluting the scattering solution is better than using grey (ND) filters. Ideal is when the decay and the IRF are recorded at the same differential count rate (and NOT at the same average count rate).

If the IRF should be measured in the UV range on a microscope system with SPAD detectors, the Raman scattering of water can be used, too. E.g. the Raman scattering can be recorded with a HQ480/40 bandpass filter, if a 405nm laser diode is used. This method is less suited for long wavelengths, as the Raman scattering decreases. In order to avoid signal contamination by any fluorescence, the water must be very pure.

Using samples with ultrafast decay

Some detectors (particularly MPD SPADs) have a wavelength dependent timing response. In this case an IRF recorded at the excitation wavelength may not be useful for precise reconvolution. The solution is to acquire the IRF at the fluorescence wavelength, or at least spectrally closer to the fluorescence emission wavelength.

General recipe

- Create a saturated aqueous KI (potassium iodide) solution. (Note, NOT KCl, but iodide.) Beware that KI is pretty well soluble in water. You will need quite a lot of KI. Our experience is that the volume of KI crystals is almost the same as the volume of the water added. The solution MUST be saturated.
- Then add any water soluble fluorescent dye with emission spectrum in the range where you need to record the IRF.
- Good luck with IRF measurements. Do not expect high count rates, but IRF must be recorded at much lower (than fluorescence) count rates anyway.

Our guess is that this quenching trick works generally. Specifically, we found that classic Fluorescein works at 480-500 emission with 440 nm excitation. Alexas or ATTOs would be perhaps even better, although more expensive...

Note: the strongly quenched solution will not look fluorescent when watched by eye. However, it must have a strong color (strong absorbtion, high dye concentration). Nevertheless, be careful when adding the dye, it must be completely dissolved.

Such a cocktail cannot be stored for a long time. Latest the KI will photochemically decompose.

IRF measurement with KI and Erythrosine B

SPAD detectors have wavelengths dependent timing response. IRF recorded at the excitation wavelength may not be useful for precise re-convolution. Using Erythrosine B, the IRF is acquired at the fluorescence wavelength.

Recipe:

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prepare 1mL of saturated water solution of KI (potassium iodide)
add 0.17 mL of saturated water solution of Erythrosine B (at least 95% of purity)
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Storage:

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keep at ~ 4°C, renew the solution after one month
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Spectra:

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excitation from 470 nm to 540 nm
emission from 500 nm to 600 nm
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Measurement:

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Put a droplet on a coverslip, measurement conditions as for fluorescence
measurement
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Two photon excitation (TPE)

Do not attempt to record an IRF at the fundamental (IR) wavelength. The resulting pulse form would be meaningless anyway.

You can try to excite (by TPE, of course) any of the above mentioned fast decay time fluorophores and record their response. The signal will be wery weak, but this is not a problem for IRF measurements.

With microscopes it is convenient to record the second harmonic signal that is generated on the surface of urea crystals. The best is to let evaporate a droplet of concentrated urea solution on a clear cover slip. The resulting

film of micro-crystals is easy to target.

Urea, aka Carbamide or Carbonyldiamide, CAS Number:57-13-6

Appropriate Count Rate for Measuring an IRF

See Differential Count Rate

How often does the IRF need to be measured?

In spectrometers, the IRF is usually measured before or after each measurement.

In microscopy-applications, this is usually not practical; and often it is sufficient to measure the IRF once during a measurement series, provided that the system has had time to warm up (~15min), and neither the repetition rate nor the intensity at the diode laser driver is changed (the current which drives the laser). If the intensity needs to be changed, the optical attenuation can be adapted.

A special case are systems with 2-Photon-Excitation (2PE). Here, usually TiSa-lasers are used which have fs-pulses, therefore the IRF is normally determined by the detector. In these cases, often the IRF can be measured once (and the excitation wavelength is not important, provided that the IRF is measured with a quenched dye and the same filterset is used as for the sample), and re-used later. Over time or upon changes of the excitation wavelength, the position of the IRF can shift slightly, but this is accounted for with the “IRF-shift”-parameter of the fitting equation.

How to compensate IRF effects in the analysis of time domain measurements

There are two major ways of compensating IRF effects:

- correct the effects in the data (**de**convolution)
- take the effects into account in your model equation (**re**convolution)

Note: All analysis packages from PicoQuant use the reconvolution method. The reason is simple: Deconvolution has many disadvantages: It is rather slow, it is an ill posed problem producing a lot of noise (at best), it does not get you any nearer to the model parameters, and it is very likely to produce artefacts without allowing to tell apart artefacts and effects.

Measuring the IRF as scattered excitation light

We do not recommend to measure the IRF as scatters light in microscopy, due to the color dependence of SPAD detectors, which are generally used in microscopy. Furthermore, even with detectors lacking color effects, it is disadvantageous, due to the many reflection peaks found along the light pathway in a microscope set up.

However, in case of cuvette based measurement like in spectrometers, this is the method of choice. The simplest procedure is to use a very diluted solution of colloidal silica. (LUDOX is often used, LUDOX is a trade mark by DuPont. It can be purchased via Aldrich or Sigma.) Do not use “non-diary cafe creamer” or glycogen, mentioned in older literature. These compunds are fluorescent. The scattering solution must be really weak, typical starting “concentration” is one droplet of the colloid from the original LUDOX bottle (as delivered with you system) diluted 100x. If the signal is too strong, dilute further.

Note that recording the IRF via scattering requires tuning the emission monochromator to the excitation wavelength. In filter based machines, e.g. FluoTime100 this means removing the emission bandpass or longpass filter. In microscopes, this is equivalent to replacing the detection filter with an OD filter (OD = optical density,

grey filter) and recording the back scattering from e.g. a clean glass cover slip. Beware, on some confocal LSMs it is simply not possible to measure at the excitation wl. due to the restrictions introduced into the system software by the manufacturer. In case of measurements of solid samples in FT100 or FT200 spectrometers, the surface scattering is usually so strong that it is not necessary to mount a special sample for IRF. Tuning the mono to the exc. wl. and attenuating the excitation beam is sufficient.

Selected literature:

Luchowski R., Kapusta P., Szabelski M., Sarkar P., Borejdo J., Gryczynski Z., Gryczynski I.
Förster resonance energy transfer (FRET)-based picosecond lifetime reference for instrument response evaluation
Measurement Science and Technology, Vol.20, 095601 (2009)
http://dx.doi.org/10.1088/0957-0233/20/9/095601

Luchowski R., Gryczynski Z., Sarkar P., Borejdo J., Szabelski M., Kapusta P., Gryczynski I.
Instrument response standard in time-resolved fluorescence
Review of Scientific Instruments, Vol.80, 033109 (2009)
http://dx.doi.org/10.1063/1.3095677

Szabelski M., Luchowski R., Gryczynski Z., Kapusta P., Ortmann U., Gryczynski I.
Evaluation of instrument response functions for lifetime imaging detectors using quenched Rose Bengal solutions
Chemical Physics Letters, Vol.471, p.153-159 (2009)
https://www.sciencedirect.com/science/article/abs/pii/S0009261409001389

Szabelski M., Iliev D., Sarkar P., Luchowski R., Gryczynski Z., Kapusta P., Erdmann R., Gryczinski I.
Collisional quenching of Erythrosine B as a potential reference dye for impulse response function evaluation
Applied Spectroscopy, Vol.63, p.0363-0368 (2009)
https://www.osapublishing.org/as/viewmedia.cfm?uri=as-63-3-363&seq=0

Szabelski M., Gryczynski Z., Gryczynski I.
Photophysical properties of novel fluorescein derivative and its applications for time-resolved fluorescence spectroscopy
Chemical Physics Letters, Vol.493, p.399-403 (2010)
https://www.sciencedirect.com/science/article/abs/pii/S0009261410007256

The dye **pinacyanol iodide** has a very fast decay time (in methanol 6 ps) and very broad emission spectrum (starting at around 550nm), thus suitable for determination of the IRF in a very broad range.

Pinacyanol iodide is also known as Quinaldine blue, 1,1' -Diethyl-2,2' -carbocyanine iodide, CAS Number:605-91-4. Pinacyanol bromide, CAS Number:2670-67-9 or Pinacyanol chloride CAS Number:2768-90-3 should work too, however, the dye must be pure, otherwise you may end up with measuring (long lifetime) impurity emission. For details see:

van Oort B., Amunts A., Borst J. W., van Hoek A., Nelson N., van Amerongen H., Croce R.
Picosecond fluorescence of intact and dissolved PSI-LHCI crystals
Biophysical Journal, Vol.95, p.5851-5861 (2008)
https://www.sciencedirect.com/science/article/pii/S0006349508820012

The dye **Allura Red** has also a remarkably short fluorescence lifetime of about 10 ps. This short lifetime does not depend on the emission (observation) wavelength (from 550 nm to 750 nm) and it is another candidate for impulse response measurements.

Allura Red is Disodium 6-hydroxy-5-[(2-methoxy-5-methyl-4-sulfophenyl)azo]-2-naphthalenesulfonate, CAS Number: 25956-17-6, Colour Index Number 16035, EC Number 247-368-0, MDL number MFCD00059526, PubChem Substance ID: 24869338

Rahul Chib, Sunil Shah, Zygmunt Gryczynski, Rafal Fudala, Julian Borejdo, Bogumil Zelent, Maria G Corradini, Richard D Ludescher, Ignacy Gryczynski
Standard reference for instrument response function in fluorescence lifetime measurements in visible and near infrared
Measurement Science and Technology, Vol.27, 027001 (2016)
http://dx.doi.org/10.1088/0957-0233/27/2/027001

Pyridinium Styryl Dyes have also been used as IRF standard:

Lei Li, Mengfang Chang, Hua Yi, Menghui Jia, Siaodan Cao, Zhongneng Zhou, Sanjun Zhang, Haifeng Pan, Chun-Wei Shih, Ralph Jimenez, Jianhua Xu
Using Pyridinium Styryl Dyes as the Standards of Time-Resolved Instrument Response
Applied Spectroscopy, Vol. 70(7), 1195-1201 (2016)
http://asp.sagepub.com/content/70/7/1195

Even dye with lifetimes that are usually in the ns range can be used, if the lifetime can be quenched to a few ps. Quenching can be achieved using a saturated iodide solution. For details see:

Mengwei Liu, Mengui Jia, Haifeng Pan, Lei Li, Mengfang Chang, Hua Ren, Françoise Argoul, Sanjun Zhang, Jianhua Xu
Instrument Response Standard in Time-Resolved Fluorescence Spectroscopy at Visible Wavelength: Quenched Fluorescein Sodium
Applied Spectroscopy, Vol. 68, p. 577-583 (2014) http://dx.doi.org/10.1366/13-07236

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