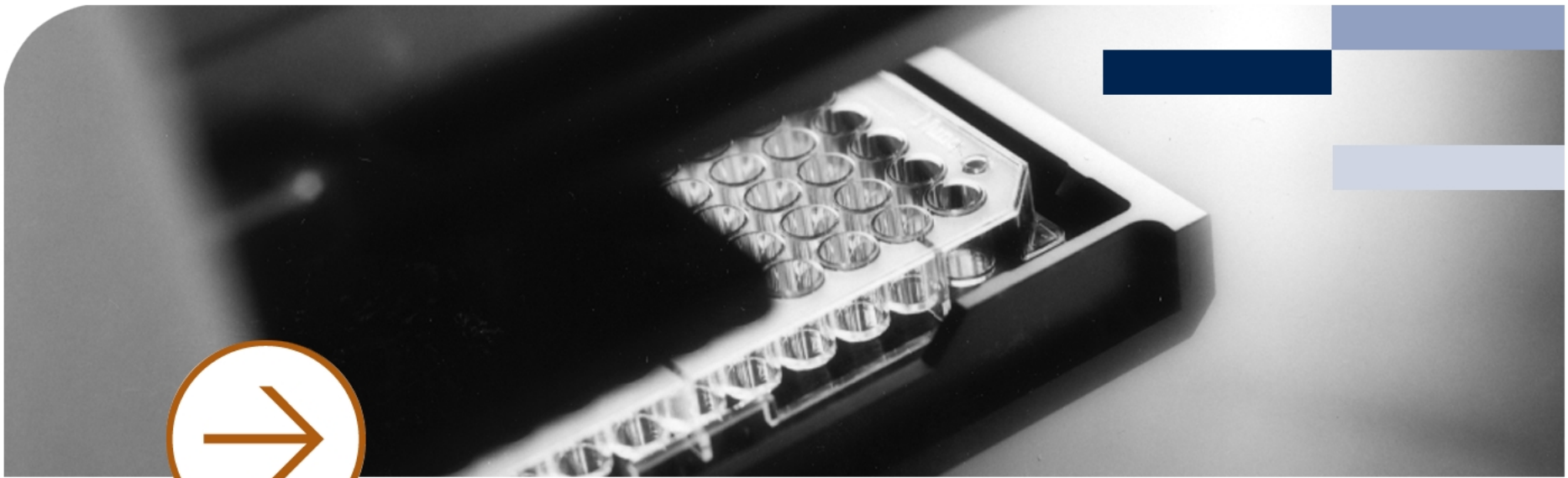
Molecular Devices

Wavelength Optimization Review

ELM; January 26, 2004



Optimization of a red fluorophore with a small Stokes shift

For this demo, I chose a fluorophore with

Ex/Em somewhere in mid 600's
Small Stokes shift

I also used a low concentration so lamp light and artifacts are more likely to interfere

Fluorophore:

UniSignal Fluor 0607 (Hilyte Biosciences.Com)
Similar to Cy5 (Amersham) and AlexaFluor 647 (Molecular Probes)

Plan - Optimize first in Cuvette, then repeat in microplate

1. Locate Ex and Em peaks (qualitative)
2. Optimize instrument settings for best quantitative analysis
   (Ex/Em wavelengths will not be lambda maxima unless Stokes shift is large)

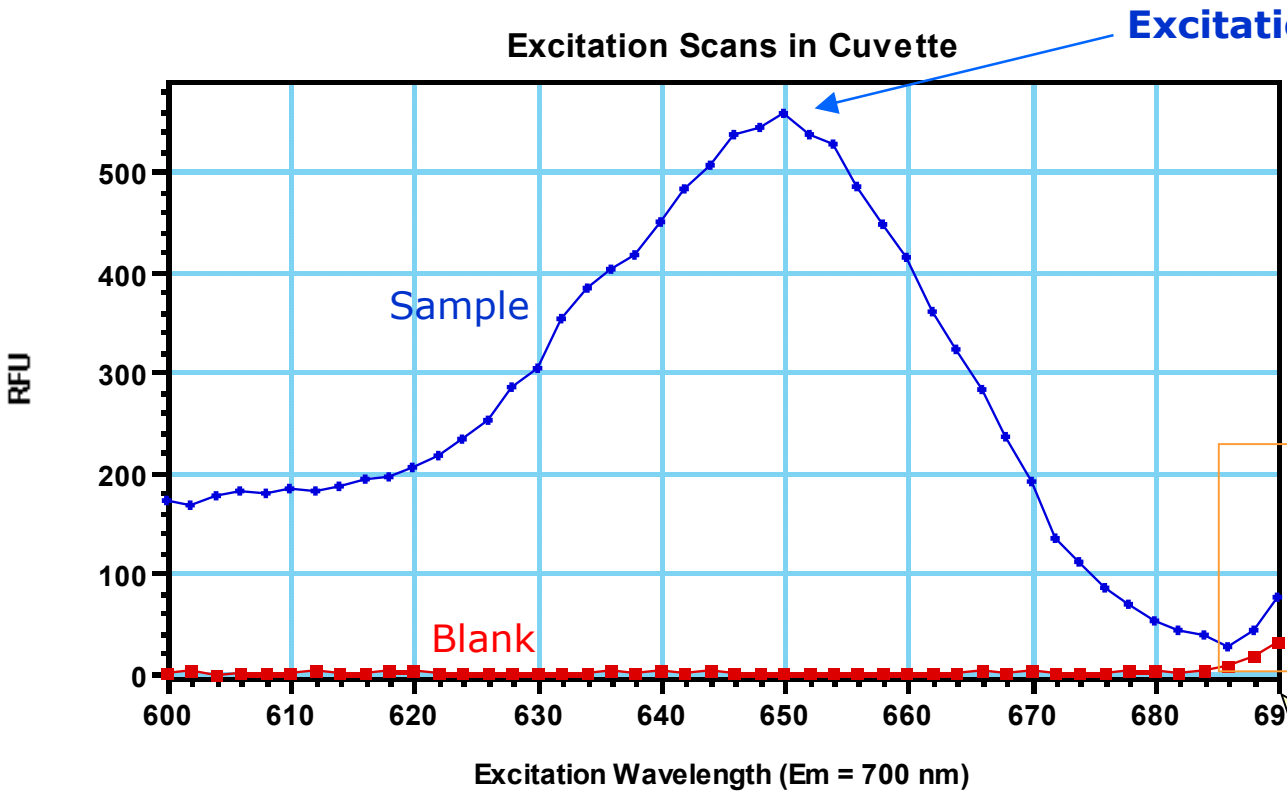
Strategy and Comments

Locate excitation peak first

- Temporarily set the emission wavelength well above its expected peak *(in this case, I chose 700 nm)*.
- Set up excitation scan, stopping ≥ 10 nm before the emission wavelength to avoid lamp light. *(I chose 600 – 690).*
- Always include buffer blank

If you do not find an excitation peak, the fluorophore may be too dilute or the wavelengths too far off.

Cuvette Excitation Scans



Lamp light

Always stop scan ***below*** Emission wavelength

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Strategy and Comments (cont)

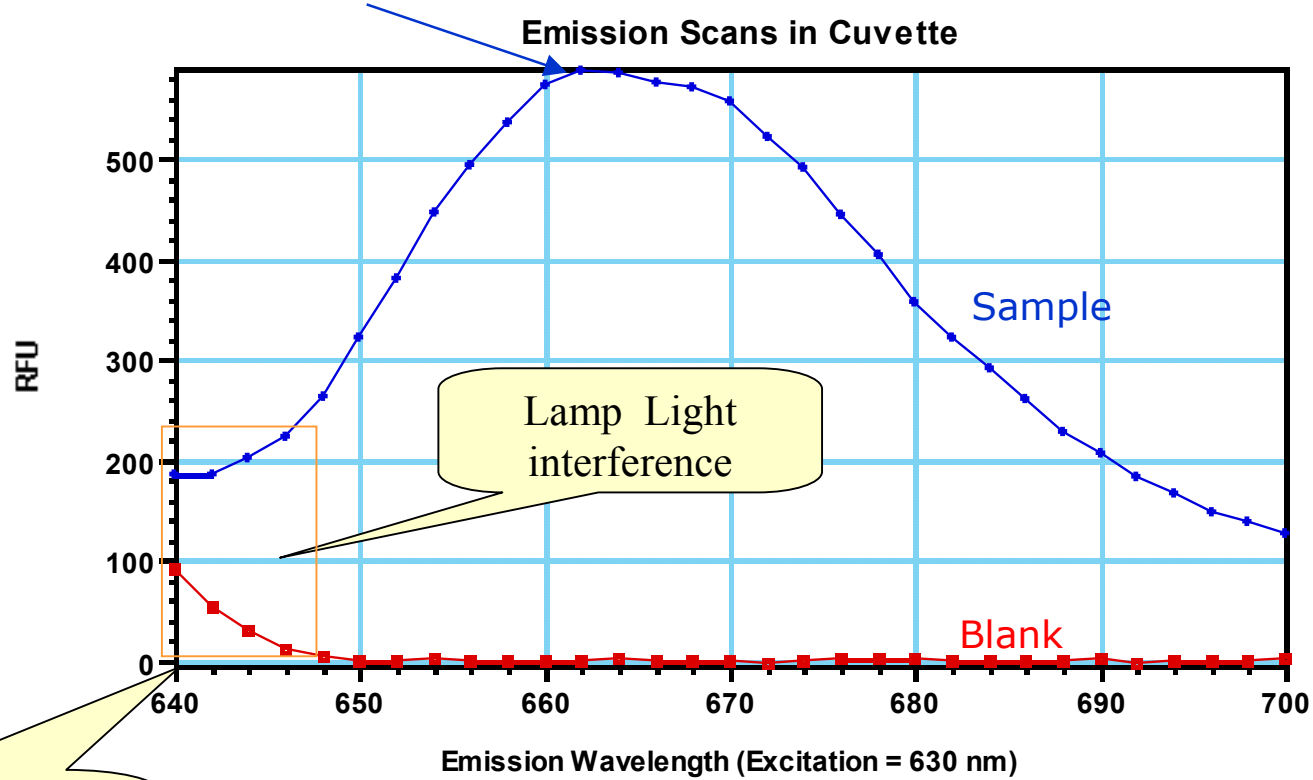
We found that the excitation peak is ~ 650 nm

Next, do emission scan to locate Em peak

- The Em peak will be at a higher wavelength than Ex peak.
  (The greater the Stokes shift, the easier it will be to measure)
- Temporarily set Ex wavelength 20-30 nm below its peak *(in this case, I chose 630 nm).*
- Set up emission scan starting ≥ 10 nm above the Ex wavelength to avoid lamp light.

Cuvette Emission Scans

Emission Peak ~ 662 nm



Always start Em scan ***above*** Ex wavelength

('!WavelengthRun@Em640-700' vs '!A2Lm1@Em640-700')
!WavelengthRun@Em640-700' vs '!Lm1@Em640-700')

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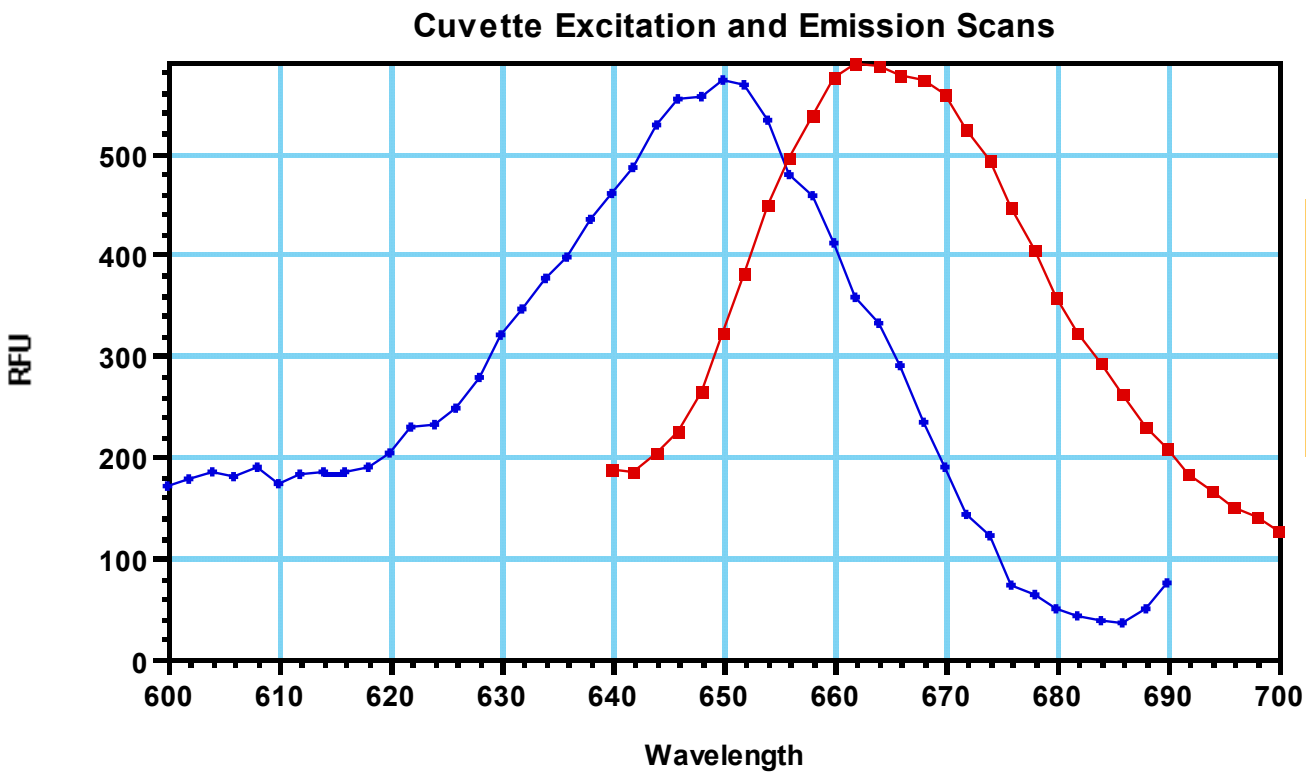
Consider the spread between Ex and Em maxima (Stokes shift)

Excitation Peak ~ 650 nm

Emission Peak ~ 662 nm



Stokes shift ~ 12 nm – quite small!!

Cuvette wavelength Optimization

- In the cuvette, Ex and Em wavelengths must be $\geq$ 15 nm apart to avoid excitation spillover.
- With a 12 nm Stokes shift, we cannot use the Lambda maxima; we must lower the Ex and raise the Em wavelength

- *Plan:*

  Lower the Ex wavelength to 90% of maximal signal (to 640 nm).

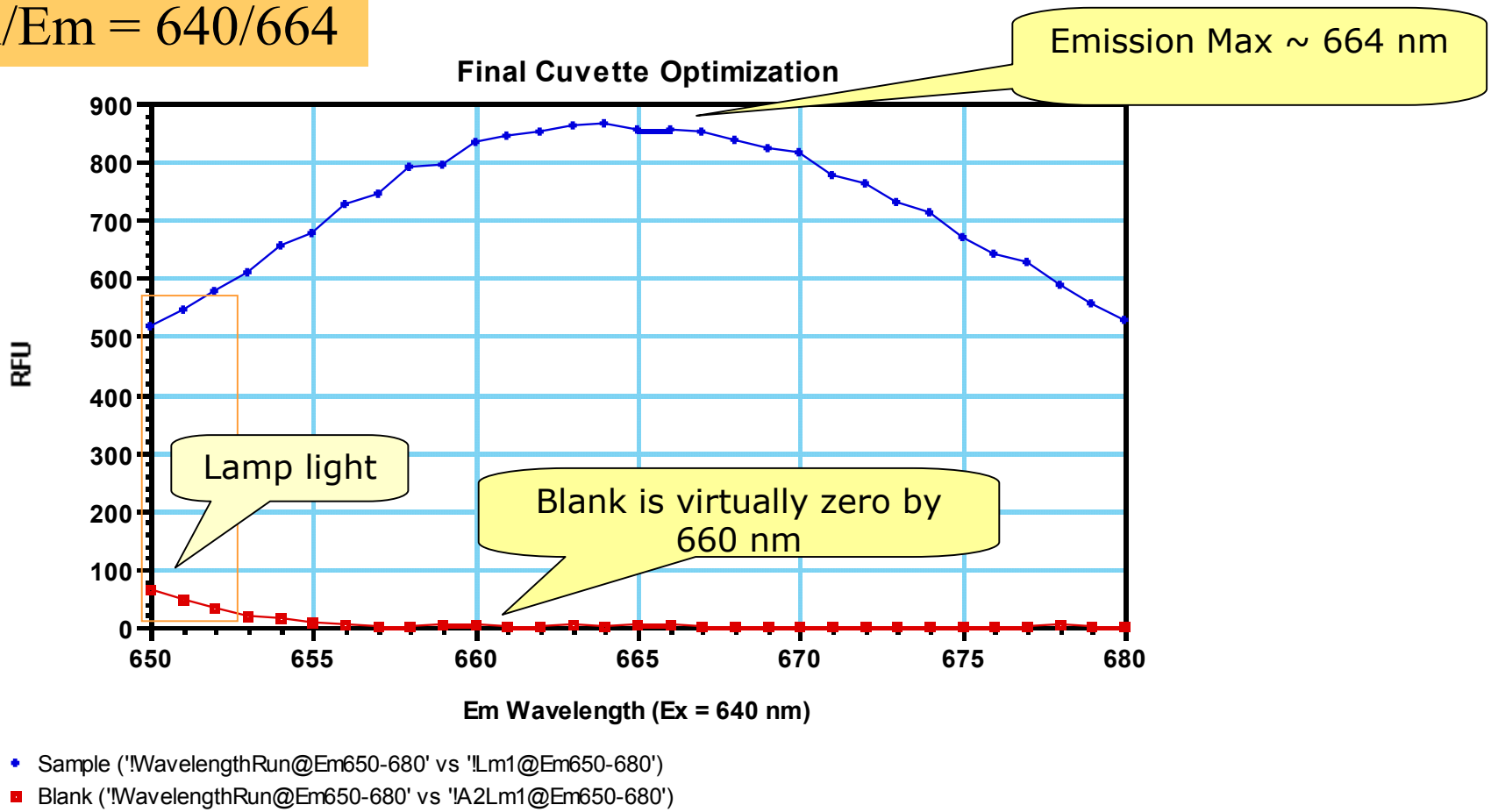
  Do emission scan (650 – 680 nm).

  Emission cutoff filter probably not necessary in cuvette.

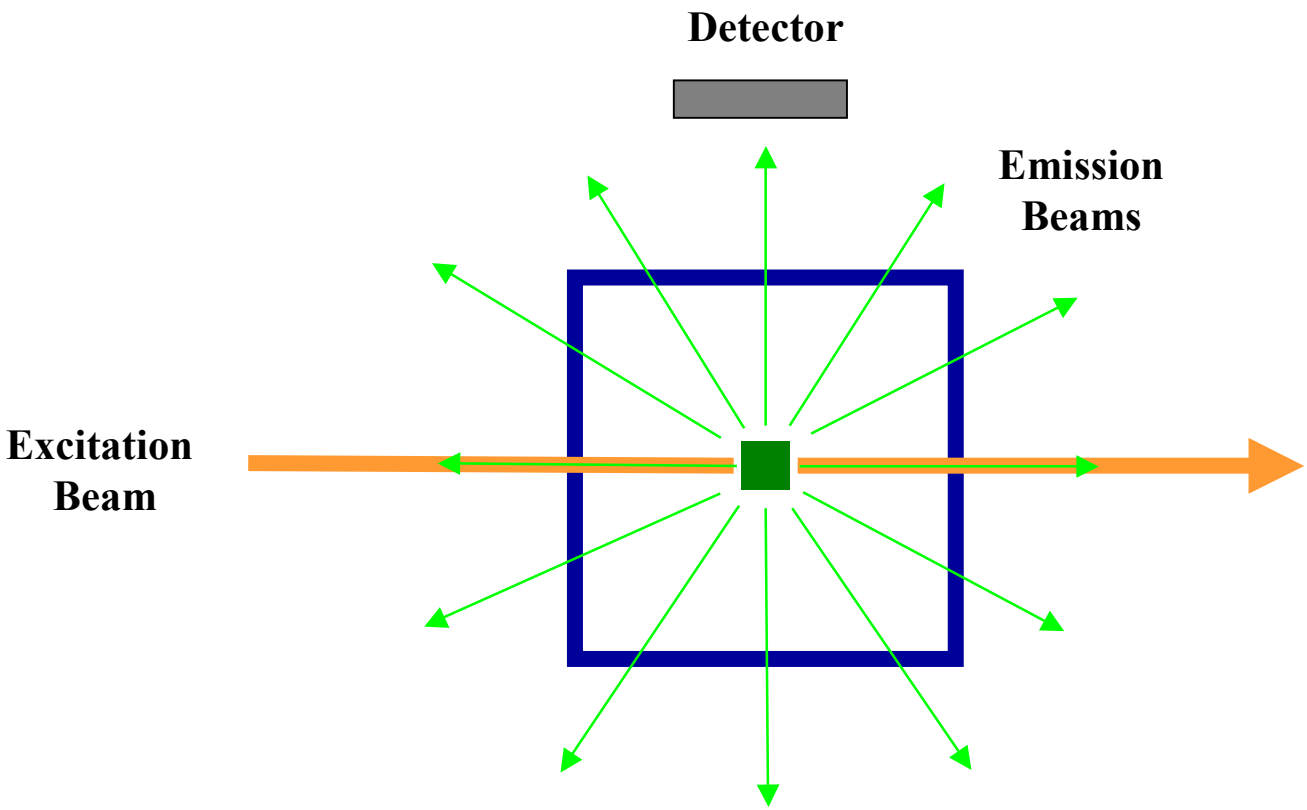
  Look for max signal and min background

Wavelength Optimization in the Cuvette

Final selection:
Ex/Em = 640/664



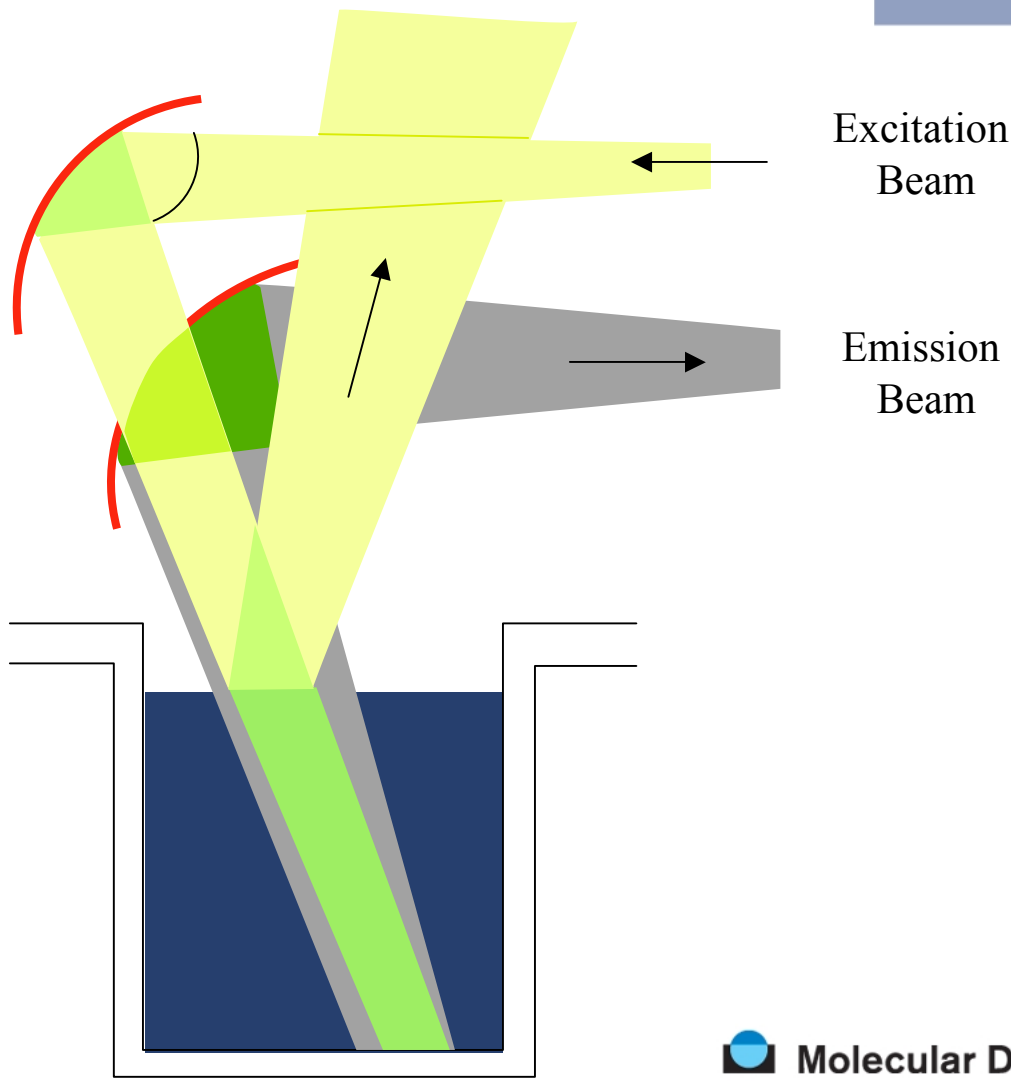
90° Fluorescence in Cuvette Cell



The excitation beam passes straight through. (except for some of light scattering).

SPECTRAmax Gemini and M2 Plate Optics

- Optics in a microplate cannot be 90°, so a considerable amount of lamp light is reflected into emission beam
- Raw background is much higher than in cuvette



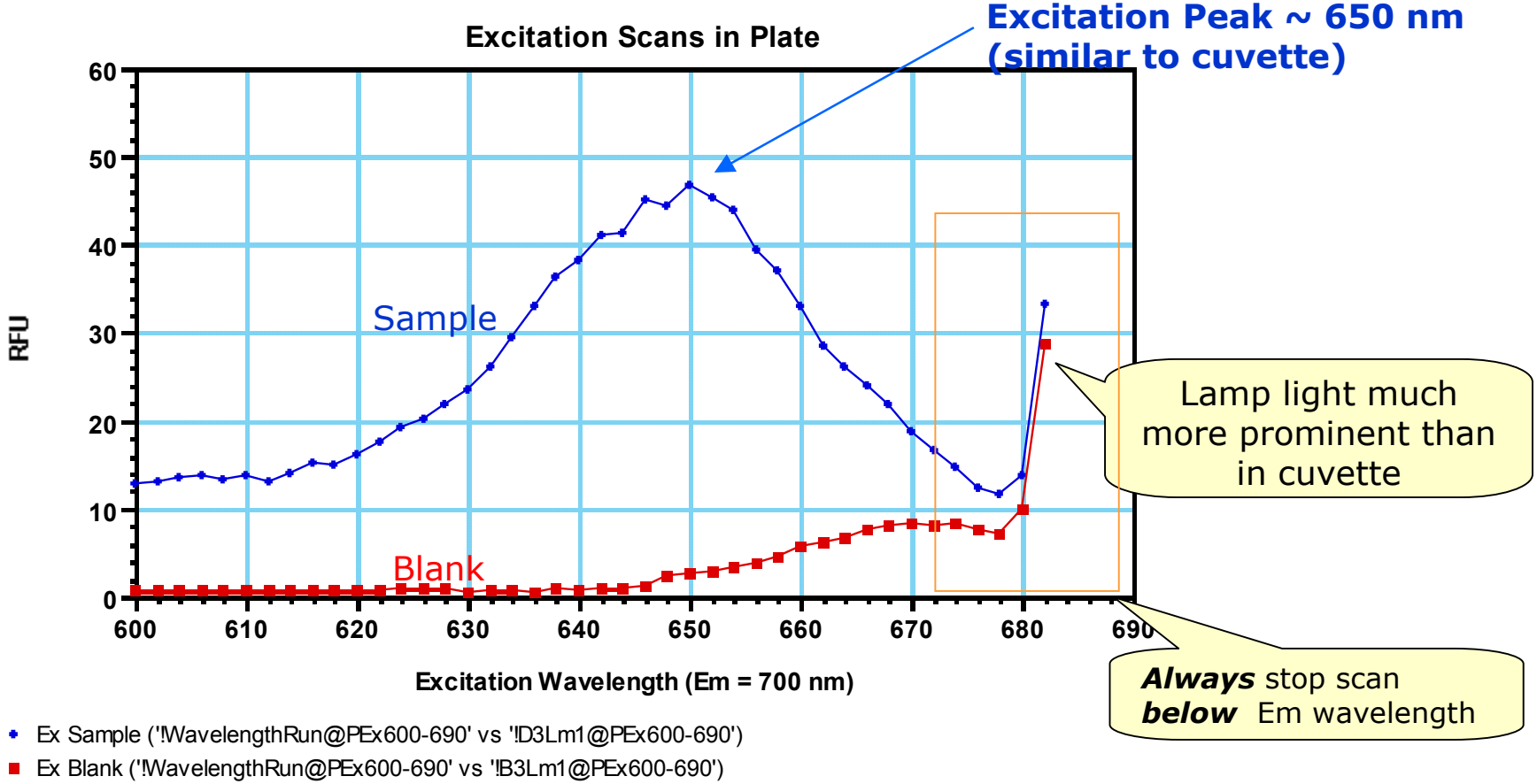
Microplate wavelength optimization in the

Because of lamp light reflection from the well,

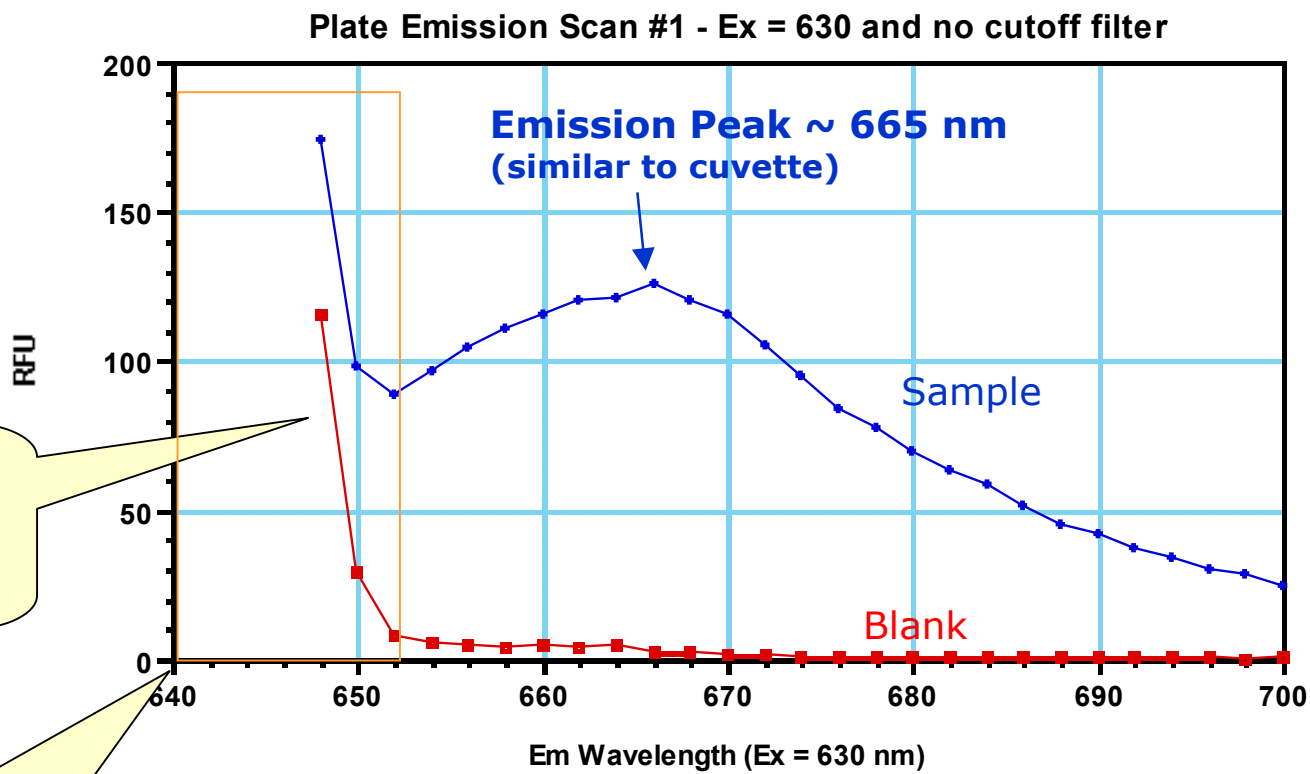
- The Ex/Em wavelength separation will have to be greater than that in cuvette.
- We will need to use an emission cutoff filter to reduce background.

We begin by running excitation scan with same settings as used for cuvette

Microplate Excitation Scans



Microplate Emission Scans



Lamp Light much more prominent than in cuvette

Always start Em scan ***above*** Ex wavelength

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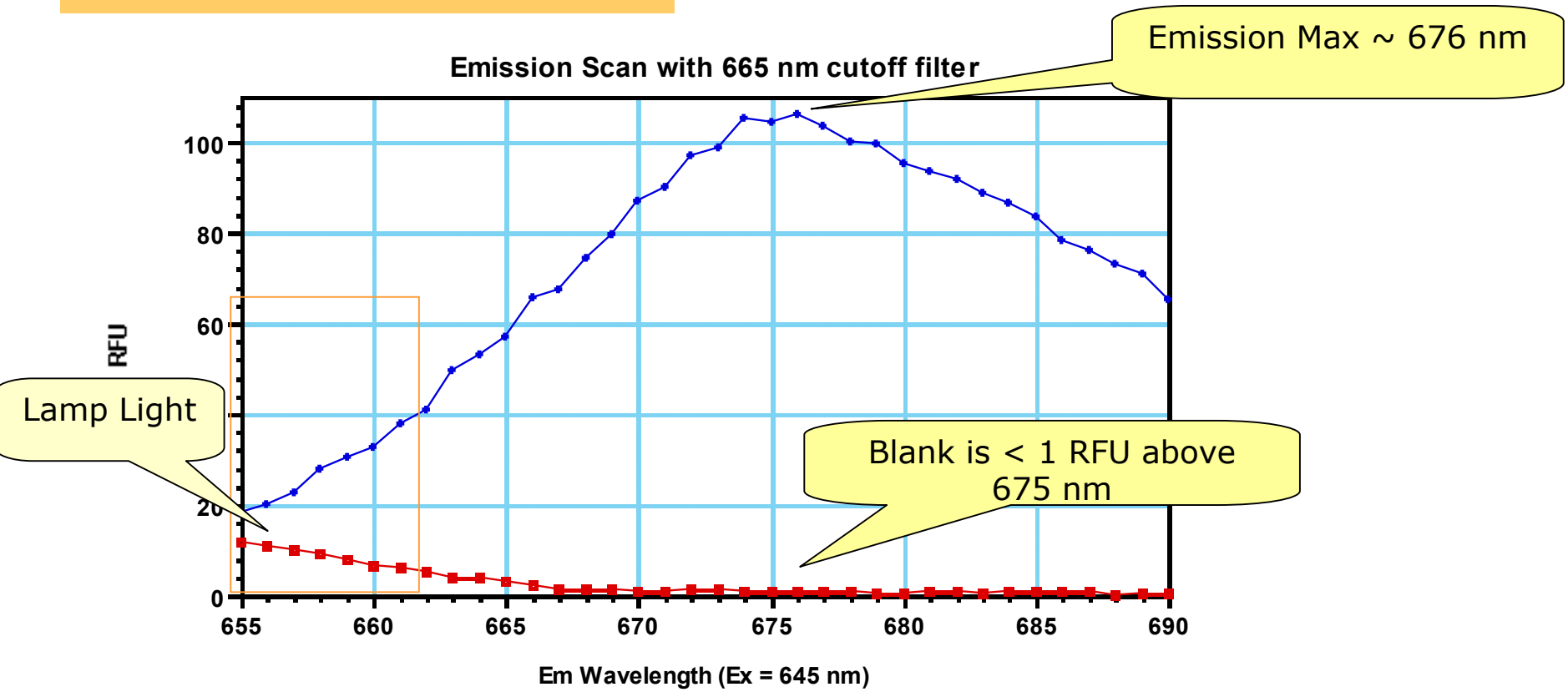
Microplate Optimization: final strategy

- Ex/Em maxima ~ ~650/665 (small Stokes shift!)
- We must use an emission cutoff filter to block lamp light
  - → The cutoff should be between the Ex and Em wavelengths
  - → A cutoff filter will lower the background and shift the apparent peak to the right
- The cutoff choices in this region are 630, 665 & 695 nm.
- The 665 nm filter looks the most reasonable because it will block lamp light (650 nm) and transmit at least half of the emission peak.

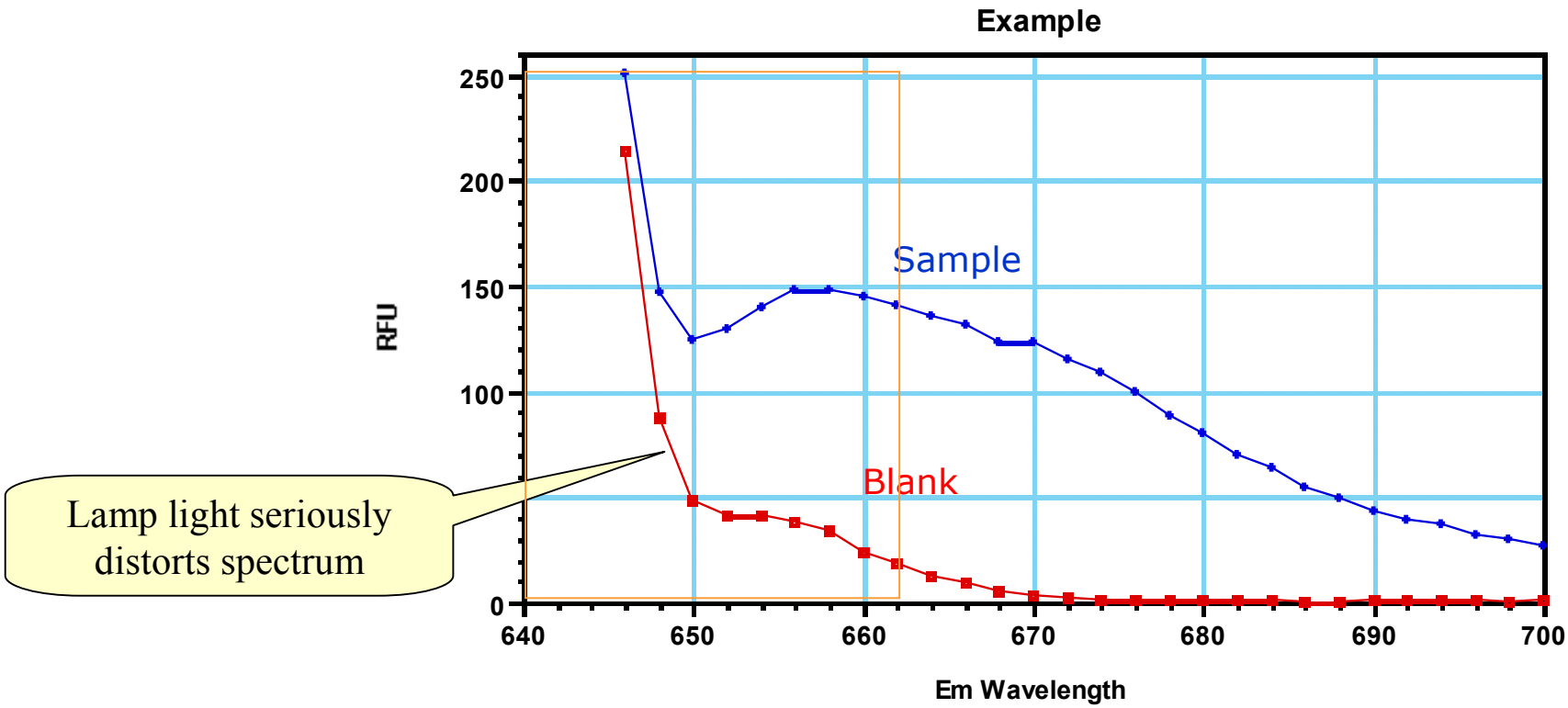
Plan: Lower the Ex wavelength to ~90% of max (645 nm) and scan emission using the 665 nm cutoff filter.

Wavelength Optimization in the Microplate

Optimal: Ex/Em = 645/676
+ 665 nm cutoff filter

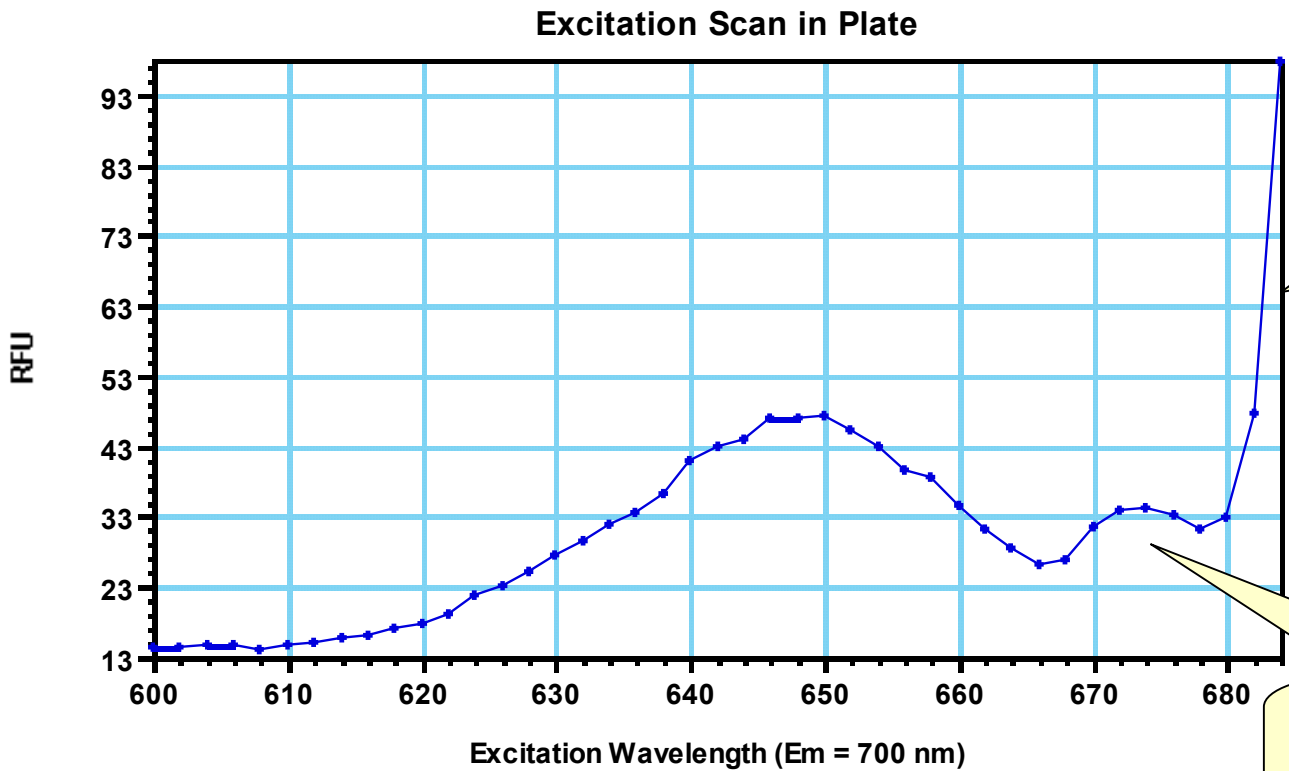


Potential problem:
What if lamp light distorts a spectrum?
Example in an emission scan



Solution: move Ex wavelength lower to eliminate interference.

Artifacts-Unexpected peaks



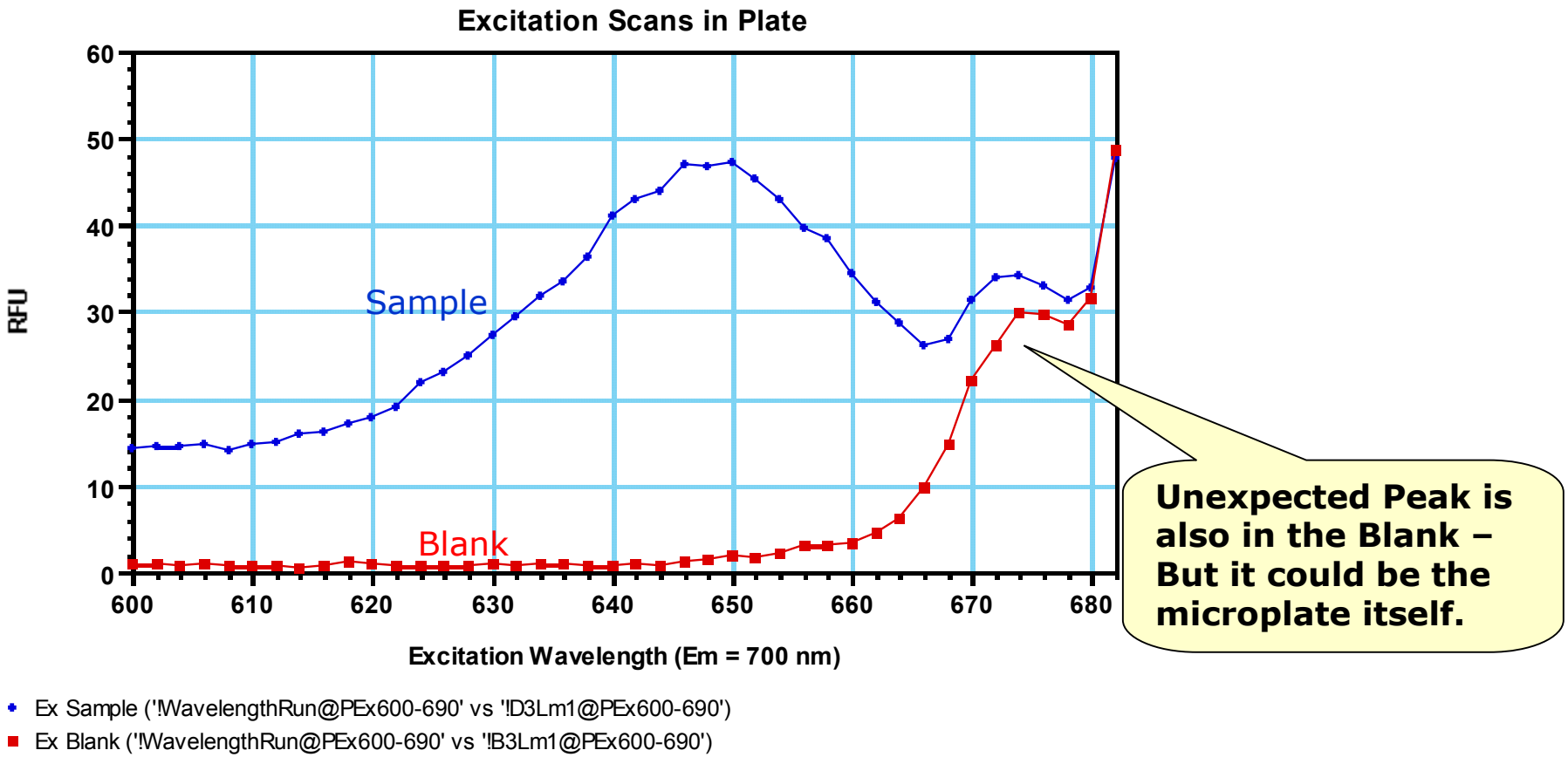
- Ex Sample ('!WavelengthRun@PEx600-690' vs '!D3Lm1@PEx600-690')

Expected lamp light as scan nears 700 nm

Peak ~ 675 nm – but is it real?

Check the blank!!

Artifacts (cont)



Artifacts can appear on edge of the lamp light

Beware of artifacts on leading or trailing edge of lamp light peak where signal intensity is changing rapidly