Increasing your confidence

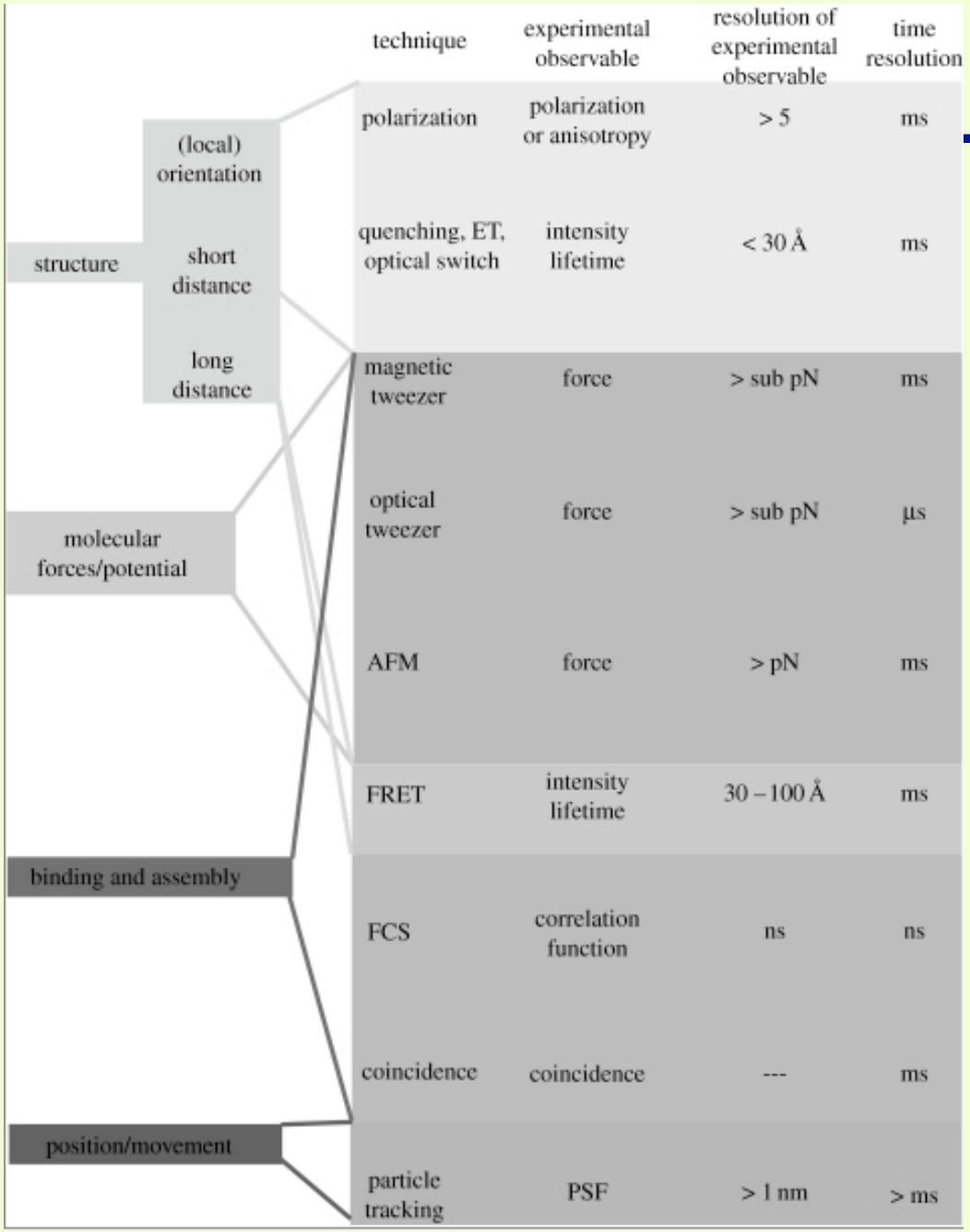
~~Proving~~ that data is single molecule

Chem 184 Lecture
David Altman
5/27/08

OUTLINE

- Brief discussion/review of single molecule fluorescence
- Statistical analysis of your fluorescence data
- Where could we go from here

Single molecule studies come in all flavors



		technique	experimental observable	resolution of experimental observable	time resolution
structure	(local) orientation	polarization	polarization or anisotropy	> 5	ms
	short distance	quenching, ET, optical switch	intensity lifetime	< 30 Å	ms
	long distance	magnetic tweezer	force	> sub pN	ms
molecular forces/potential		optical tweezer	force	> sub pN	µs
		AFM	force	> pN	ms
		FRET	intensity lifetime	30 – 100 Å	ms
binding and assembly		FCS	correlation function	ns	ns
		coincidence	coincidence	---	ms
position/movement		particle tracking	PSF	> 1 nm	> ms

Why single molecules?

1. For your system, you gather a distribution as opposed to an ensemble average.
   - gives you information about inhomogeneities in your system, including the "nanoenvironment"

2. No need to synchronize a system when you are measuring a time dependent process.

3. Can observe new effects.

Important moments in single molecule optical detection

T. Hirschfield, Appl. Opt. 15, 2965 (1976).

Detected a single antibody labeled with 80-100 fluorophores

Moerner and Kador, Phys Rev Lett. 62, 2535 (1989).

M.Orrit and J. Bernard, Phys. Rev. Lett. 65, 2716 (1990).

Detection of a single dopant molecules in a host molecular crystal at cryogenic temperatures - pentacene in p-terphanyl crystals.

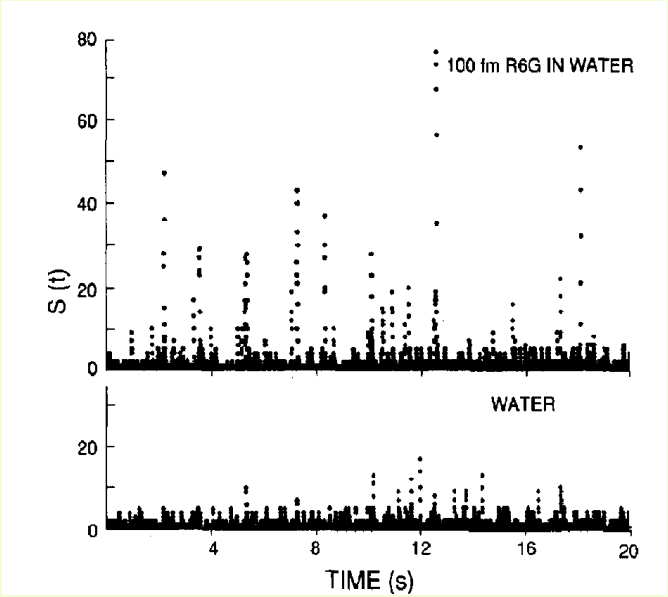
Moerner et al - absorbance

Orrit et al - fluorescence

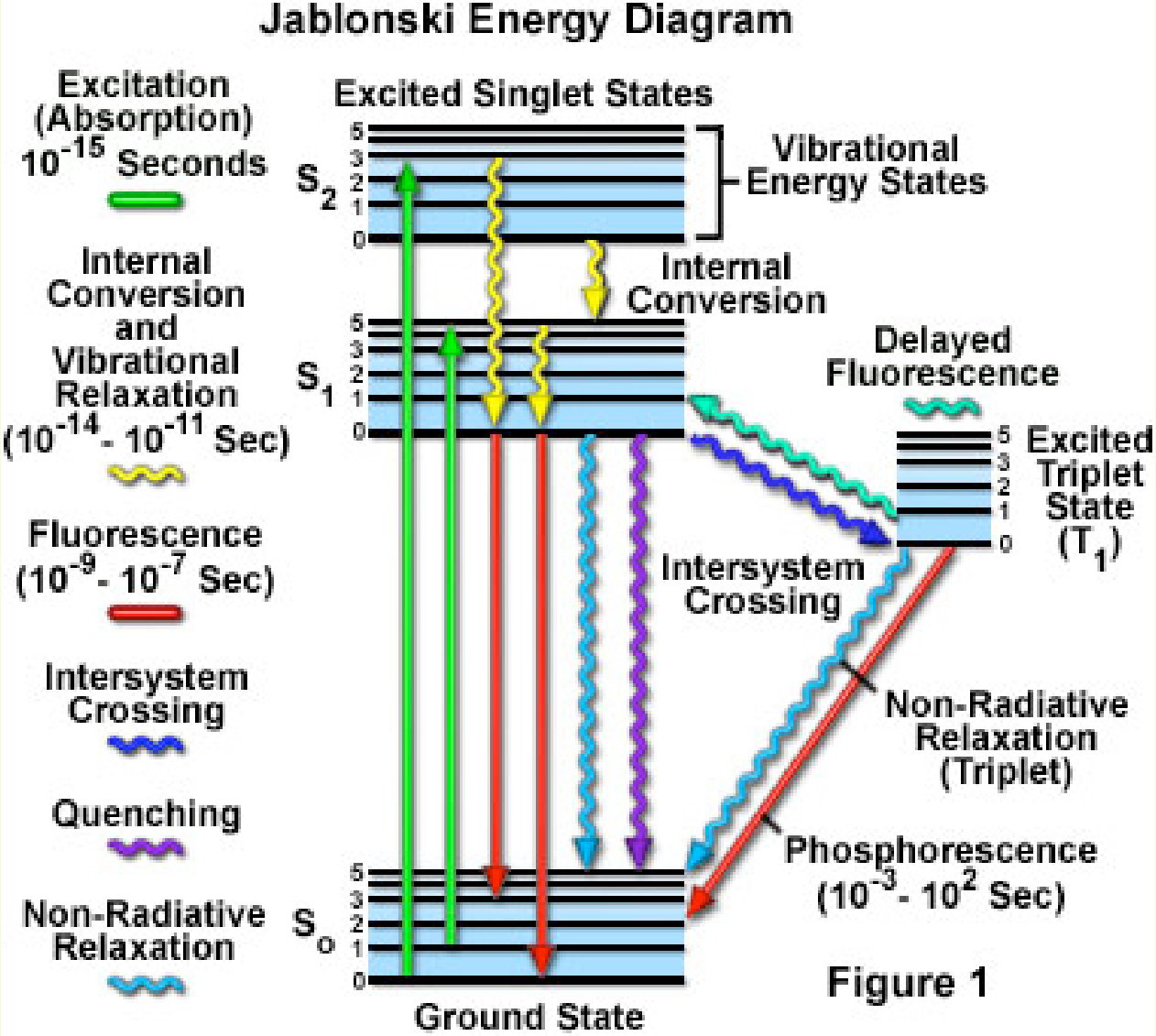
Important moments in single molecule optical detection

E. B. Shera, N. K. Seizinger, L. M. Davis, R. A. Keller and S. A. Soper, *Chem. Phys. Lett.* 174, 553 (1990).

Observed Rhodamine-6G in aqueous solution at room temperature using a pulsed laser



Single molecule fluorescence



Single molecule fluorescence

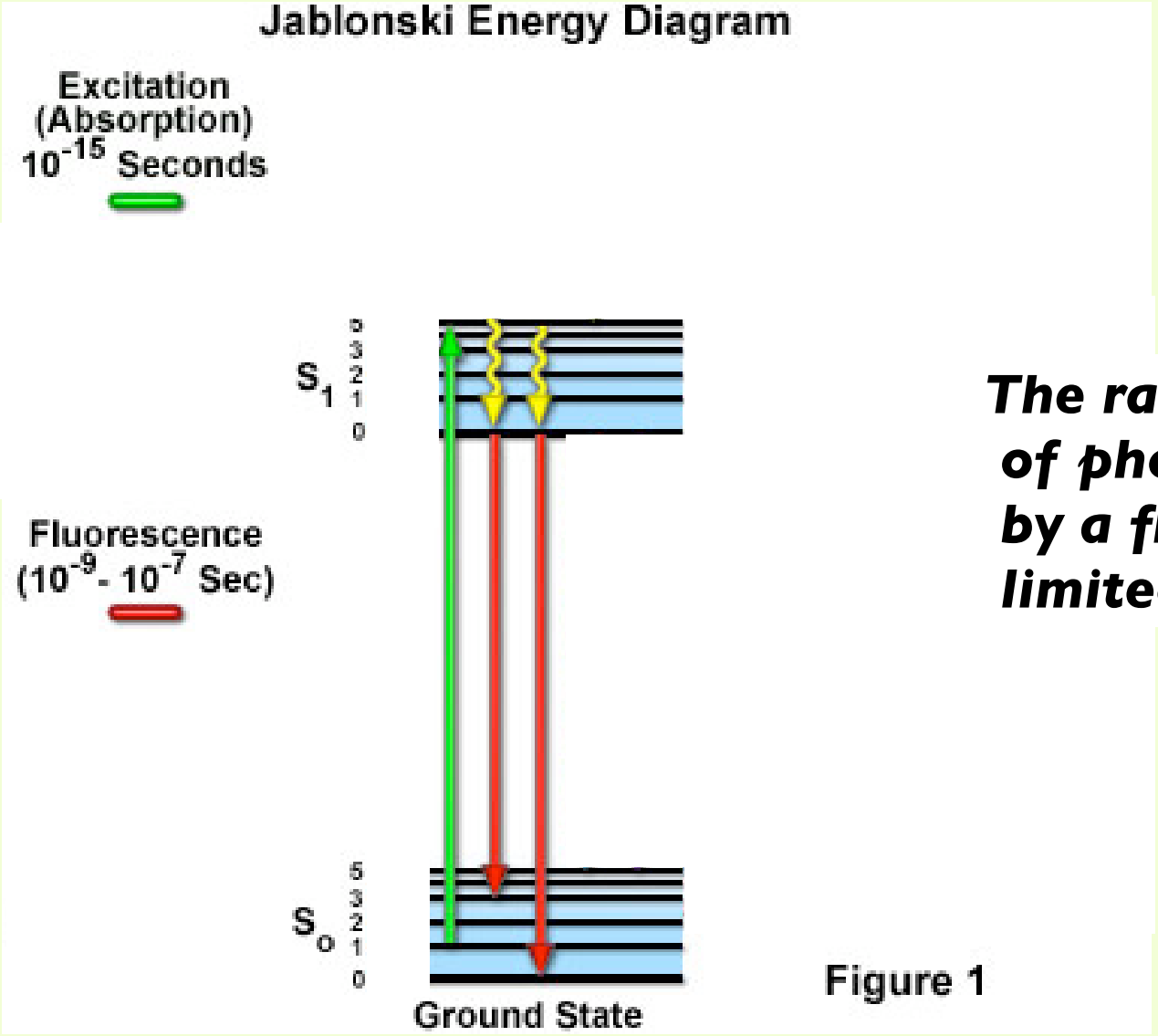


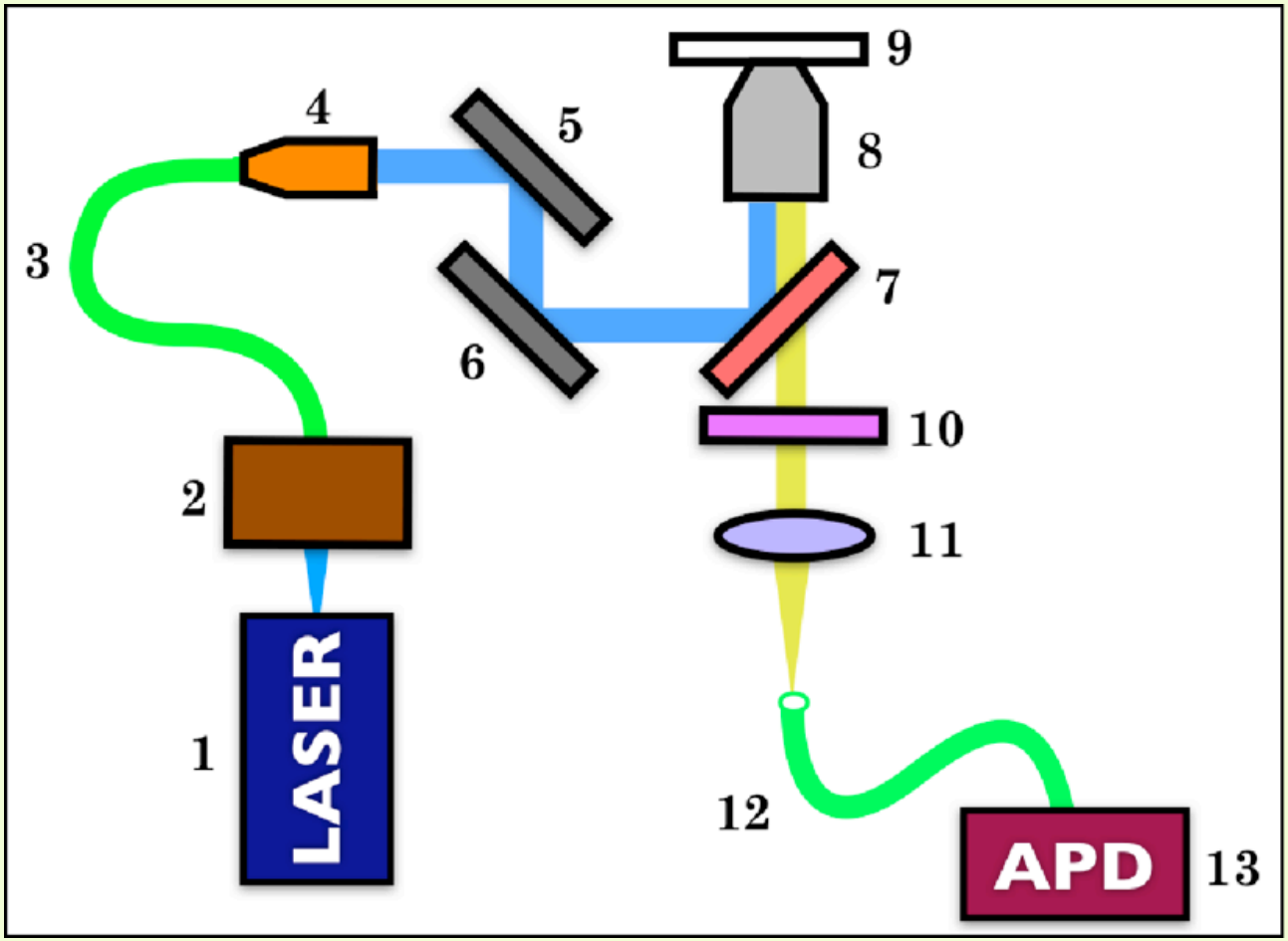
Figure 1

The rate and number of photons emitted by a fluorophore is limited.

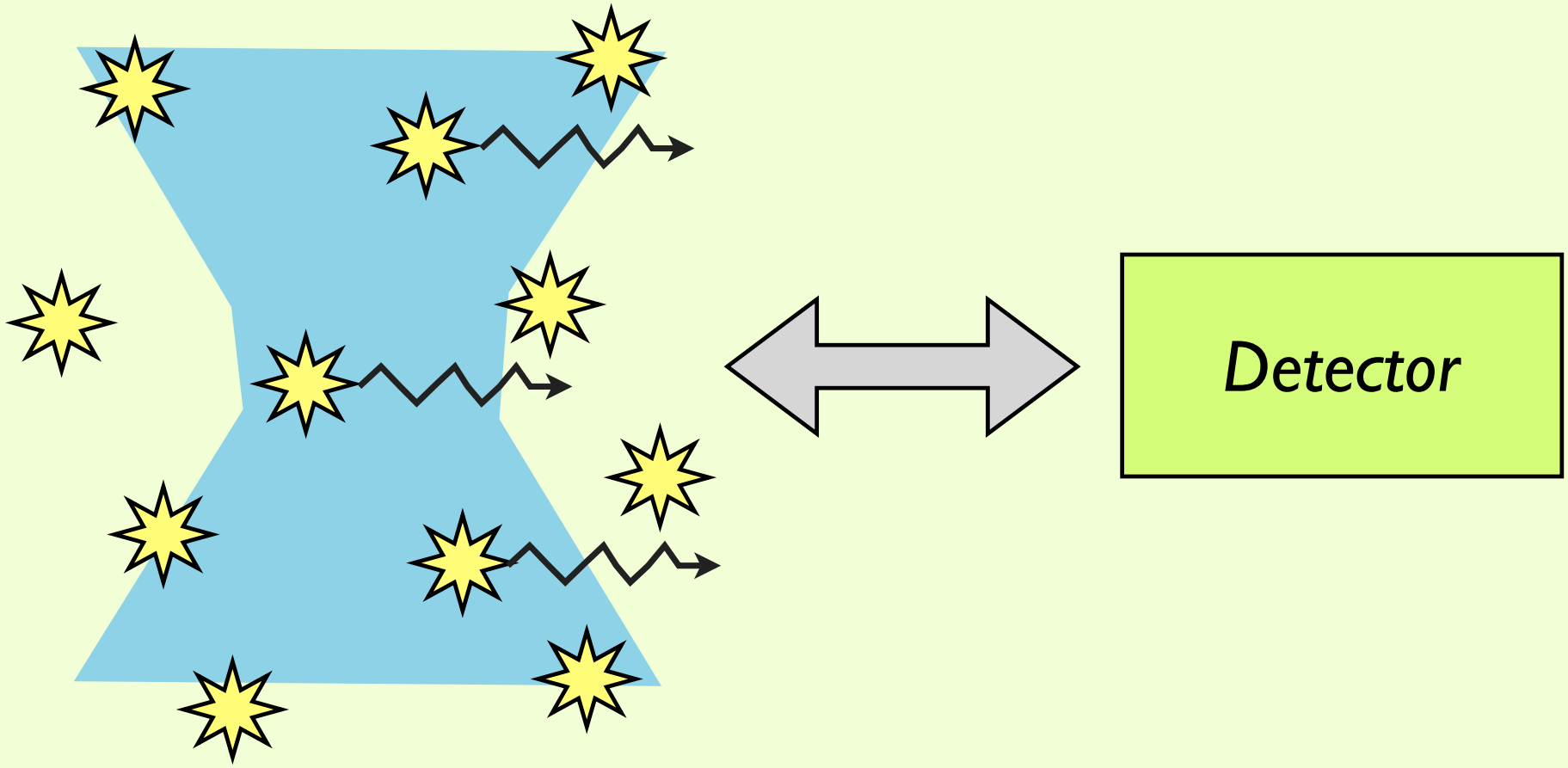
How to make single molecule fluorescence possible

- High-efficiency, low background fluorescence detection
  - *APD single photon counting module.*
- Bright (high quantum efficiency) dyes
  - *Alexa 488 dye.*
- High efficiency optics (objectives, filters, lenses) - remove Raman scattering and scattered excitation light
  - *Nikon optics, Chroma filters.*
  - *High numerical aperture objectives collect a significant number of the emitted photons.*
- Minimal detection volume (B*ackground photons are a function of the detection volume)*
  - *Confocal microscopy*

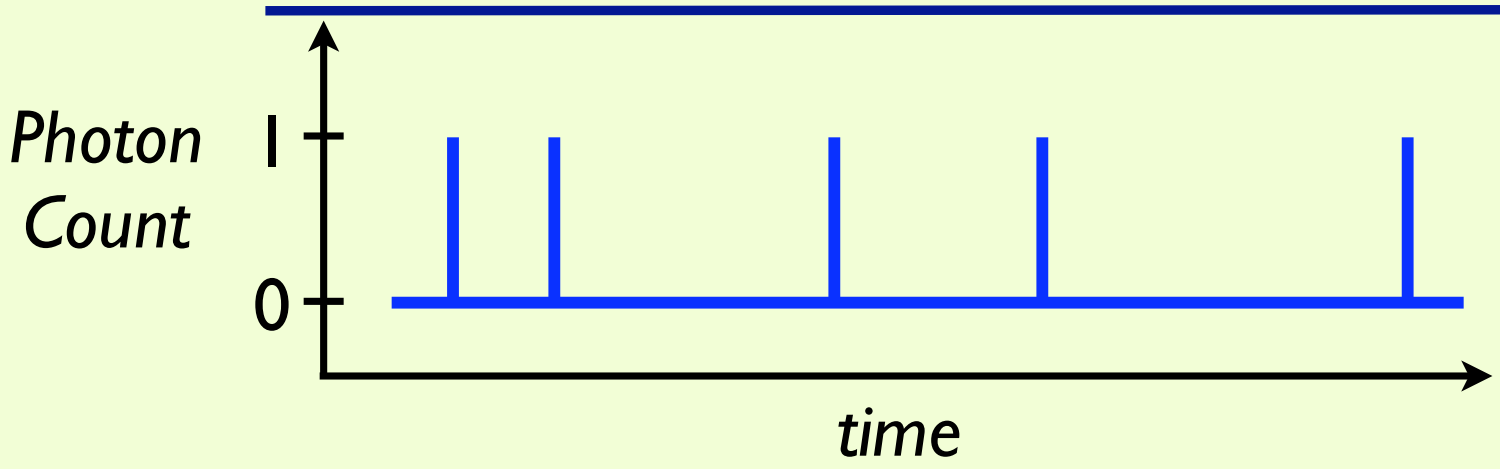
Your experimental setup



Your experimental setup



Experimental time trace



What affects the frequency of photon counts?

1. How often a fluorophore enters the confocal volume.
2. How often a fluorophore absorbs and emits a photons.
3. How often a fluorophore exits the confocal volume.
4. Background - "*Dark counts" from detection device, light from the rooom, scattered light.*

We assume these are Poisson processes

Random independent process - the occurrence of one event has no effect on the occurrence of another.

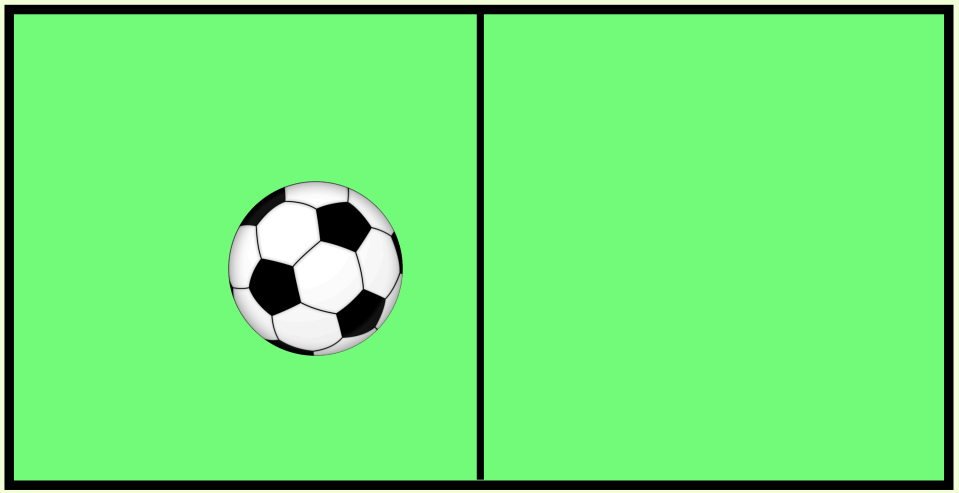
- N events are detected in measurement time interval dT.
- The expected rate of a Poisson process is μ.

$$\left\langle \frac{N}{dT} \right\rangle = \mu$$

How can you tell whether a process is Poissonian?

Measure N/dT again and again.
Does the rate of occurrence change?

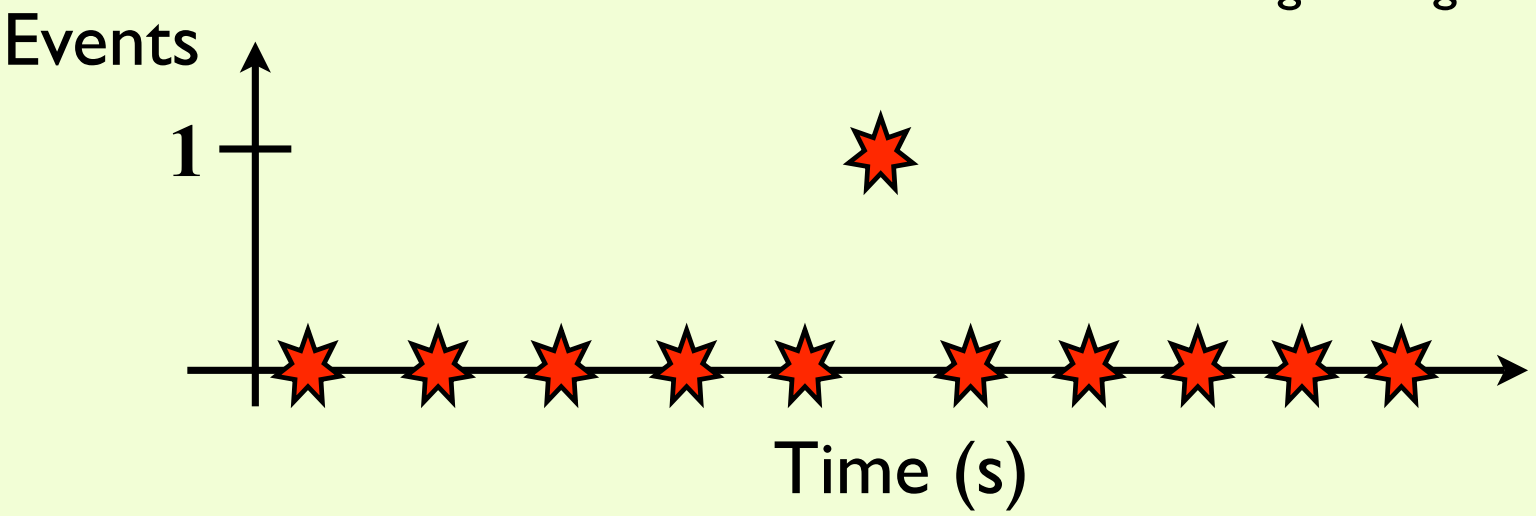
The time scale is important



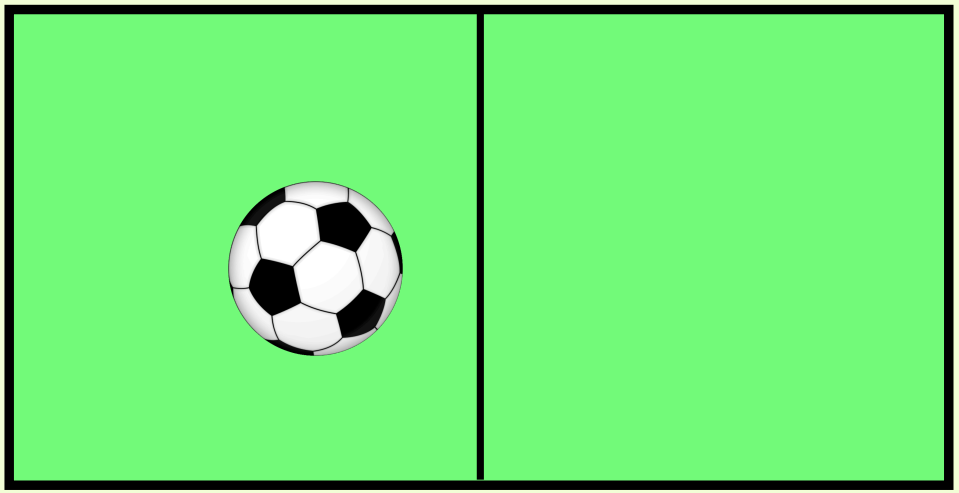
Soccer game analogy redux:

EVENT = soccer ball crosses the center line

Appears Poissonian if you look long enough.

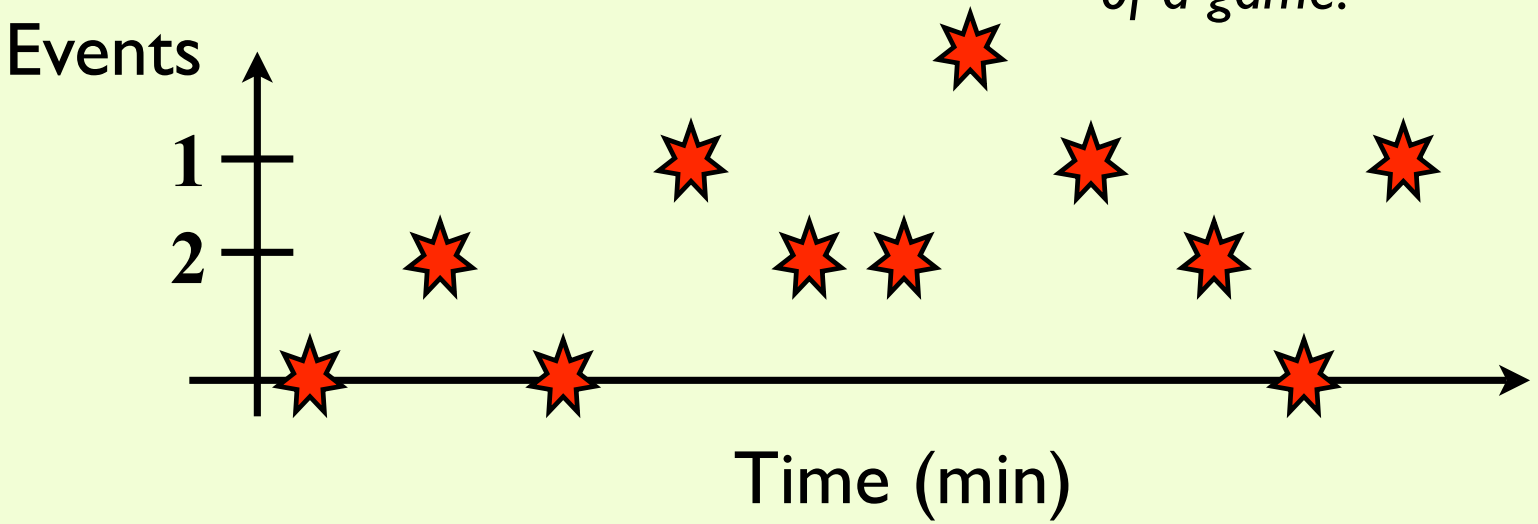


The time scale is important

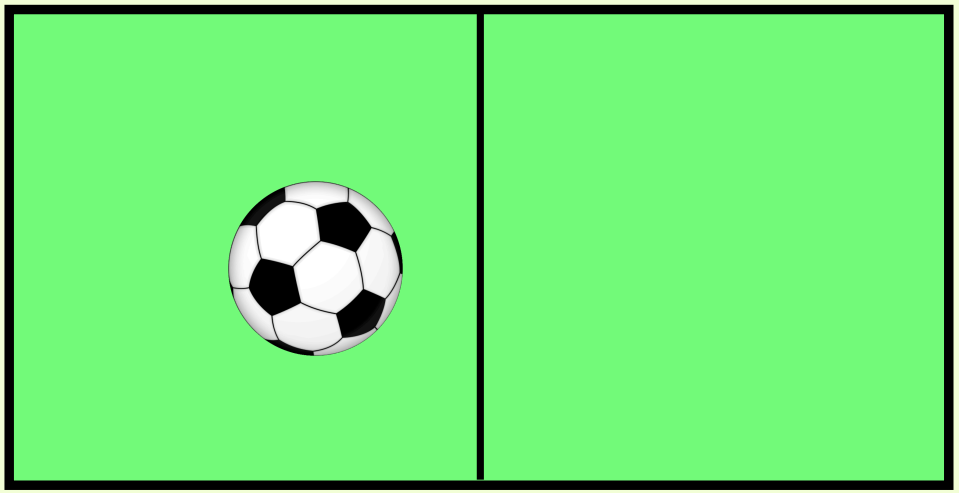


Soccer game analogy redux: How often does the ball cross the center line?

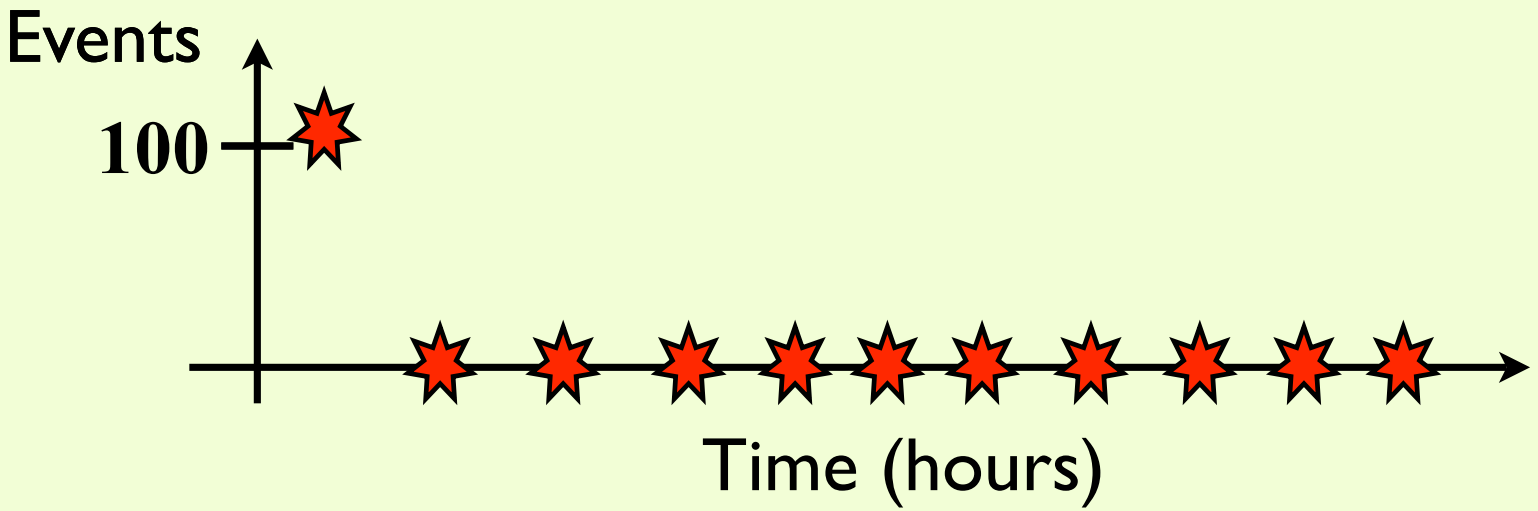
No longer appears Poissonian when you reach half-time or the end of a game.



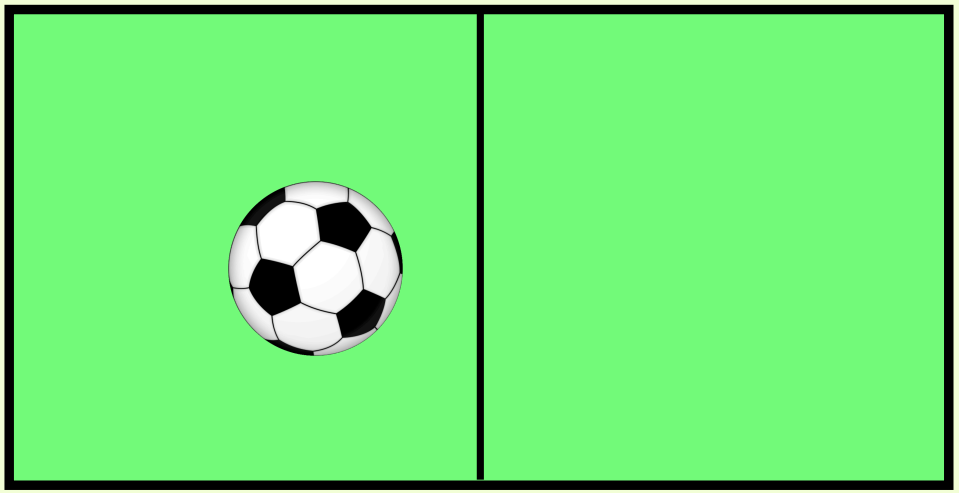
The time scale is important



Soccer game analogy redux: How often does the ball cross the center line?

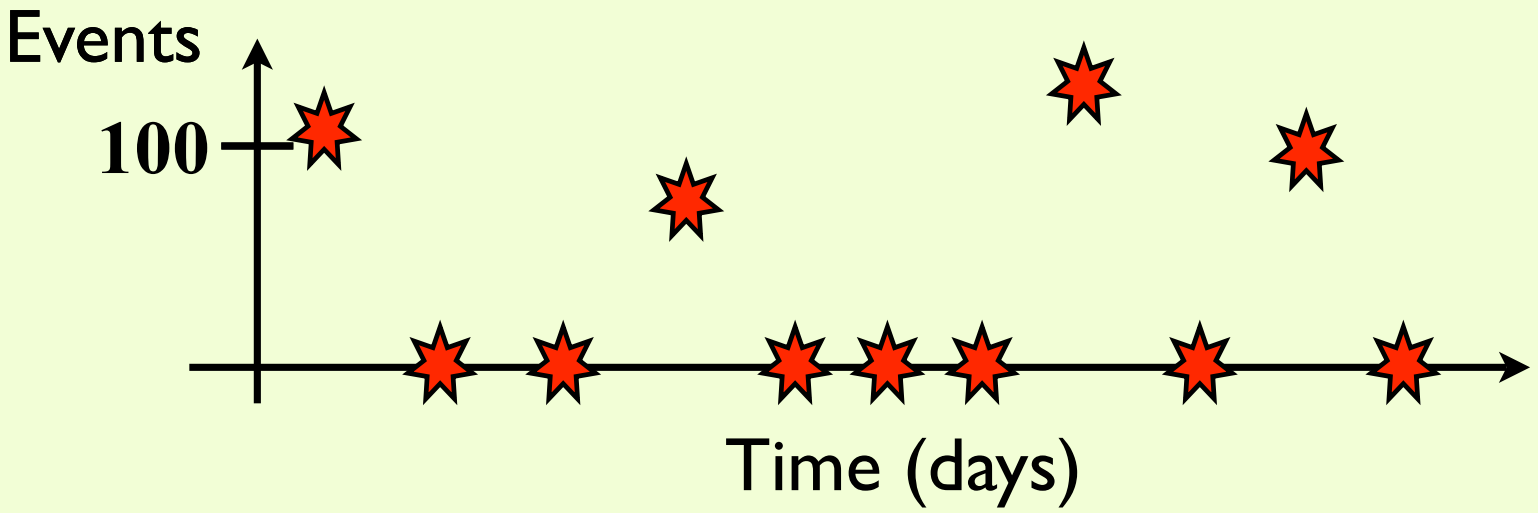


The time scale is important

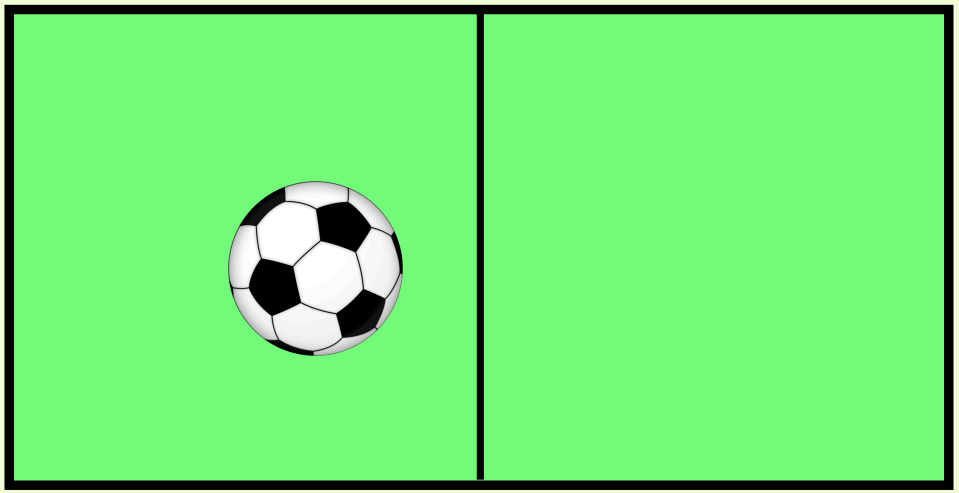


Soccer game analogy redux: How often does the ball cross the center line?

No longer appears Poissonian when you change seasons.

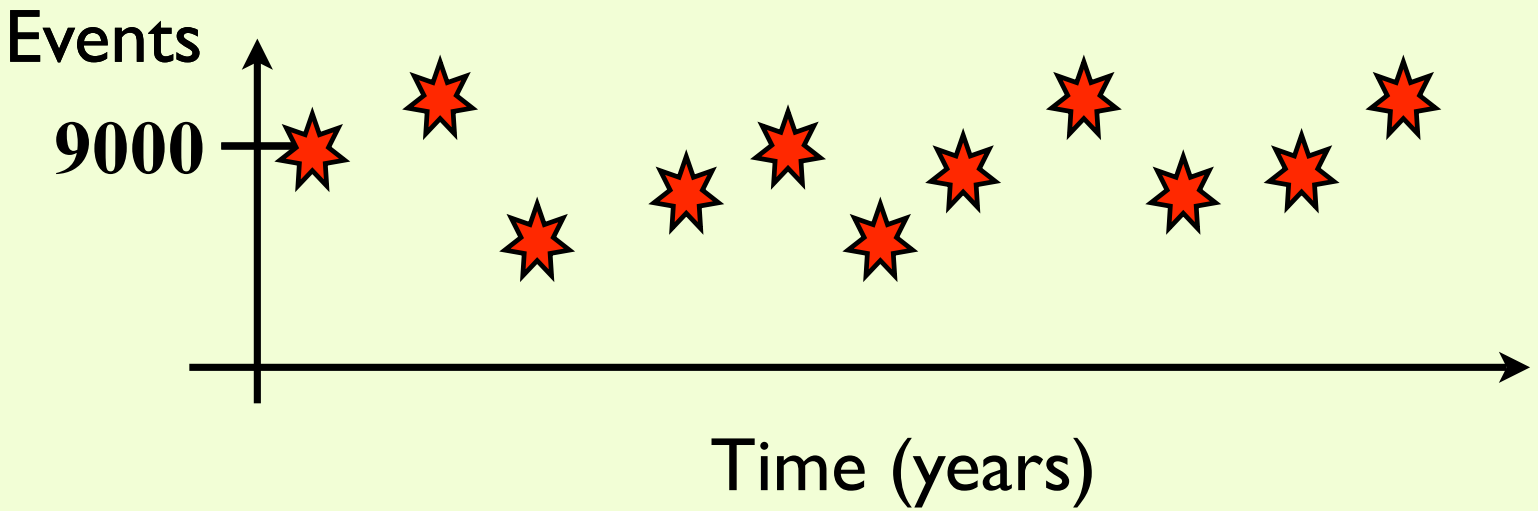


The time scale is important



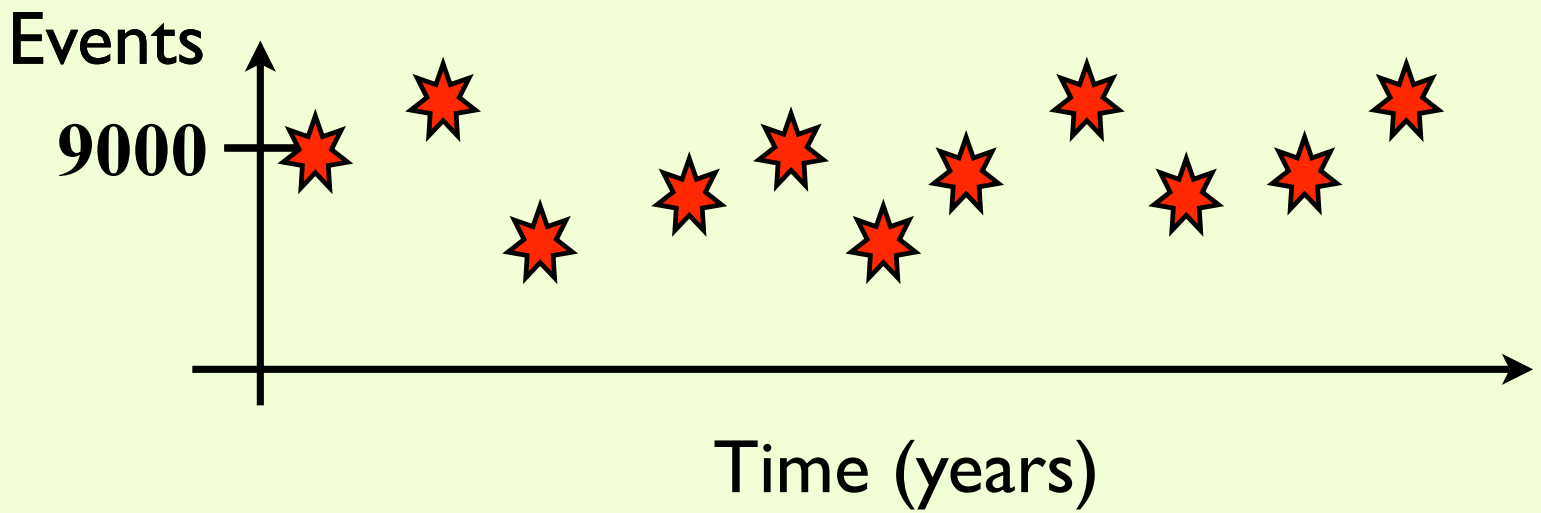
Soccer game analogy redux: How often does the ball cross the center line?

No longer appears Poissonian when you the zombies take over.



What is the probability of a particular value of N?

Even though the rate appears constant over time, the value of N is not always the same.



Poisson processes

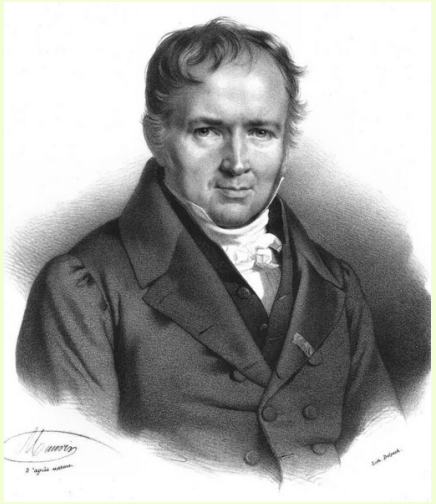
k - number of events occuring during *dt*

P(k) - probability of *k* events during *dt*

dT - measurement time interval

$$P(k) = \frac{e^{-\mu}\mu^{k}}{k!}$$

$$\left\langle \frac{N}{dT} \right\rangle = \mu$$

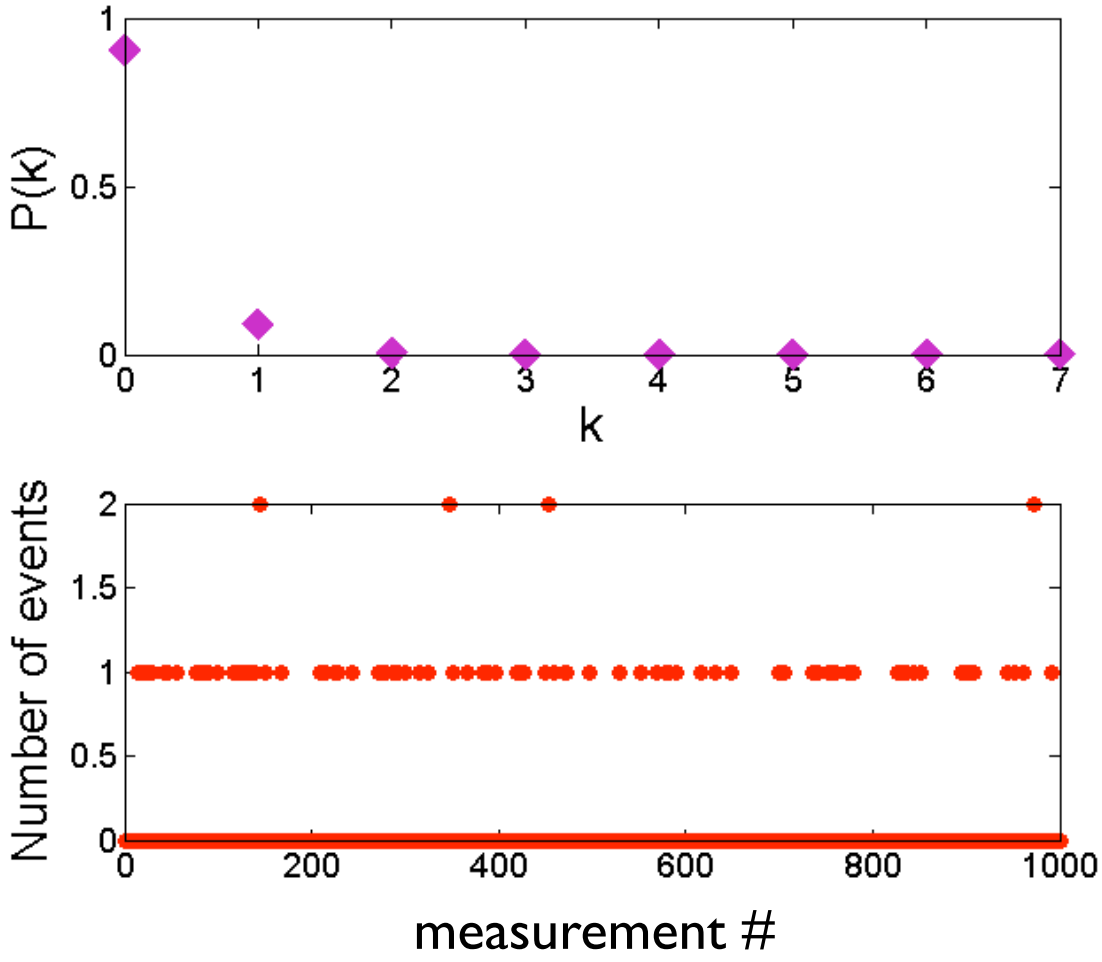


Siméon Poisson

"Research on the Probability of Judgments in Criminal and Civil Matters"

variance = mean

Sampling bin is an important variable

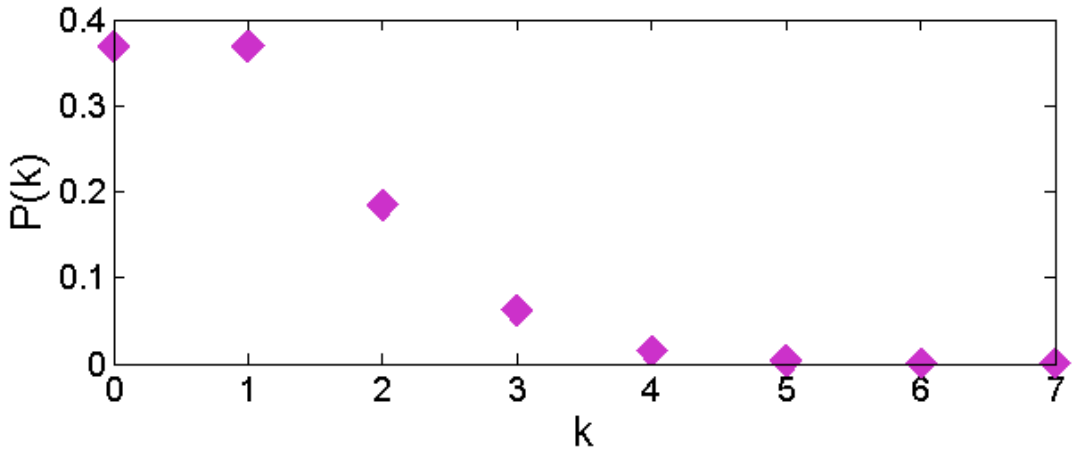


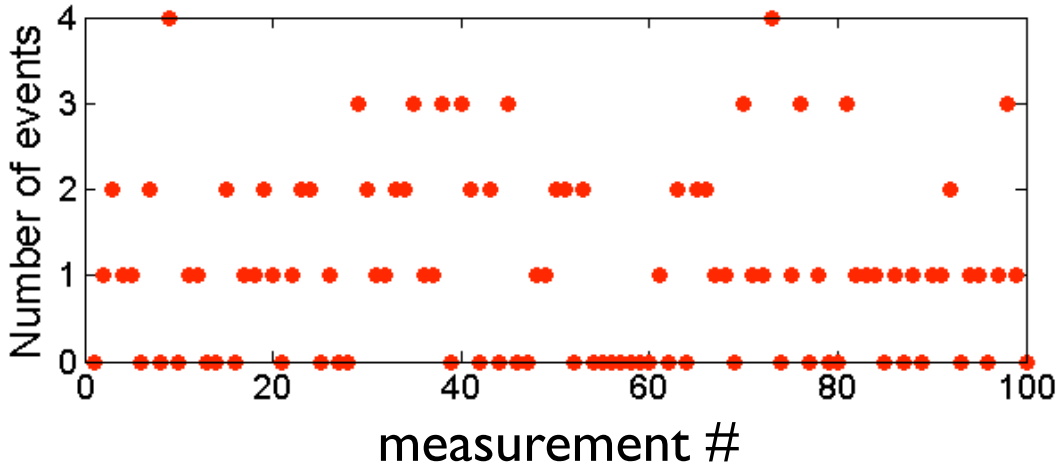
λ =1 event every 1 μs

dT = 100 ns

Total time = 100 μs

Sampling bin is an important variable



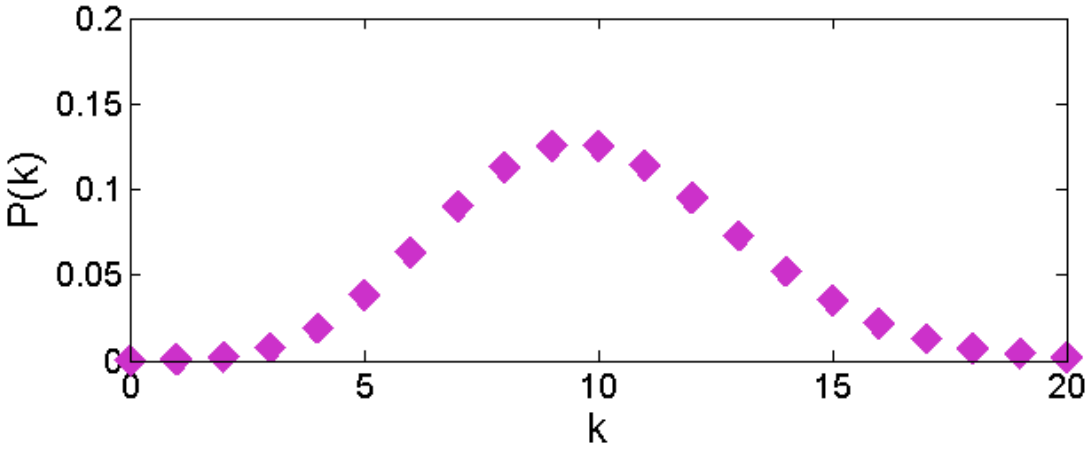


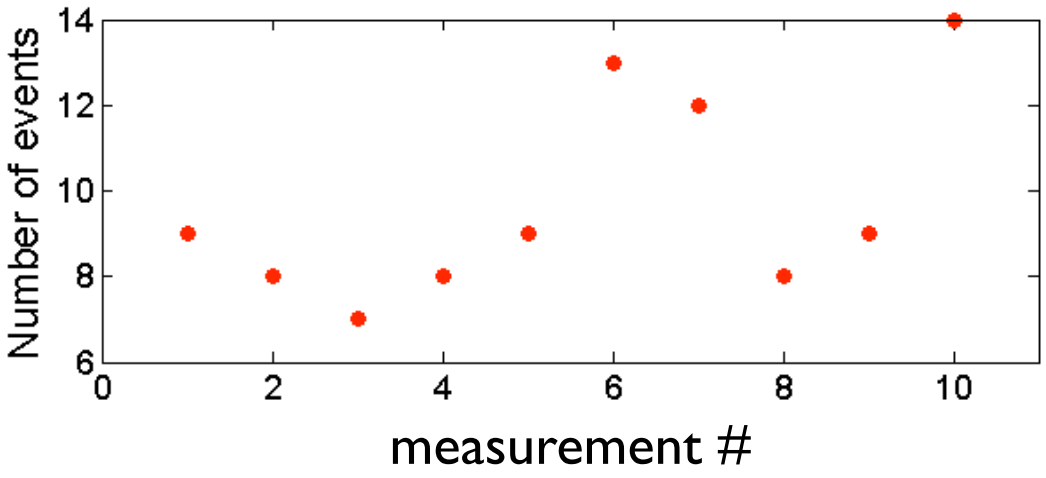
λ = 1 event every 1 μs

dT = 1000 ns

Total time = 100 μs

Sampling bin is an important variable



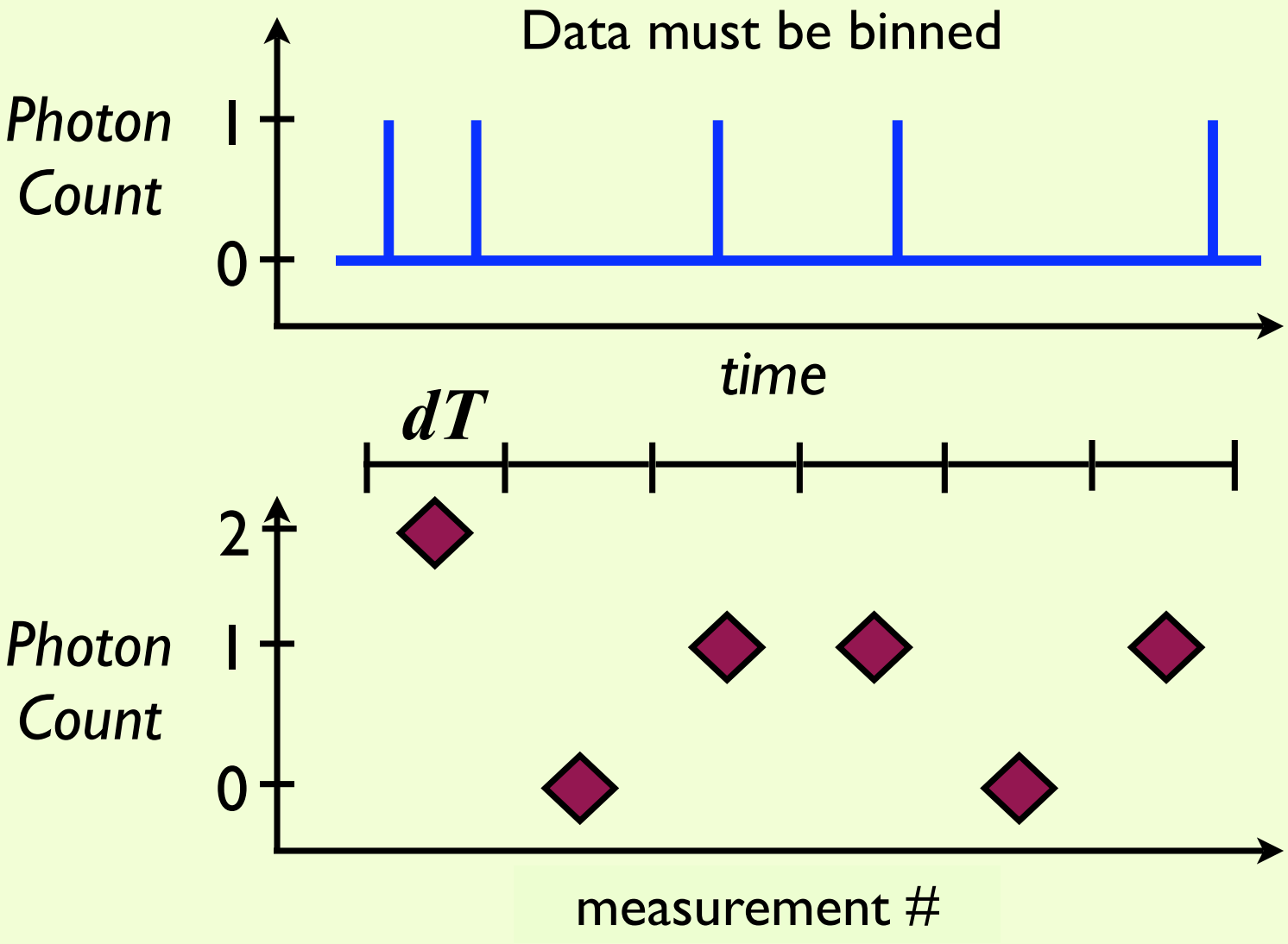


λ = 1 event every 1 μs

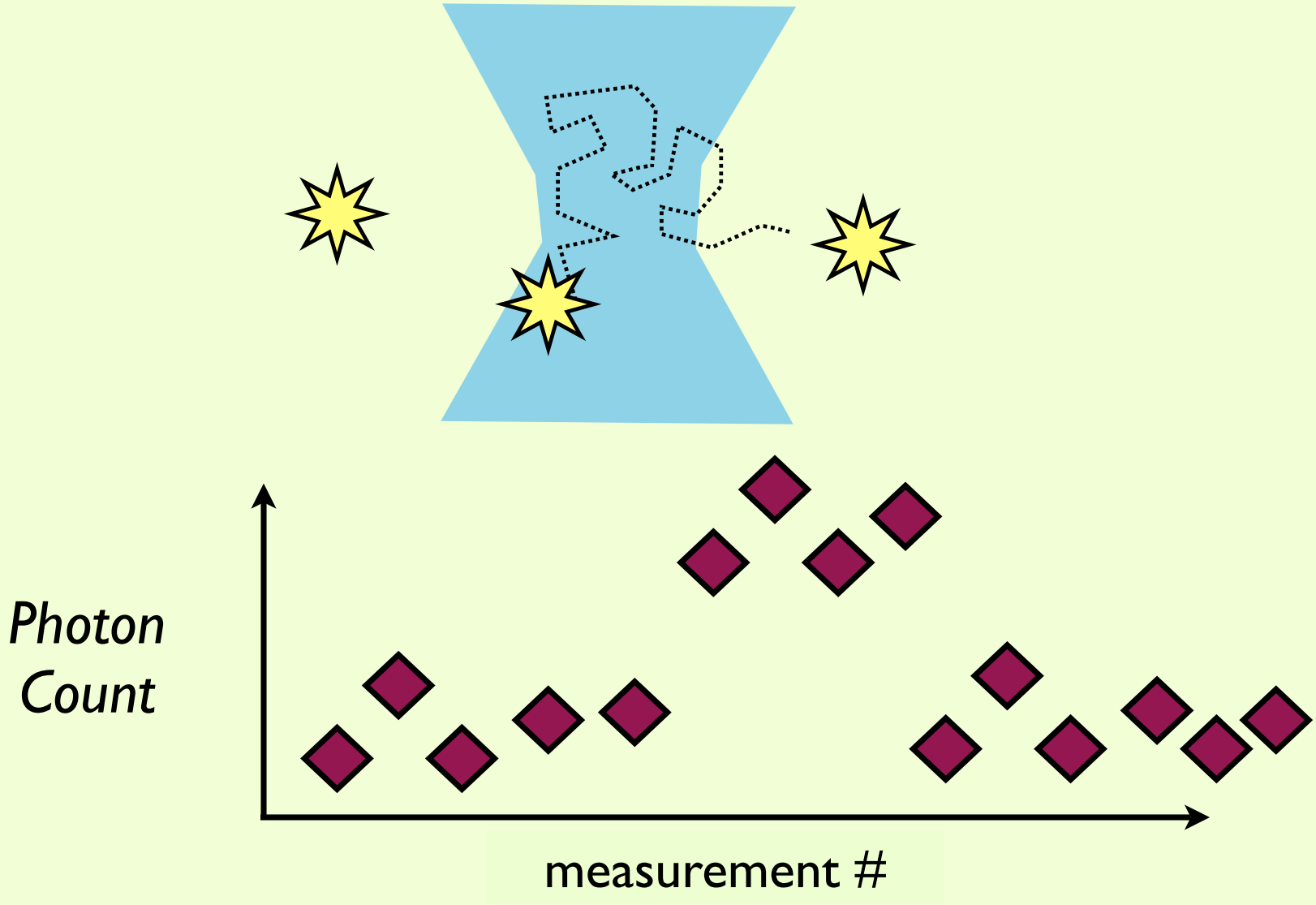
dt = 10,000 ns

Total time = 100 μs

Modeling our data as multiple poisson processes



We would like information about how long the fluorophore stays in the confocal volume



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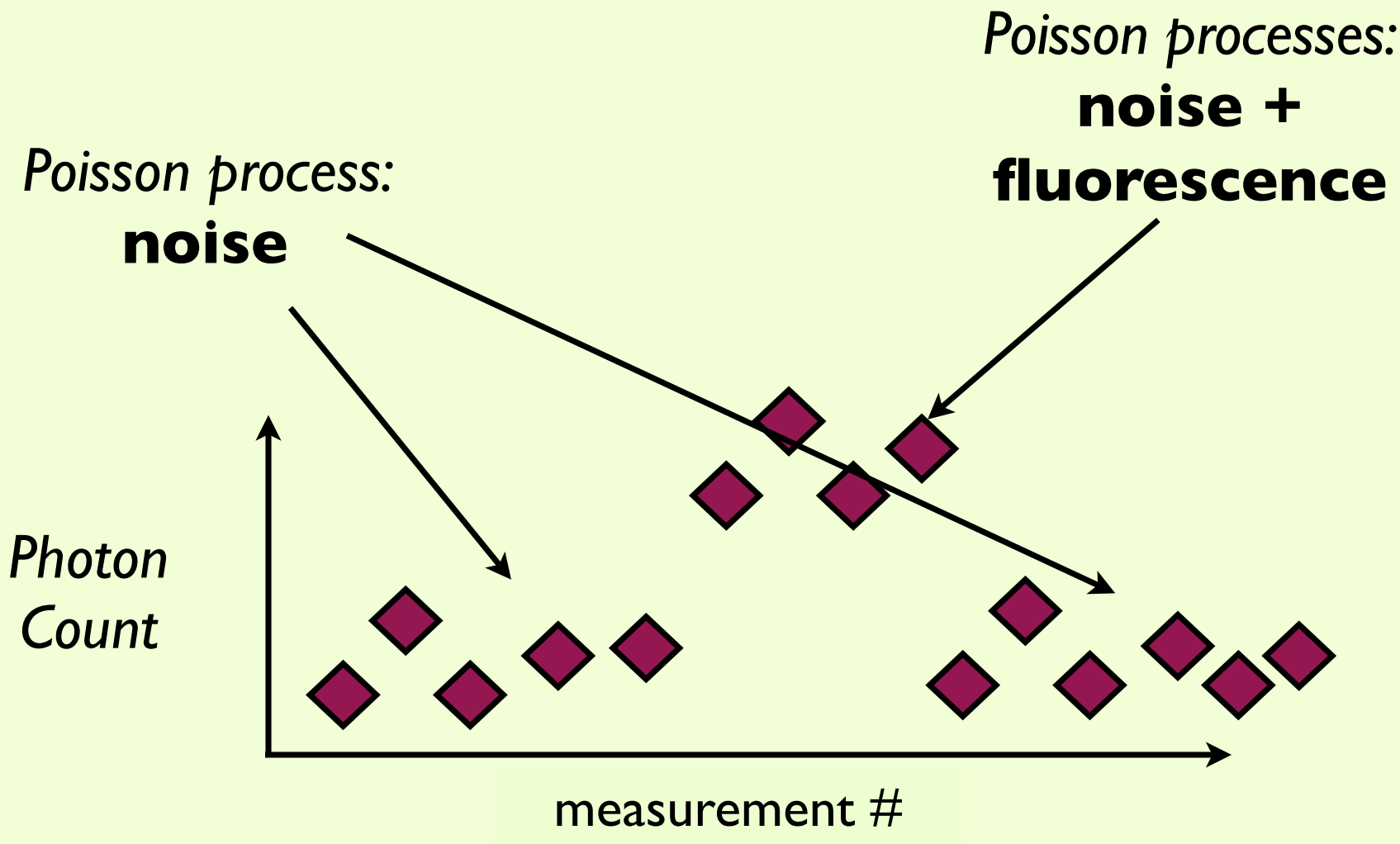
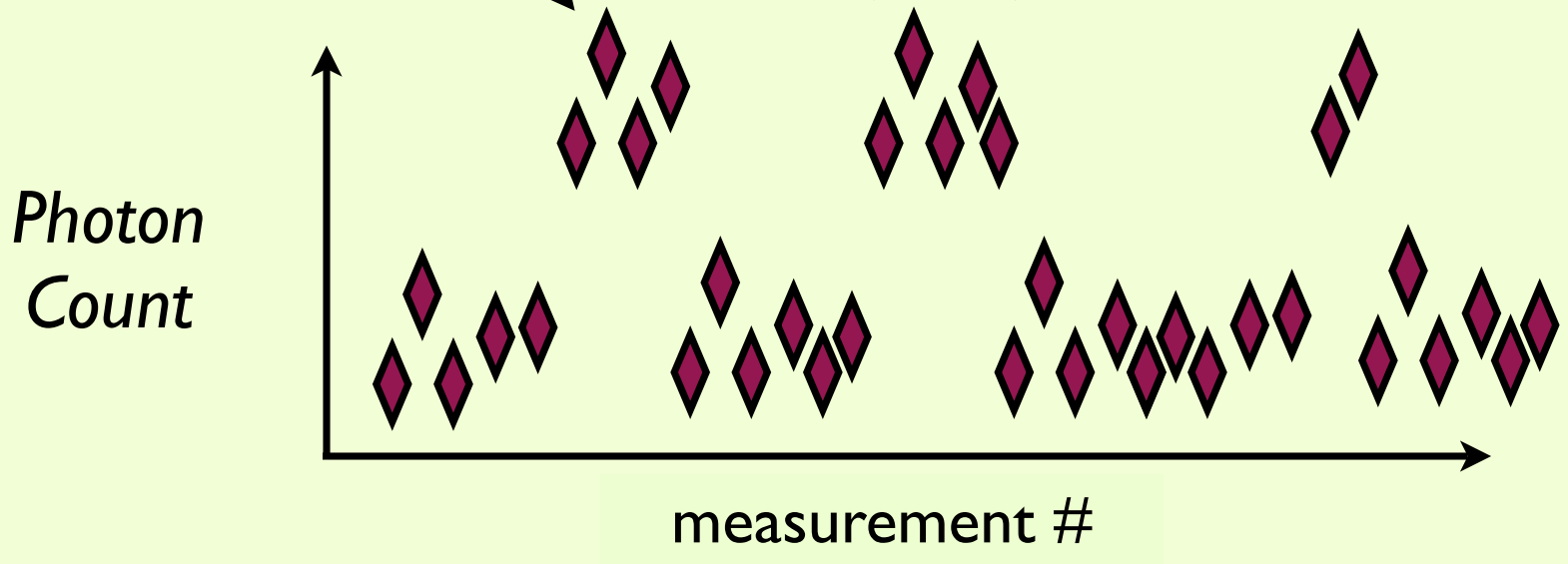


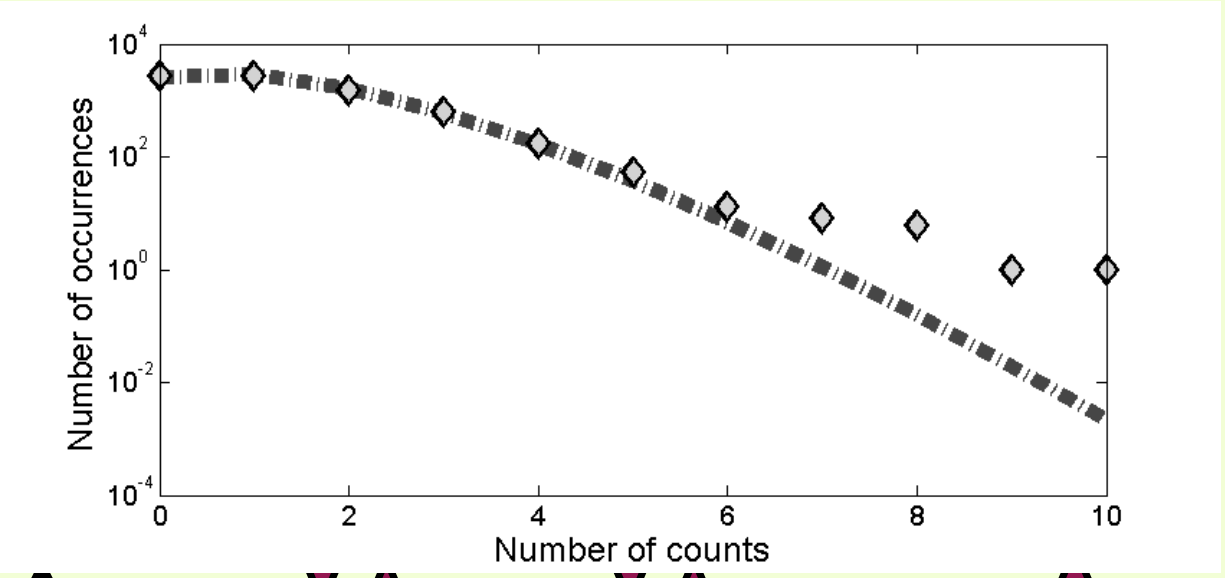
We would like information about how long the fluorophore stays in the confocal volume

Poisson process:
frequency of events

Diffusion



Our simplistic approach: can our data be described by a single Poisson distribution?



Photon Count

measurement #

Our simplistic approach: can our data be described by a single Poisson distribution?

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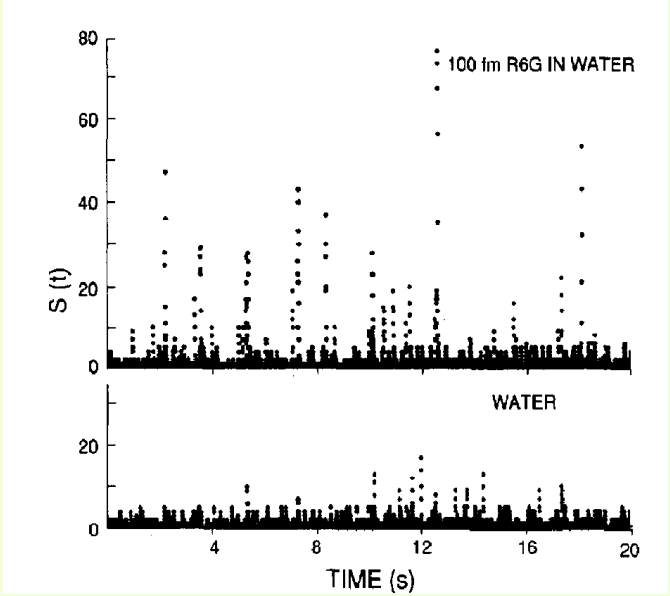
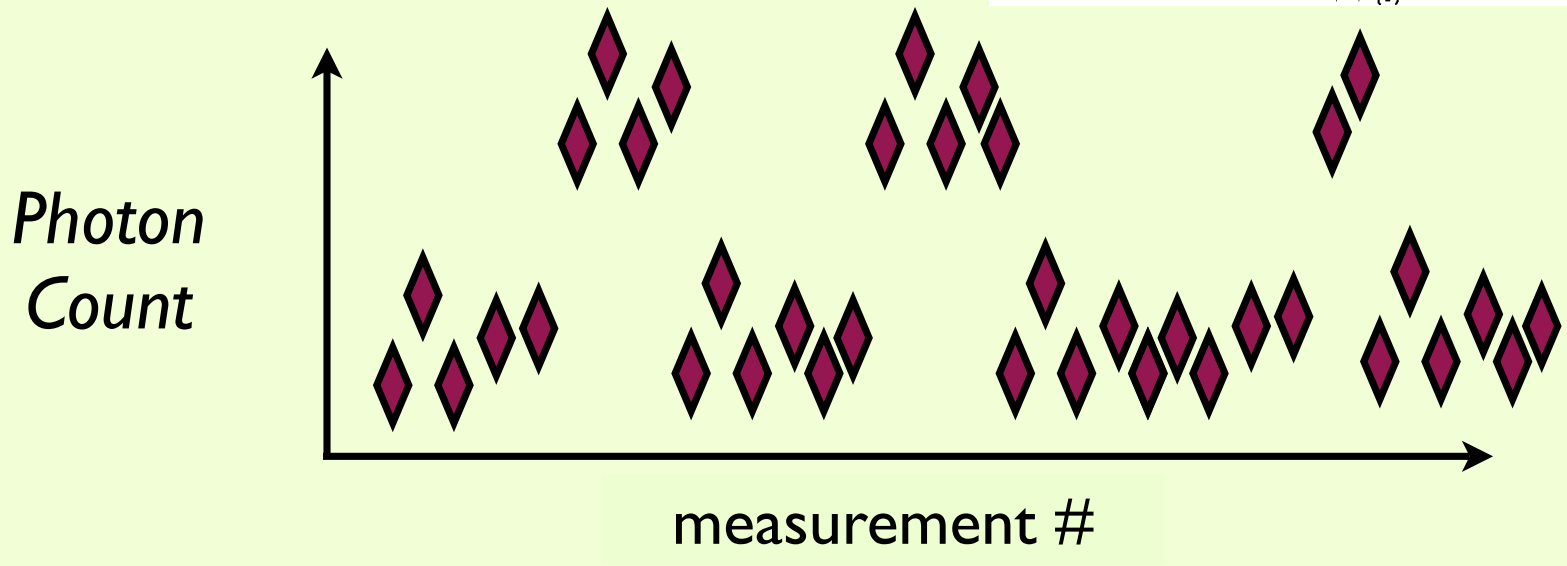




What are we missing?

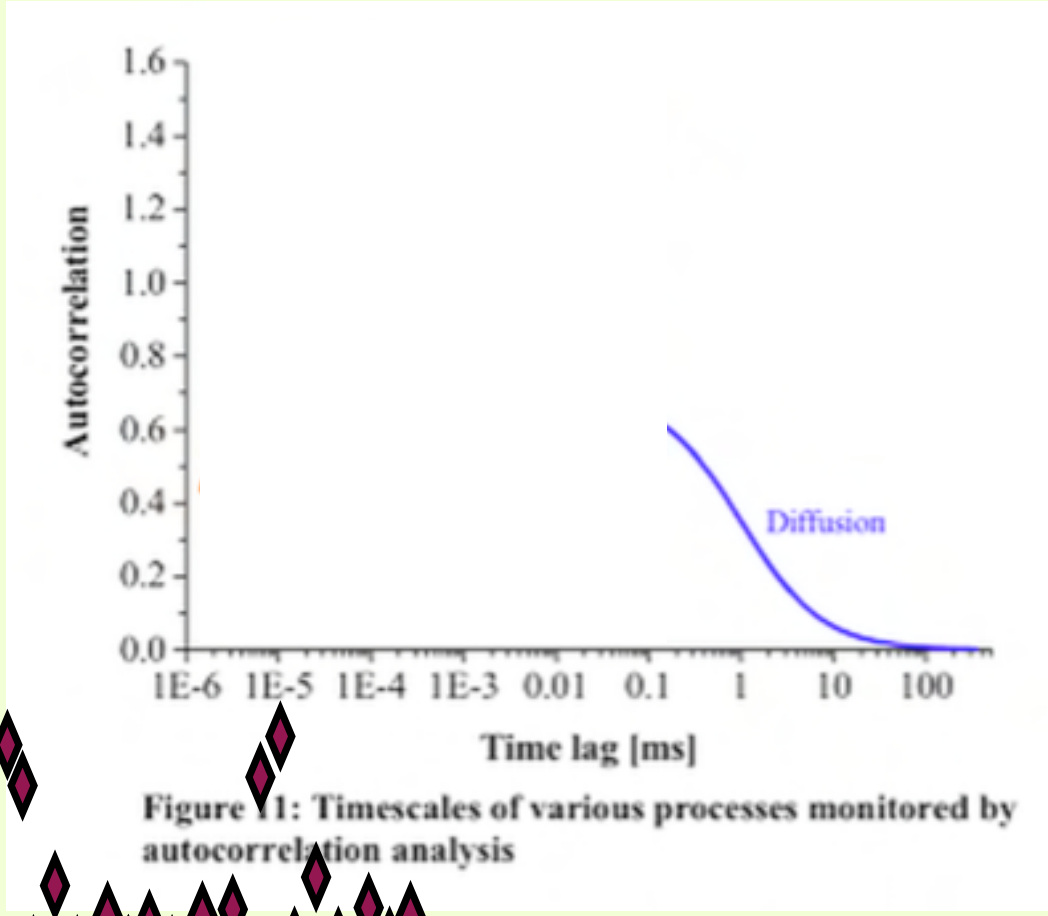
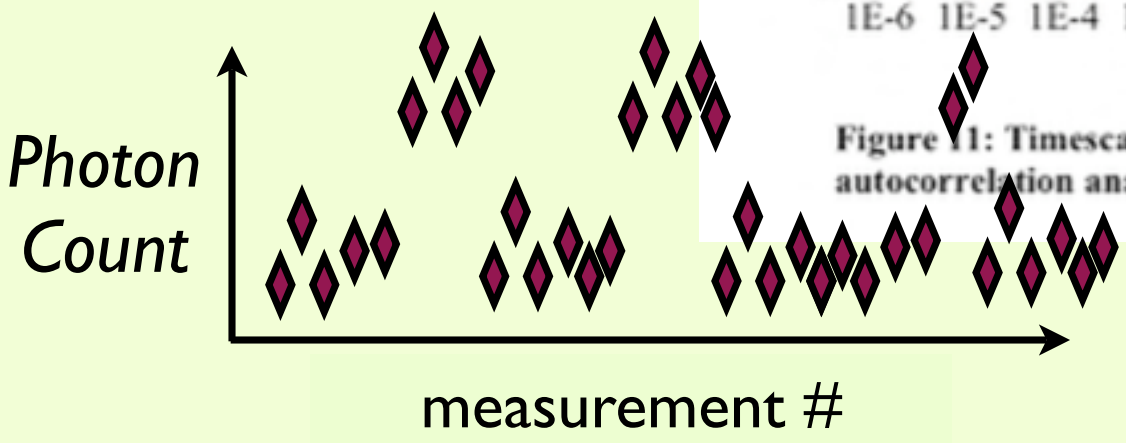


Figure 11: Timescales of various processes monitored by autocorrelation analysis



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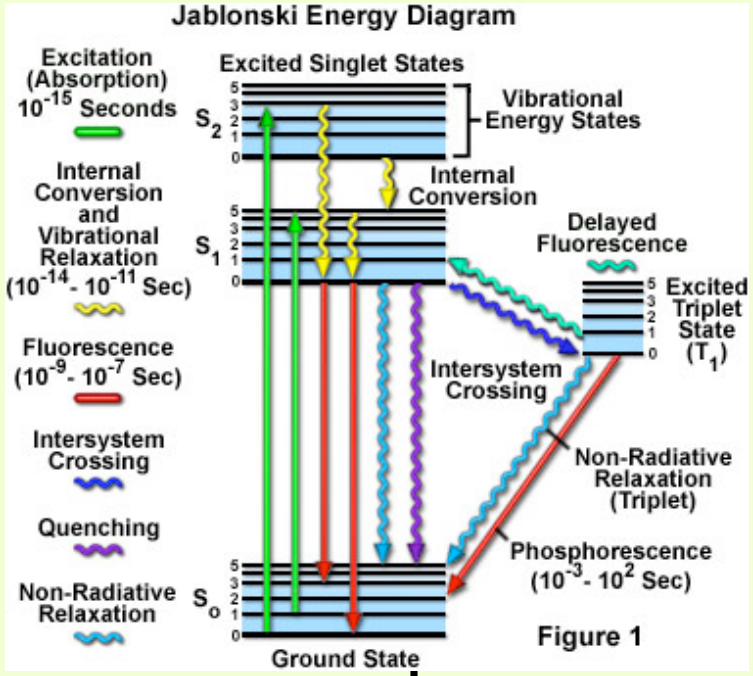

Poisson processes:
noise + fluorescence