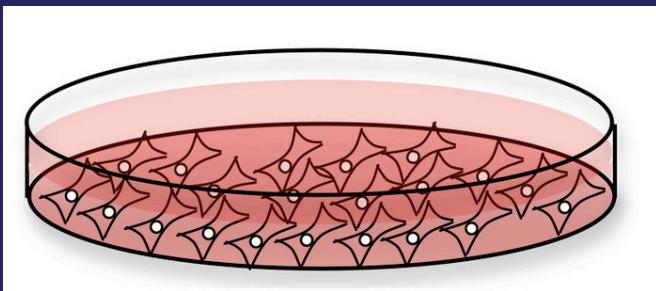


*Gene expression at the single cell level*

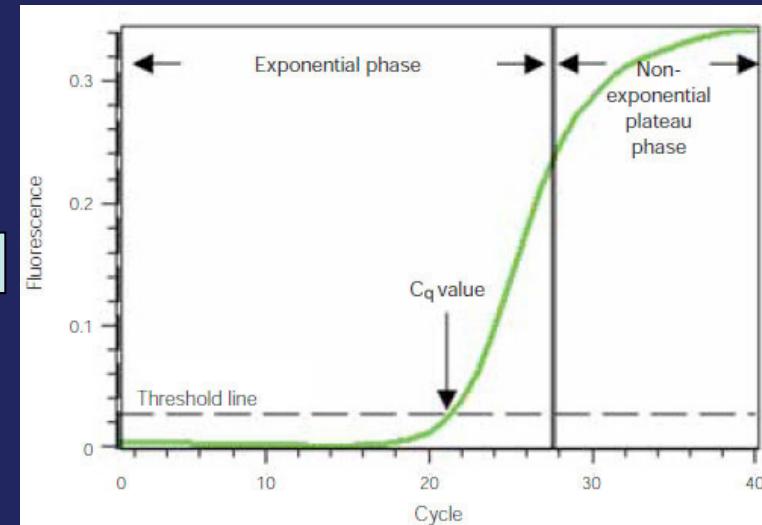
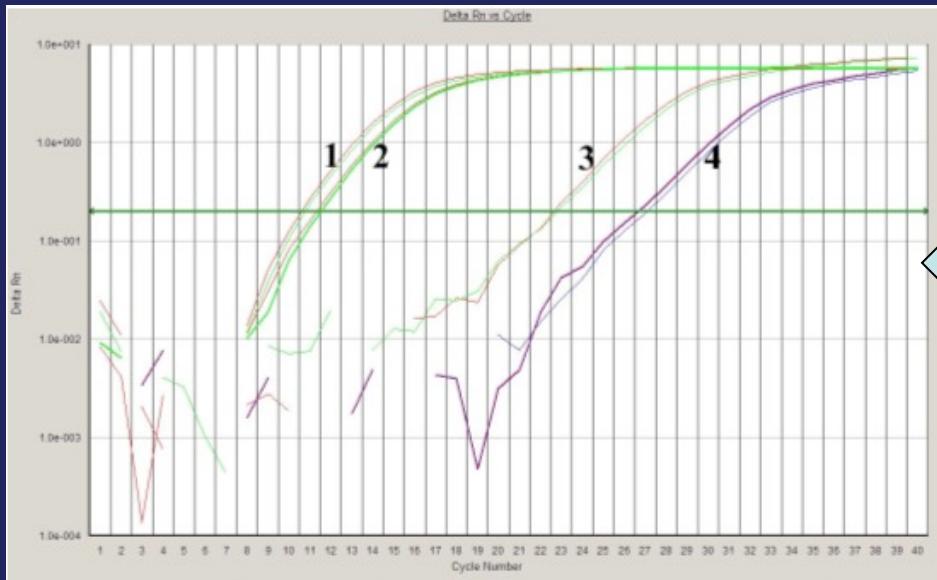
# How to quantify gene expression



Extract mRNA, convert into cDNA



Compare samples



→ Relative values from a mixture of cells

# A typical gene expression analysis experiment



Will it blend ?

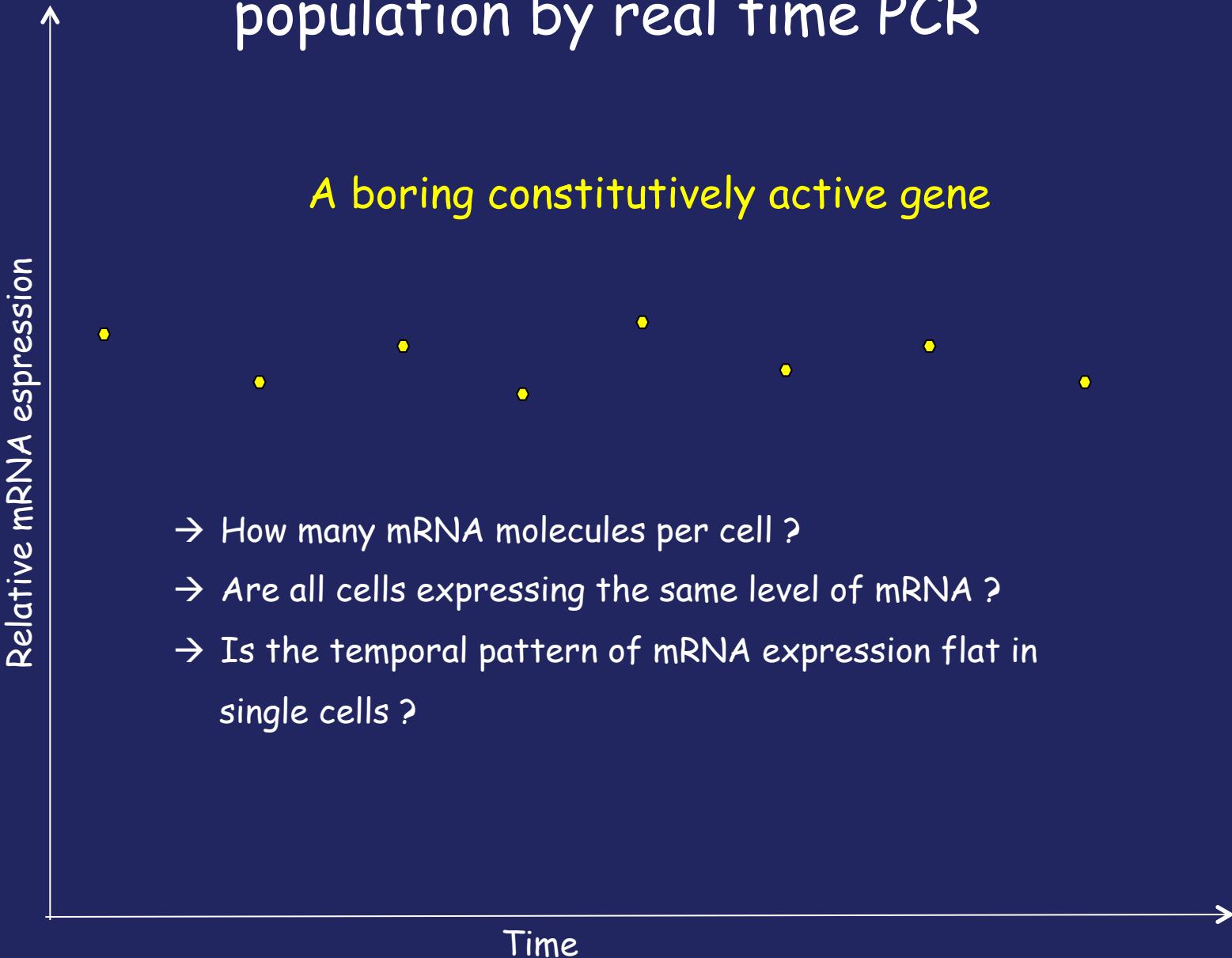


Nexus

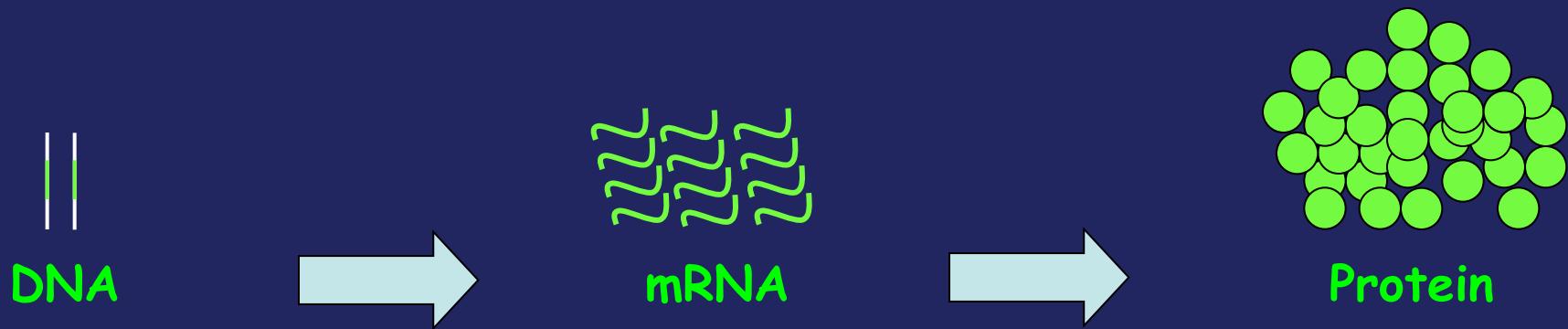
Kindle

iPad

# Quantification of gene expression in a cell population by real time PCR



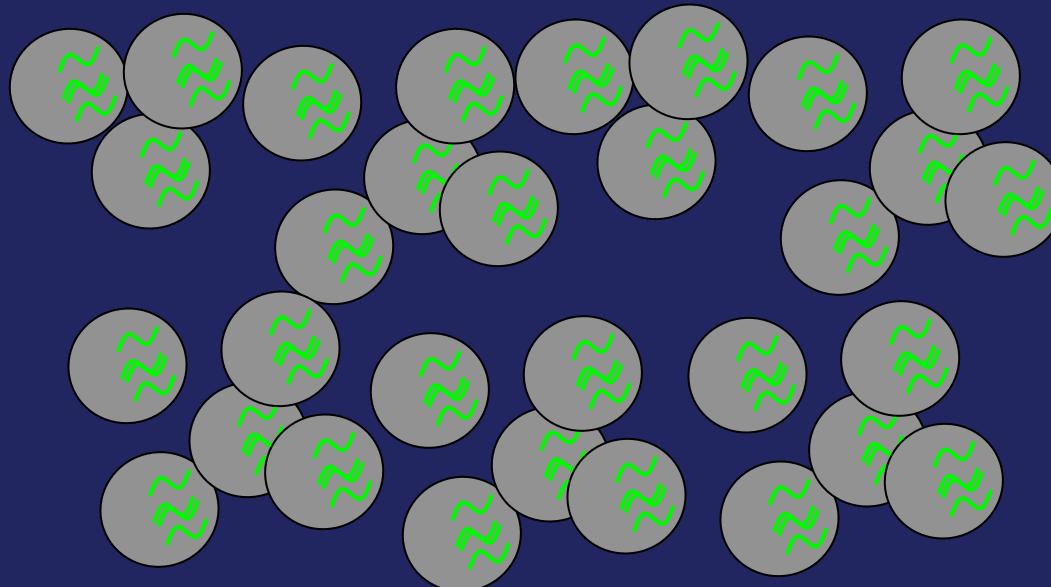
# Gene expression at the single cell level



Do population measurements  
reflect single cell behaviour ?

If population analysis reflects single cell behaviour:

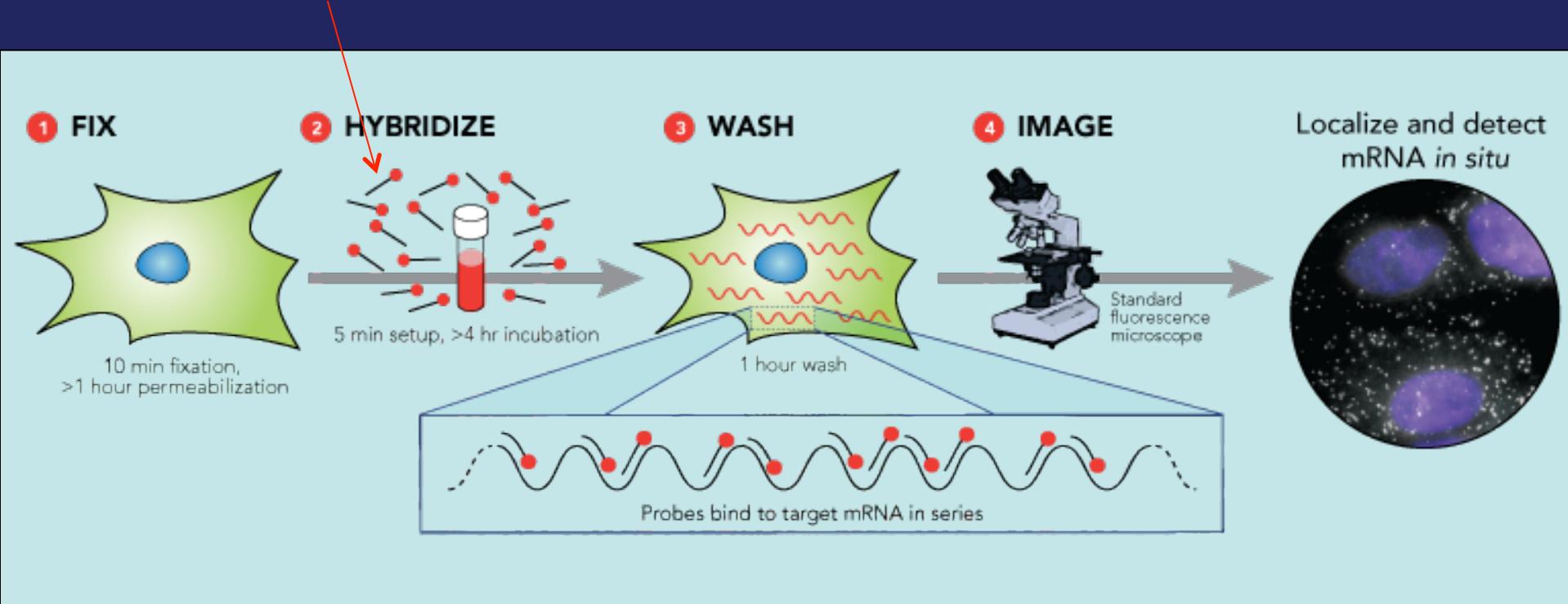
~ mRNA molecule of constitutively active gene



Isogenic cell population

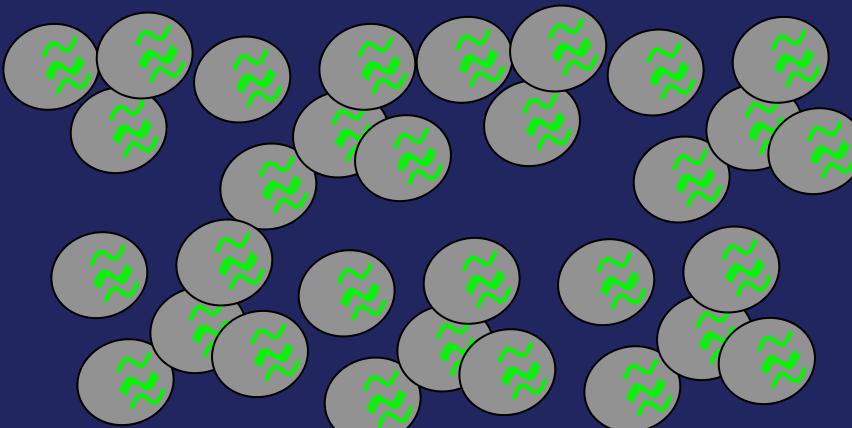
# Can we count mRNA molecules in single cells ?

Fluorescent probes complementary to a given mRNA

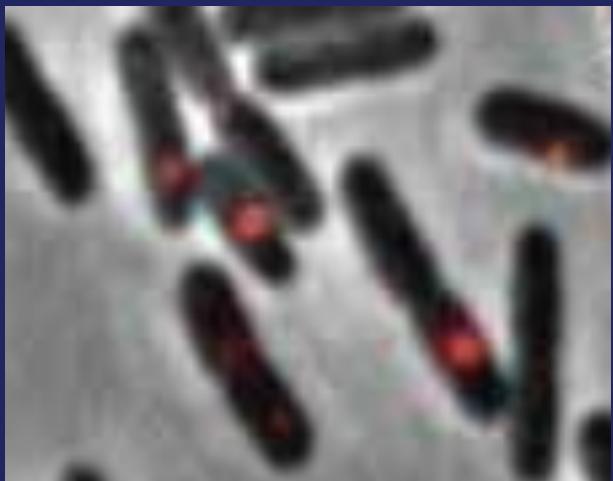


→ Yes !! Using RNA fluorescence in situ hybridisation (RNA FISH)

# Do we see this ?

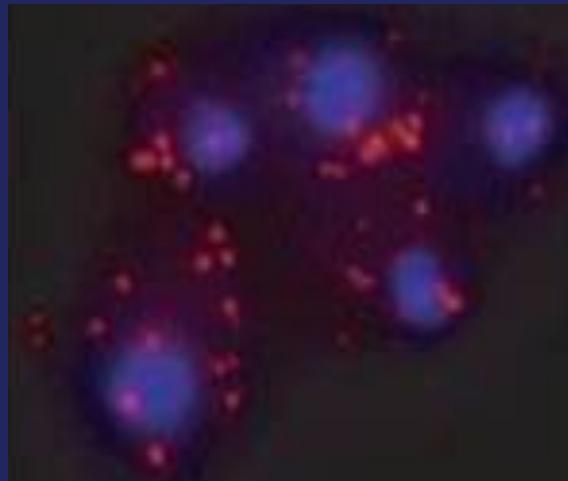


*E.Coli*



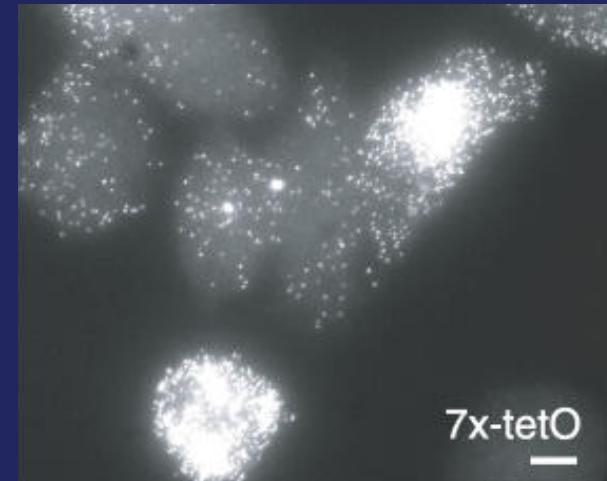
So et al., Nat. Genetics 2011

*Yeast*



Zenklusen et al., NSMB 2008

*Mammalian cells*

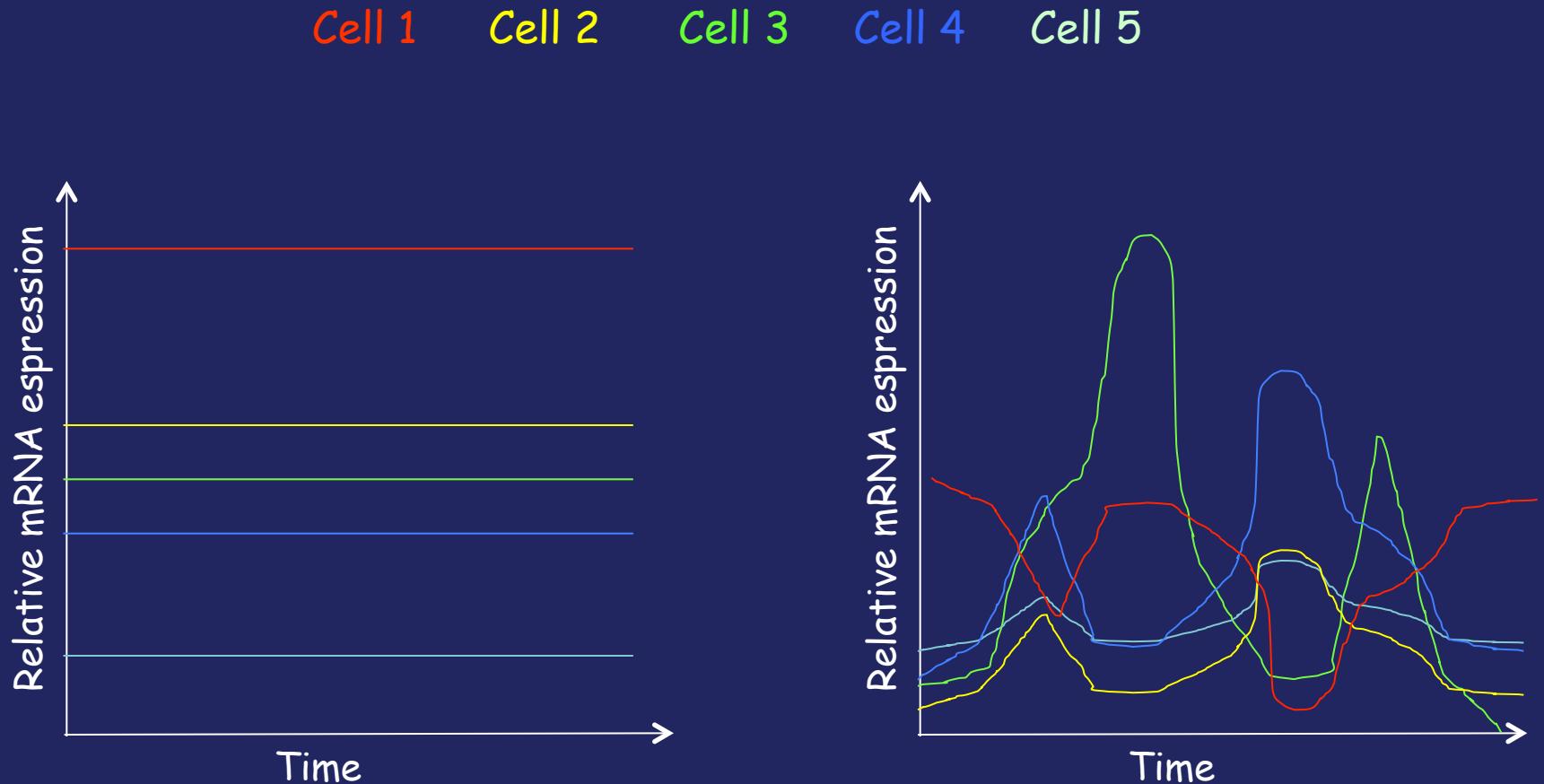


7x-tetO  
—

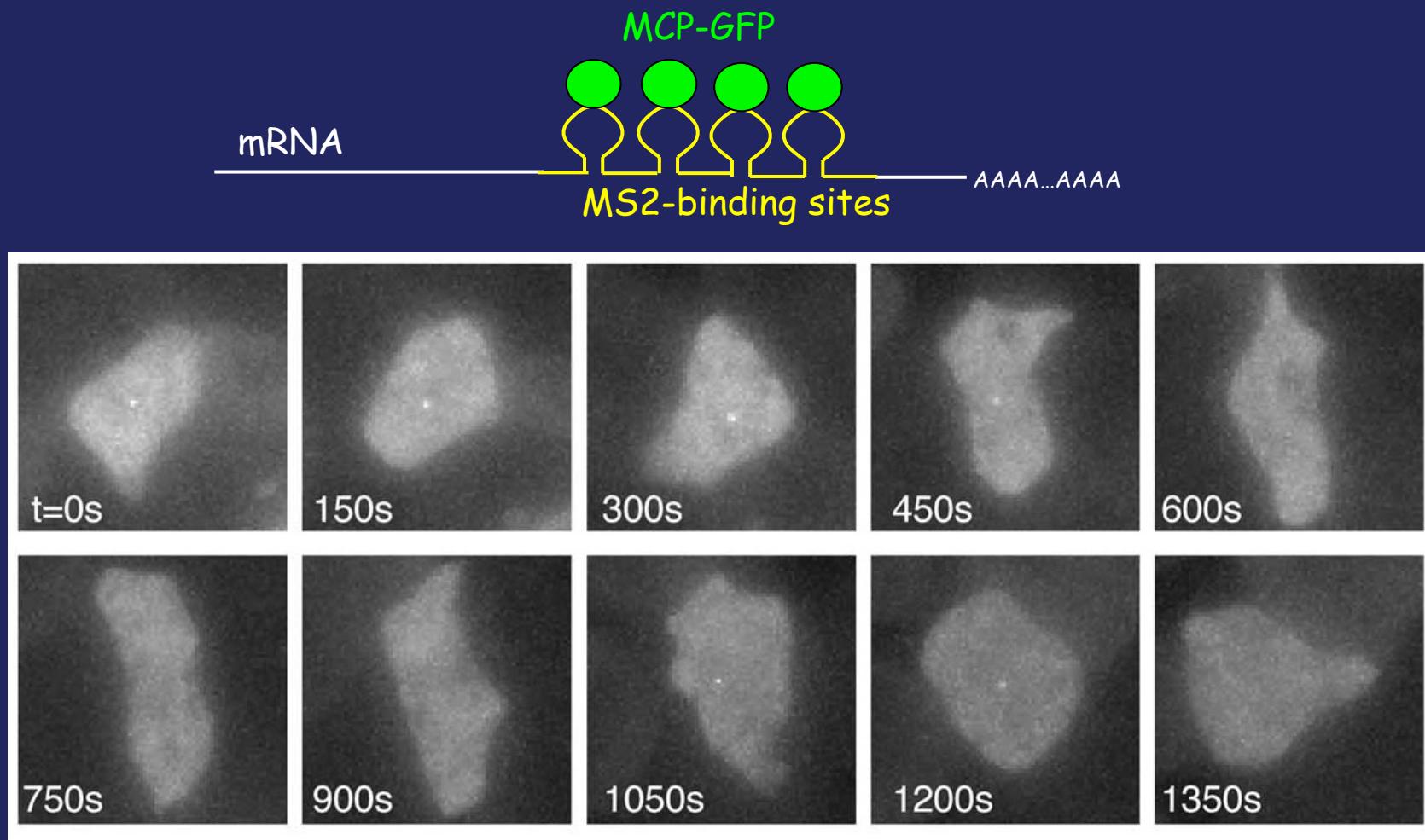
Raj et al., PLoS Biology 2006

No !!!

# What is going on ?



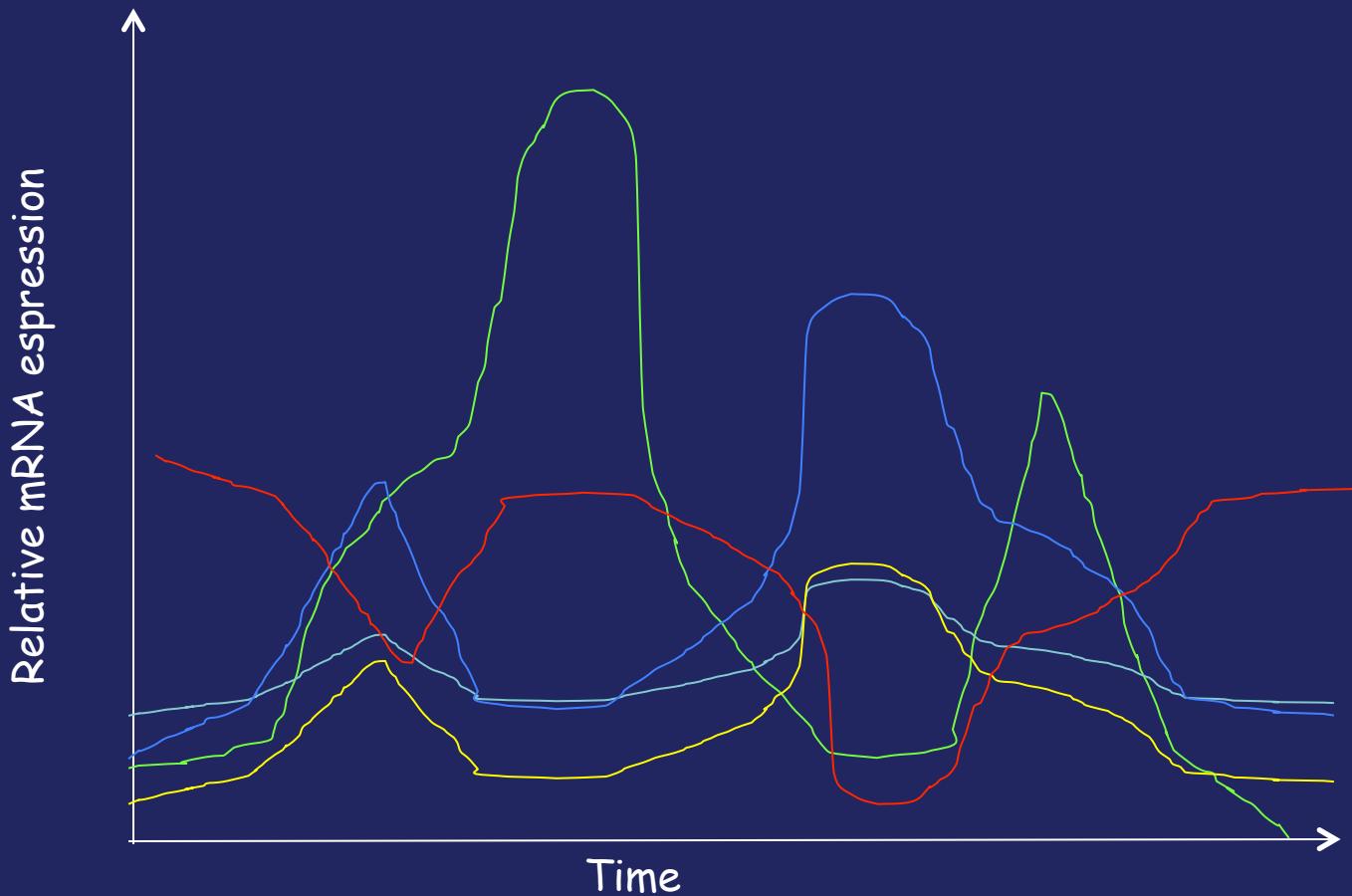
# Live imaging of transcription



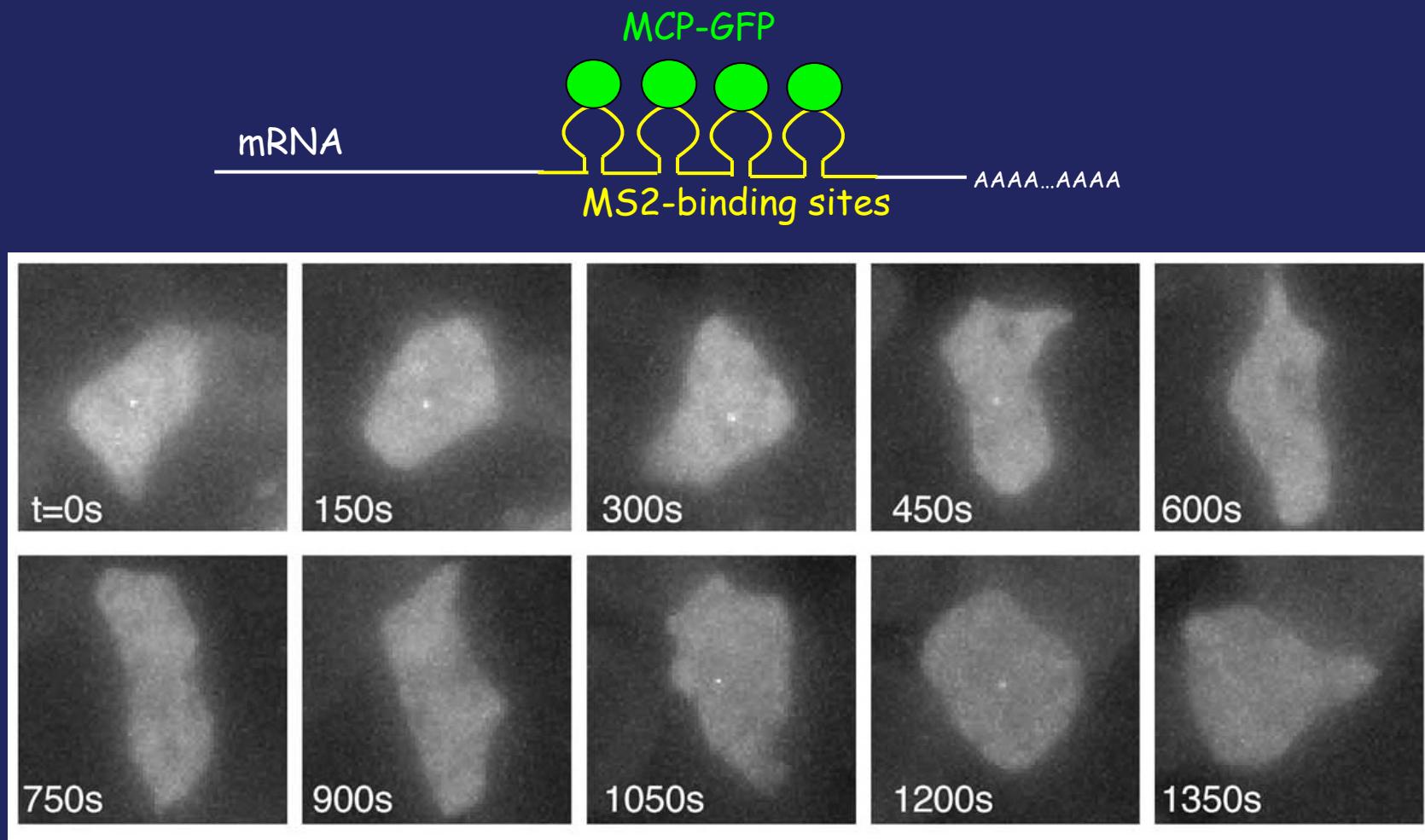
*Chubb et al., Current Biology 2006*

Transcriptional bursting

This is what is going on !



# Live imaging of transcription



*Chubb et al., Current Biology 2006*

Low signal to noise ratio, toxicity → poor quantification

→ We need a new experimental approach allowing:

- High resolution monitoring of transcription
- Absolute quantification of protein copy numbers and mRNA copy numbers
- Deconvolution to obtain temporal pattern of on/off switching of gene activity

# Experimental approach

→ Record transcription by live imaging of a short-lived reporter inserted as one copy in the genome of mammalian cells under the control of different regulatory sequences



Accumulation of mRNA and protein ÷ stability

→ Need a very sensitive system

→ Use luciferase as a reporter, monitored by luminescence microscopy

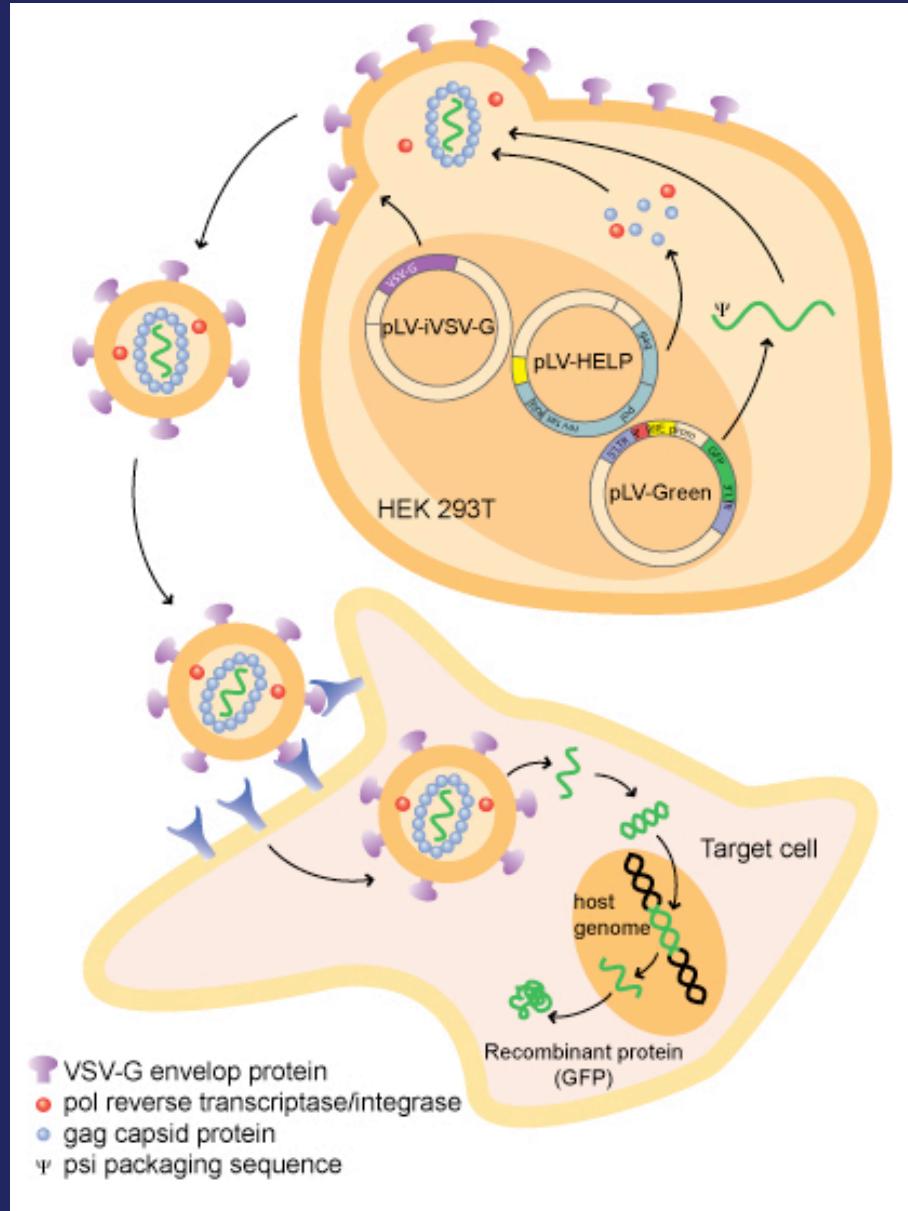
# Gene trapping

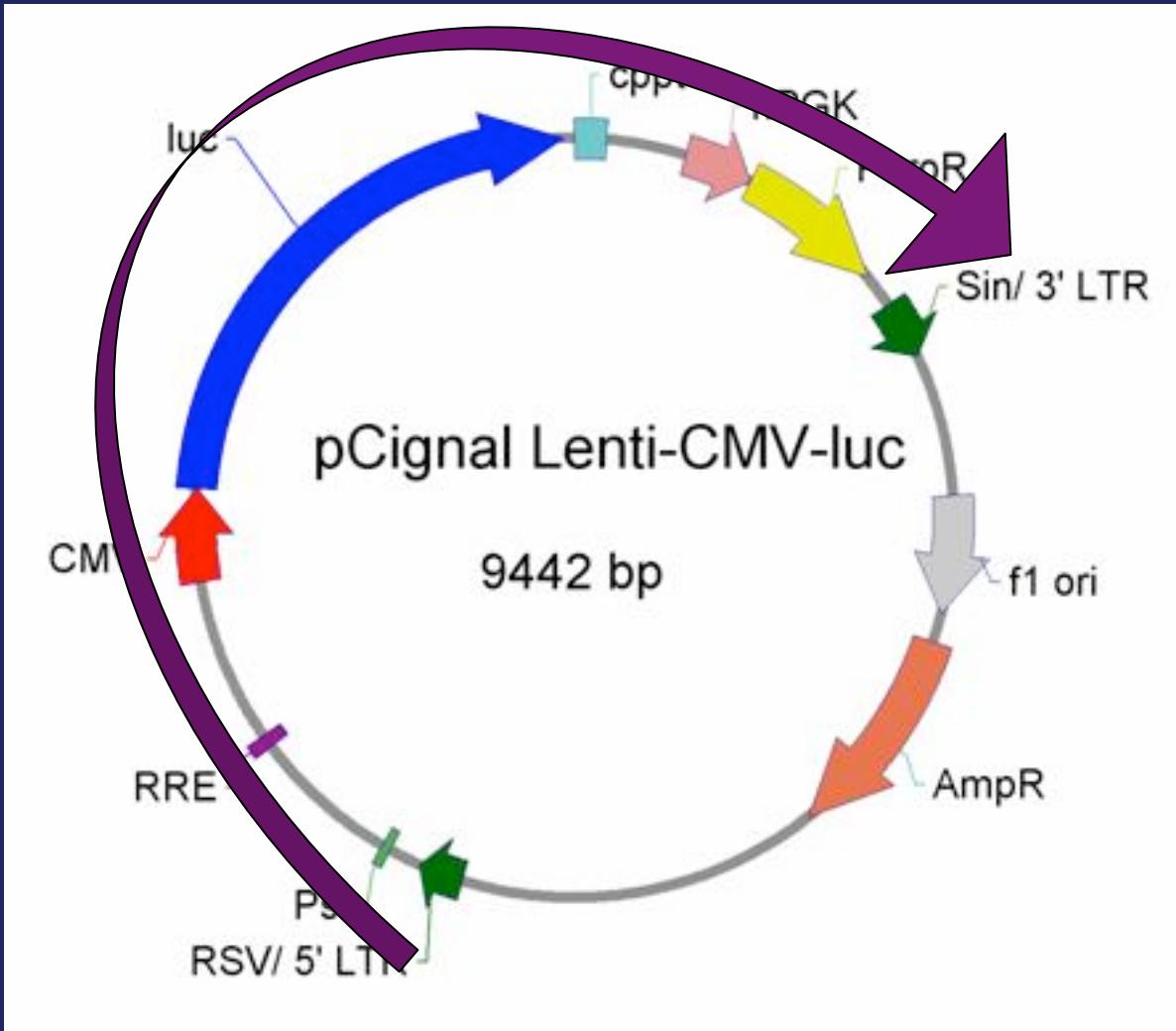
→ Insertion of a reporter (for example GFP) randomly in the genome, so that the reporter expression is controlled by endogenous sequences



- In principle, reporter expression should reflect endogenous gene expression
- Viral vector delivery of genes is generally used to ensure single copy insertion

# Lentiviral vector-mediated gene delivery





RNA packaged into the lentiviral particle

# Gene trapping to monitor transcription kinetics

In principle we need something like this:



- Issues:**
- 1/3 of short-lived luciferase in the correct reading frame
  - Fusion of protein from gene X can alter half-life of luciferase
  - How to select cells with a single insertion event
  - mRNA can have a very long half-life

# Gene trapping to monitor transcription kinetics

STOPs



Long terminal repeats of the lentiviral vector

Allows to stop synthesis of upstream protein to avoid fusion with downstream sequence

Splice acceptor: allows to connect reporter with upstream Exon if insertion occurs in Intron

Internal Ribosomal entry site: allows translation to start at a defined point in the insert

Antibiotic resistance (blasticidin): allows to select cells with an active insertion

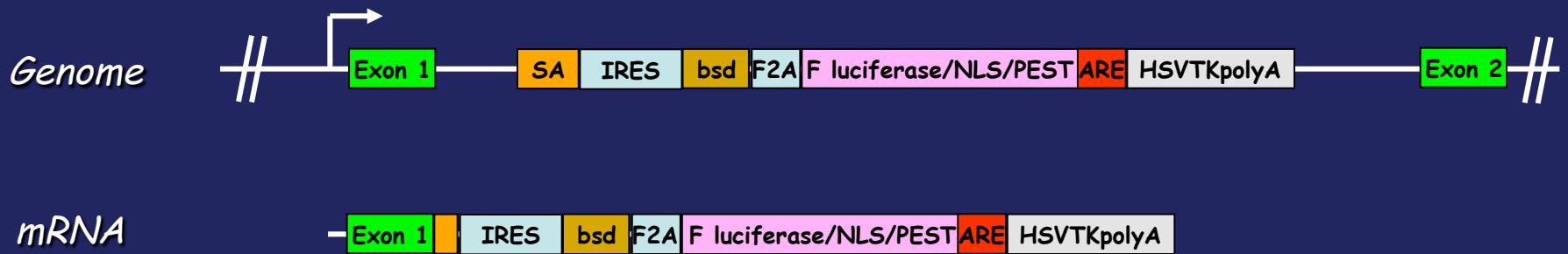
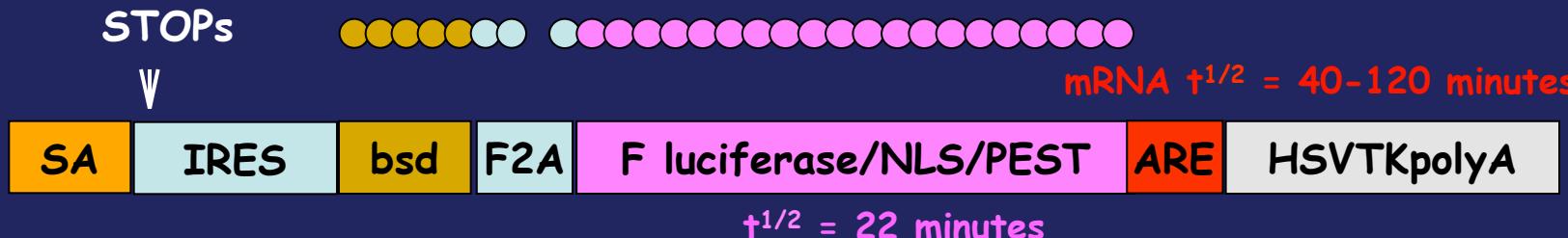
Small peptide allows to separate bsd and Luciferase polypeptide chain by « ribosomal skipping »

Reporter, with nuclear signal for better signal quantification and element to destabilize it for better time resolution

AU-rich element, allows to destabilize the mRNA

polyA signal, allows to terminate the mRNA

# Gene trap: monitoring of endogenous gene transcription (lentivector)



- ❖ Luciferase expression reflects transcription of the endogenous locus
- ❖ Translation is occurring via the IRES → Different kinetics should be independant of translation

## Recording of luminescence profiles

- Dilute luminescent cells 1:50 in non-luminescent cells at very high density to minimize cell divisions and migration
- Imaging for 48-72 hours, 1 picture every 5 minutes in the lumiview microscope
- Manual tracking of cells that don't divide during the movie



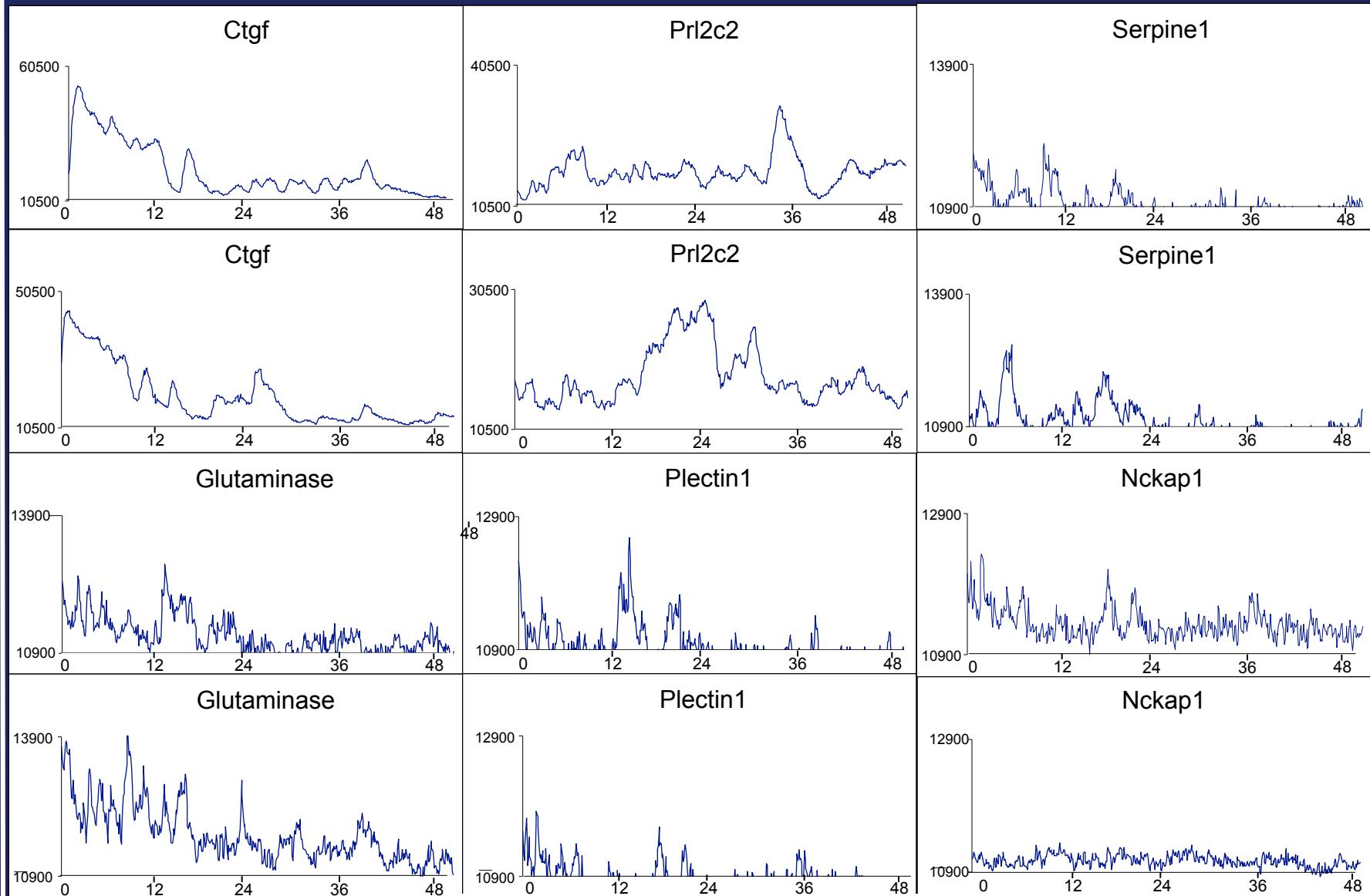
Olympus LV200 microscope



24 frames/sec

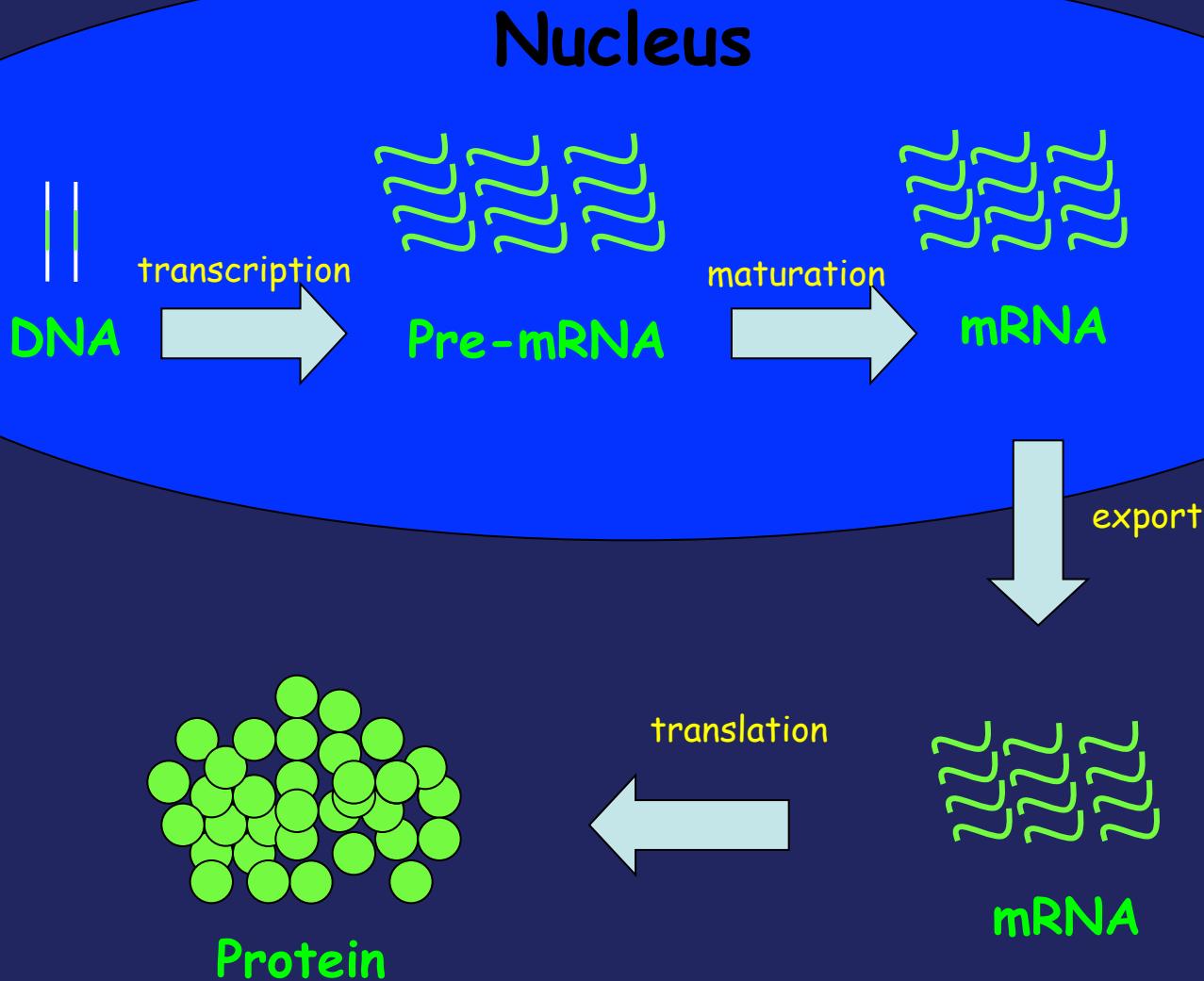
# Single cell traces

Luminescence (gray levels)

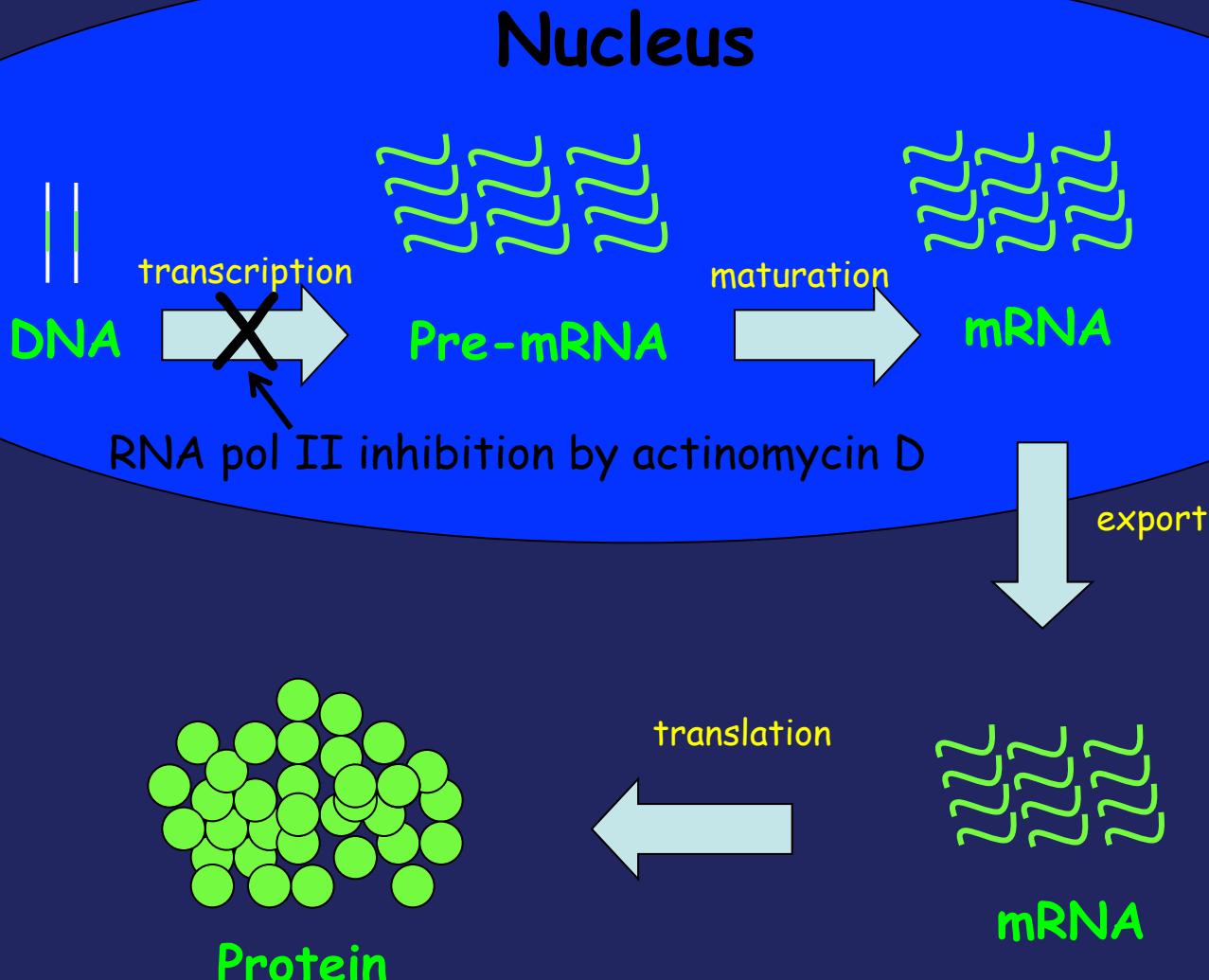


Time (hours)

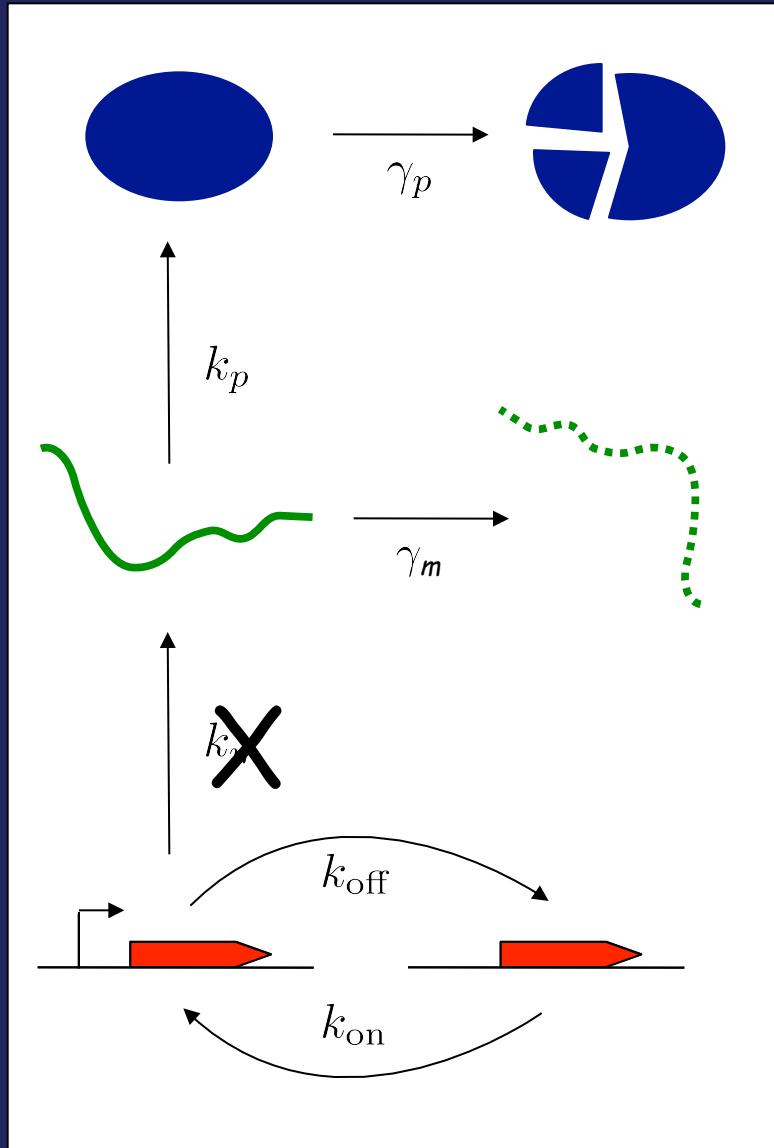
How can we prove this bursting is at the transcriptional level ?



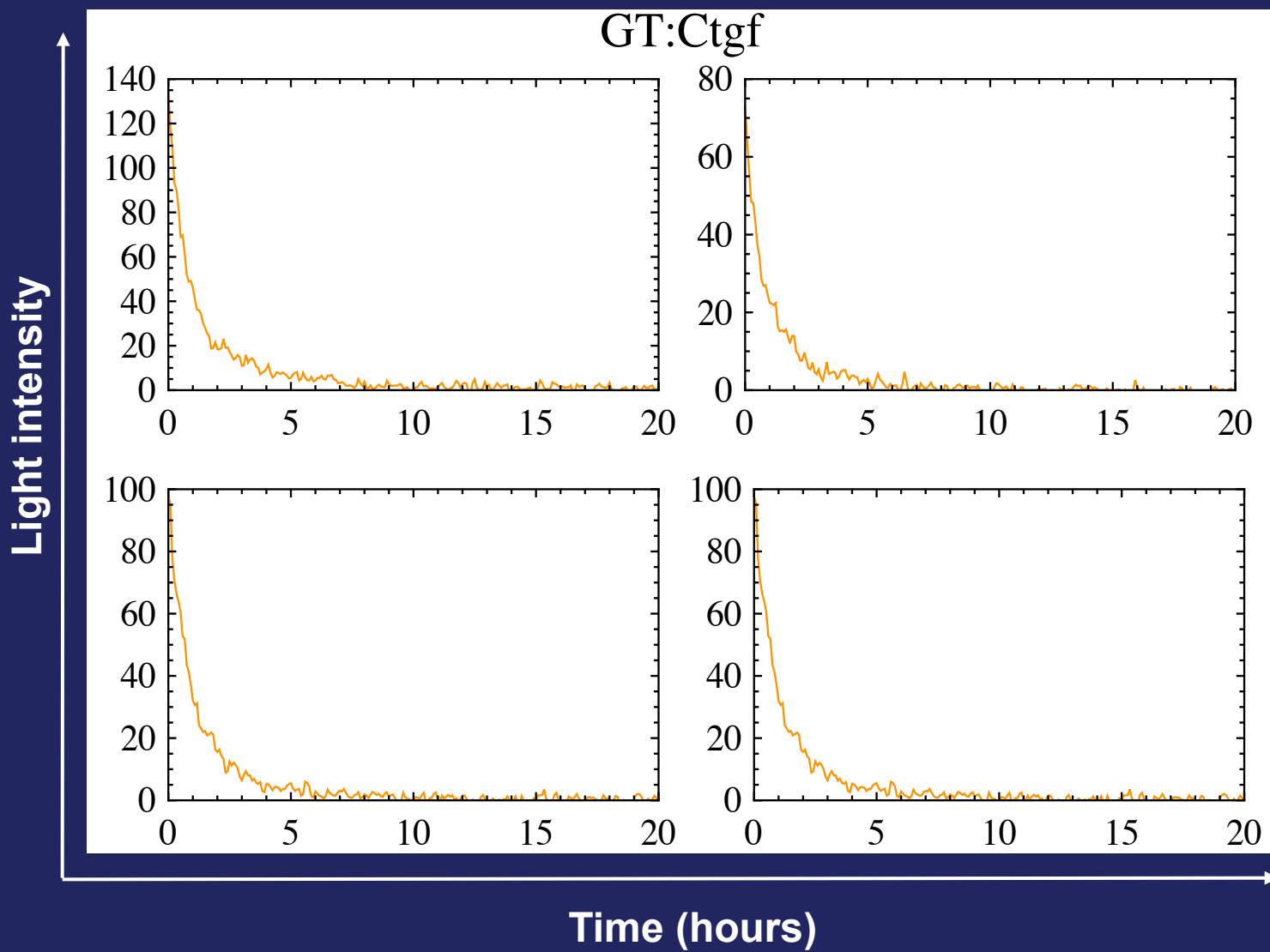
How can we prove this bursting is at the transcriptional level ?



→ If everything downstream of transcription is continuous, we should see a smooth decline of the signal that depends on the translation rate, the mRNA and protein degradation rate

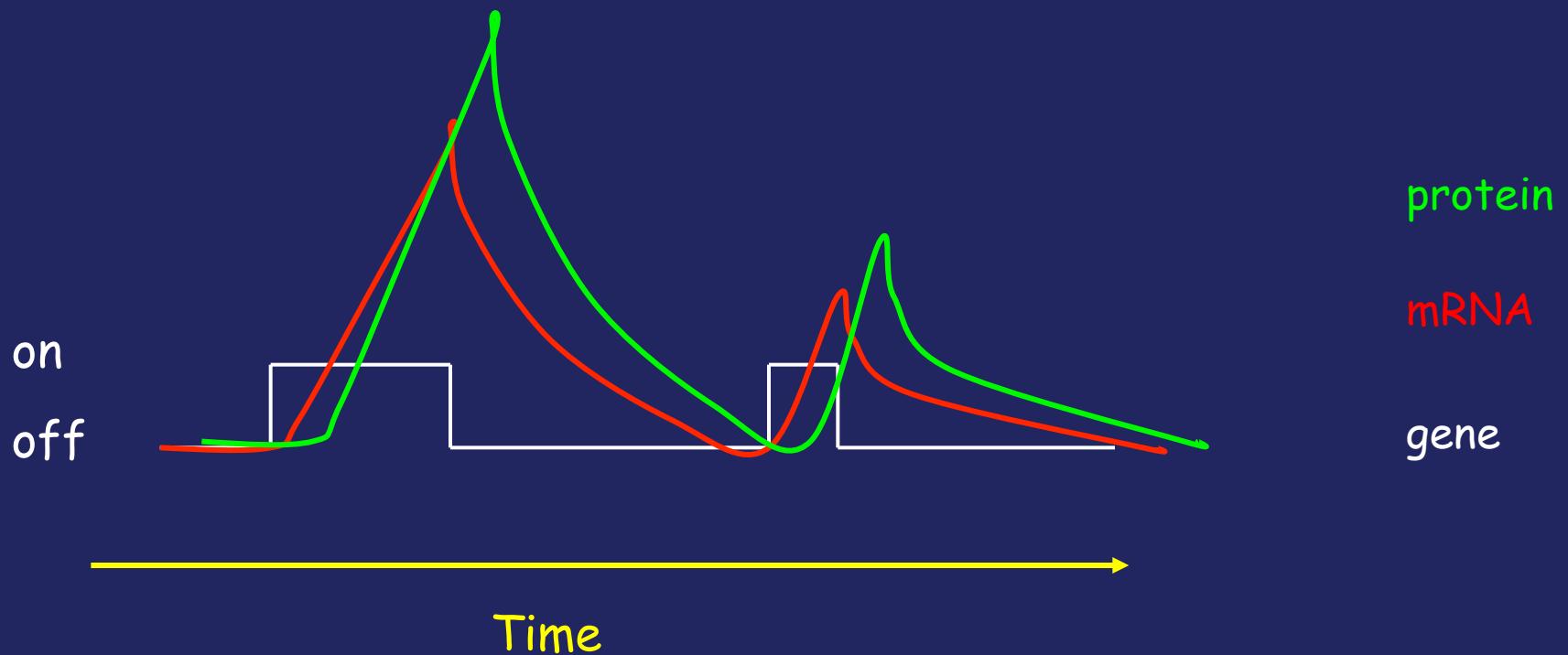


# Bursts are lost after transcription inhibition with actinomycin D



→ Bursts are of transcriptional origin

Ultimate goal: recover protein, mRNA and gene activity trace



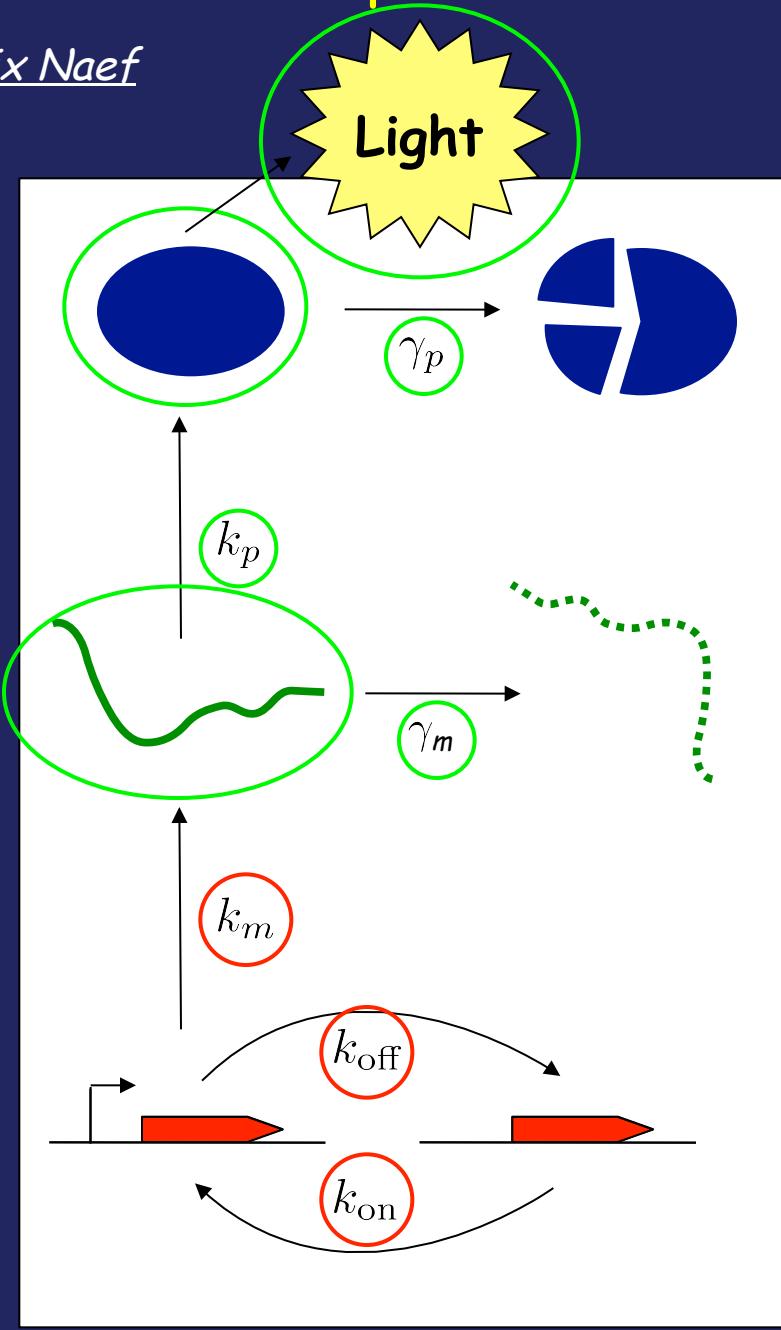
# Stochastic Biophysical Model of Gene Expression

Nacho Molina and Félix Naef

Assumptions:

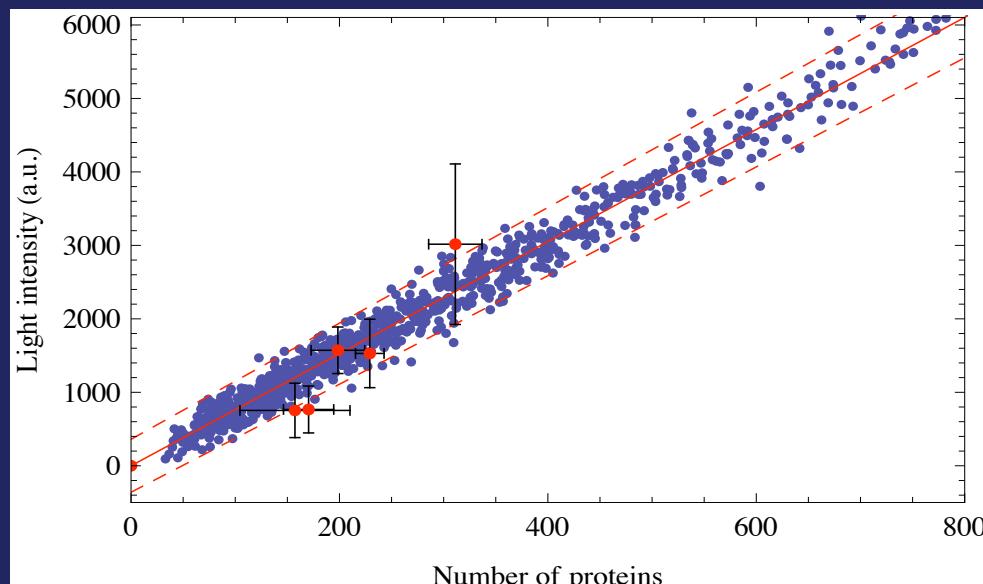
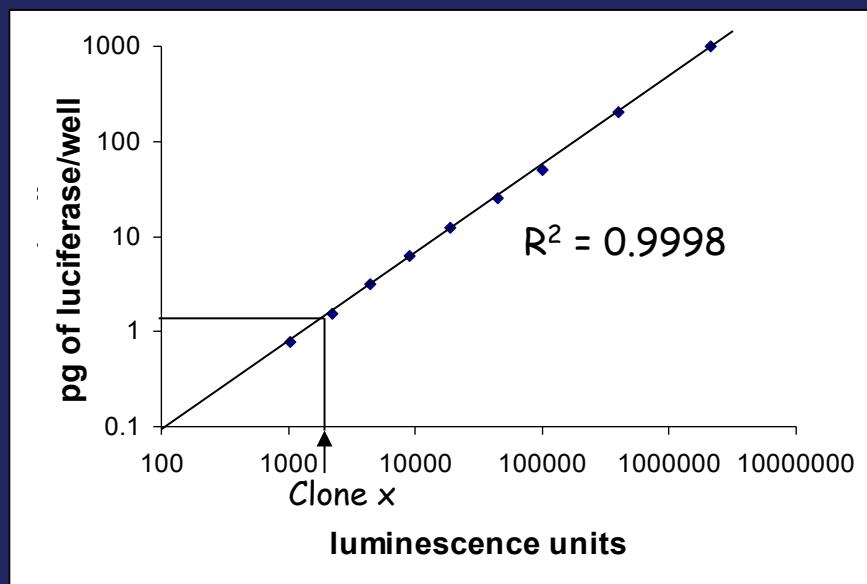
- The gene can be in two states: active and inactive. Constant rate of switching.
- If the gene is active there is a constant rate of mRNA production.
- There is a constant rate of protein production per molecule of mRNA
- Constant rates of mRNA and protein degradation per molecule.
- The system is described at each time point by three variables:
  1. protein copy number
  2. mRNA copy number
  3. gene activity

Can be experimentally determined  
Have to be inferred



# Calibration of protein (luciferase) to photon emission

- Comparison of luminescence emitted from known amounts of a recombinant luciferase to total luminescence emitted from a known number of cells
- The mean number of luciferase molecule per cell is known and compared to the mean of single cell luminescence levels

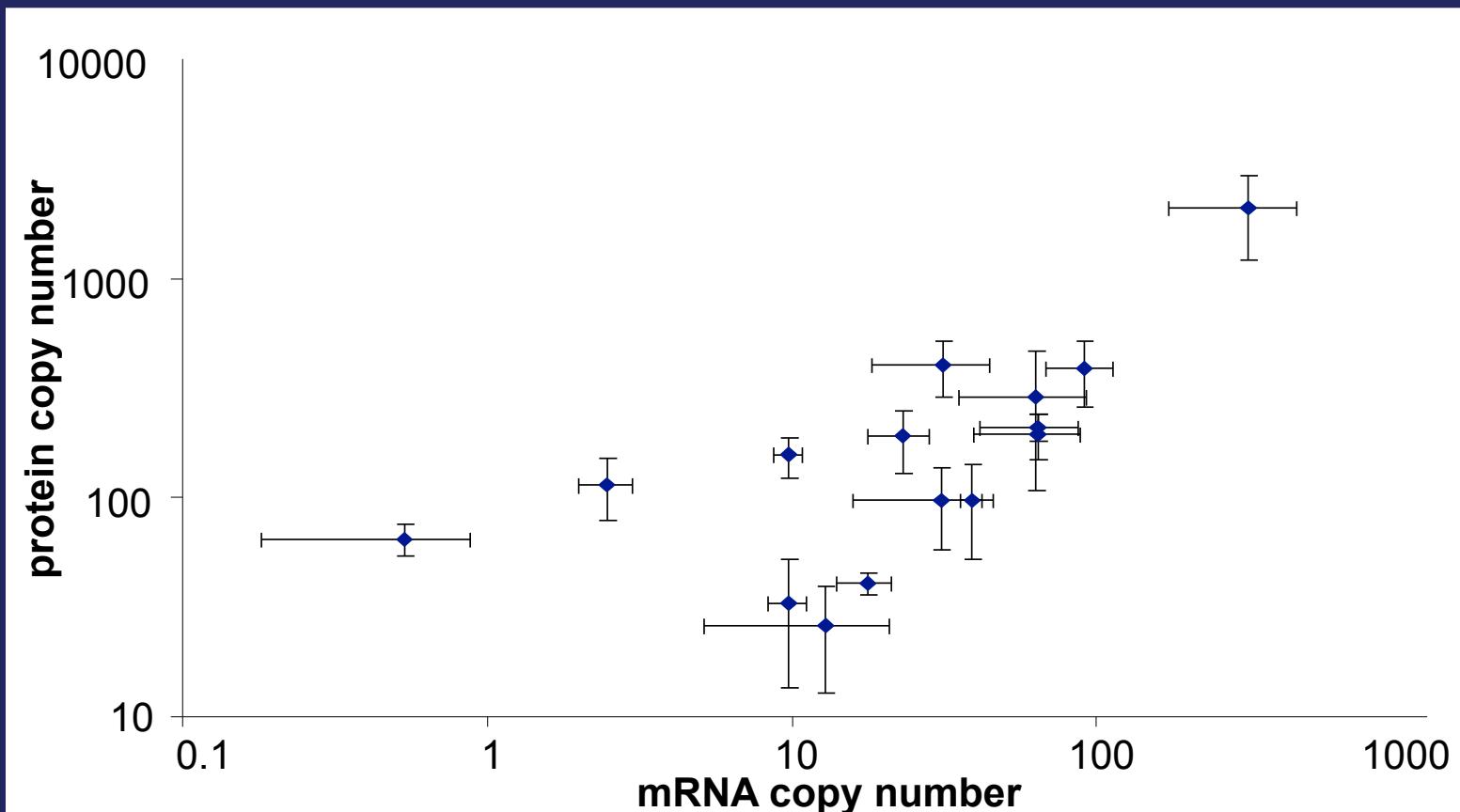


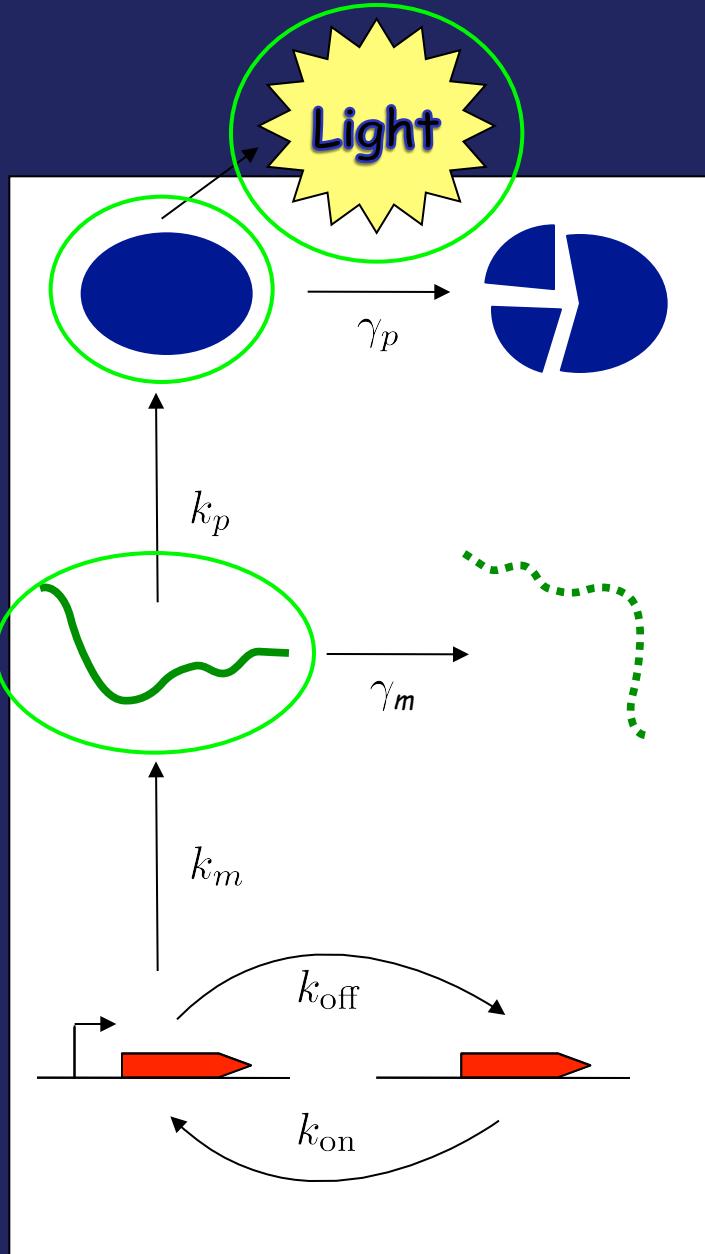
$10^6$  cells of Clone X have N pg of luciferase →  
We can calculate how many luciferase molecules  
we have in each cell

These numbers are then compared to the mean  
light intensity of cells in the microscope  
→ We know how many luciferase molecules make  
how much light

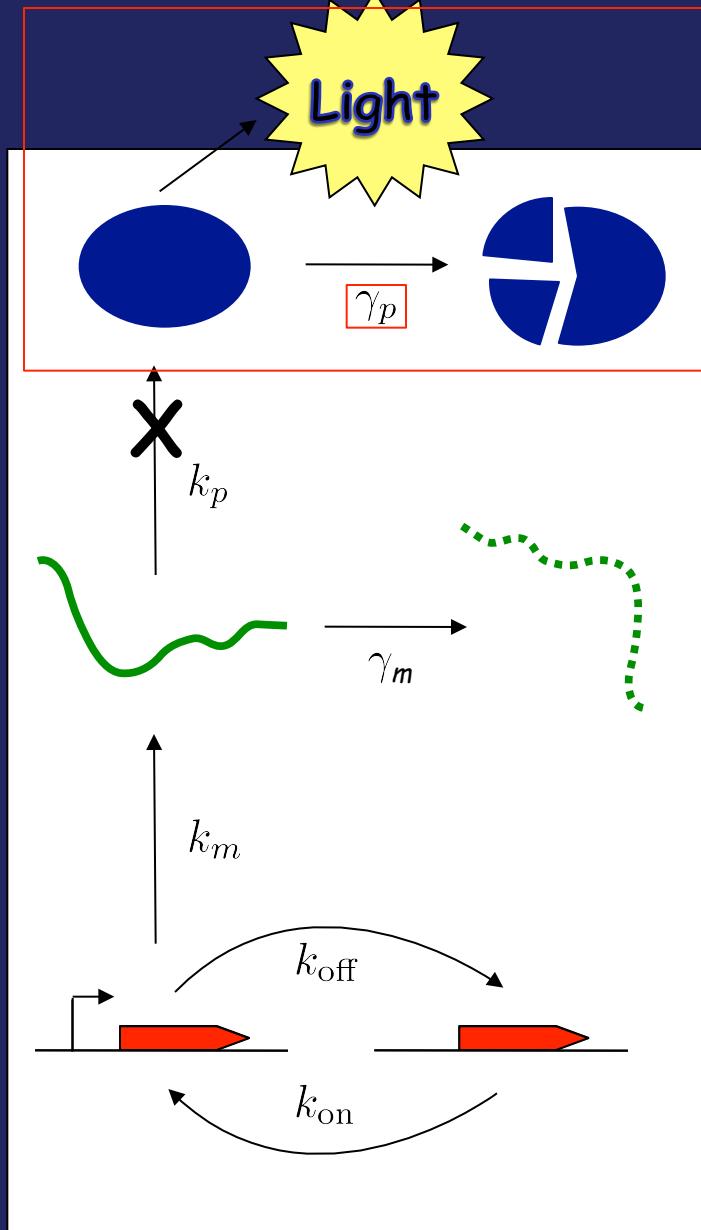
# Calibration of mRNA to photon emission/protein

→ Real Time PCR on cDNAs from a known number of cells, compared to known amounts of in vitro transcribed luciferase RNA

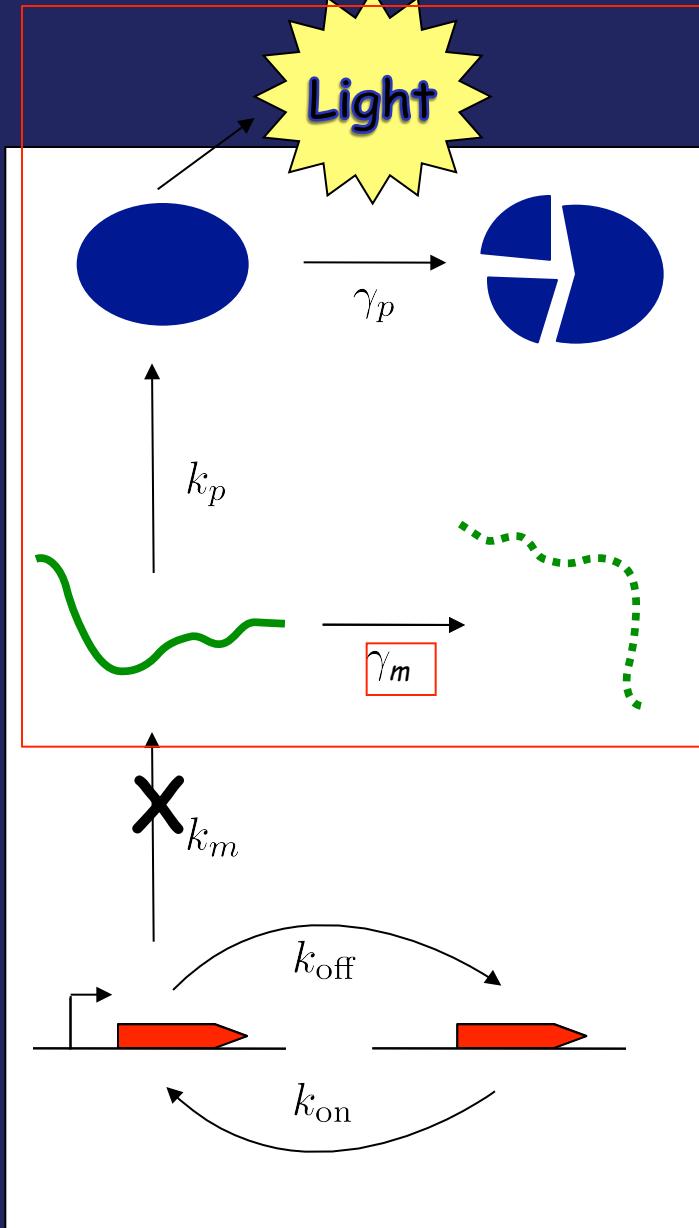




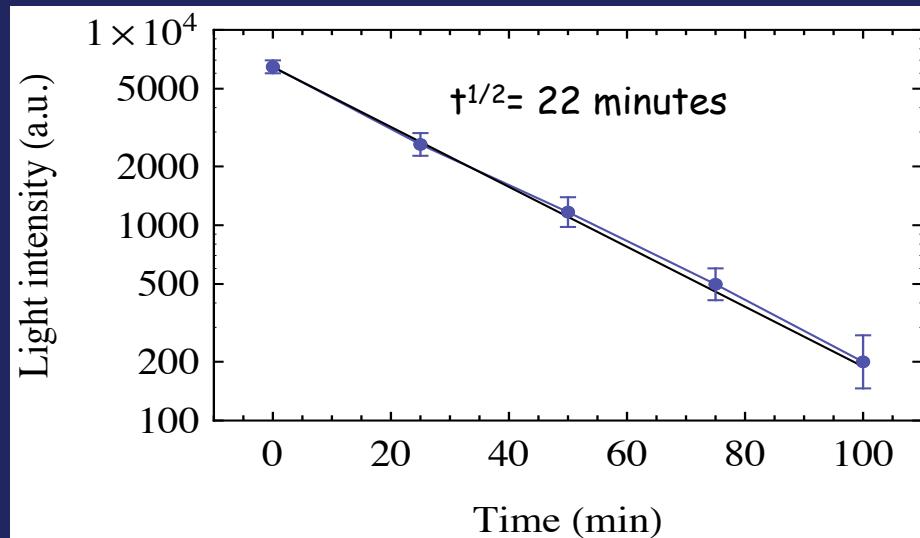
# Measuring protein stability: block translation and follow luminescence decay



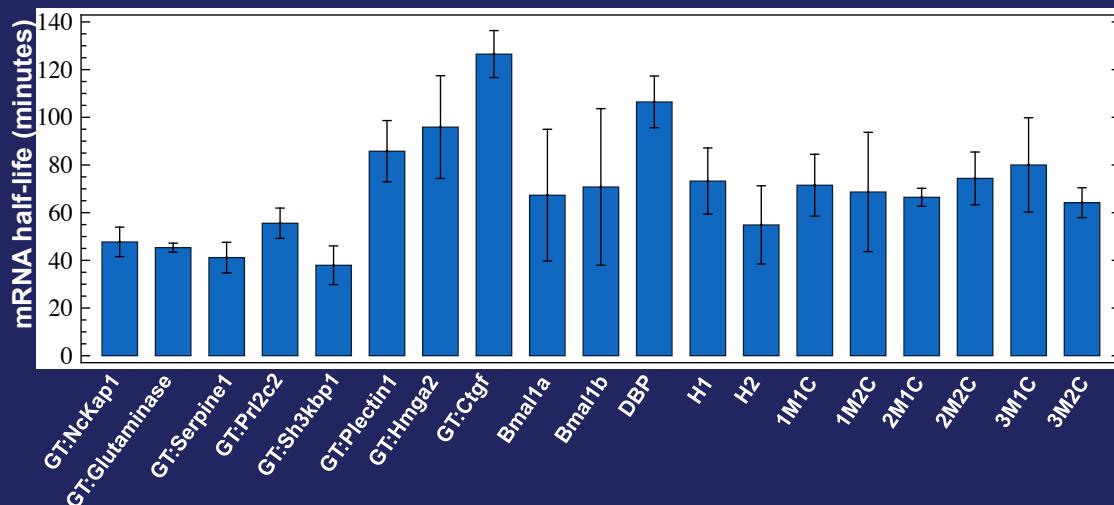
# Measuring mRNA stability: block transcription and follow luminescence decay



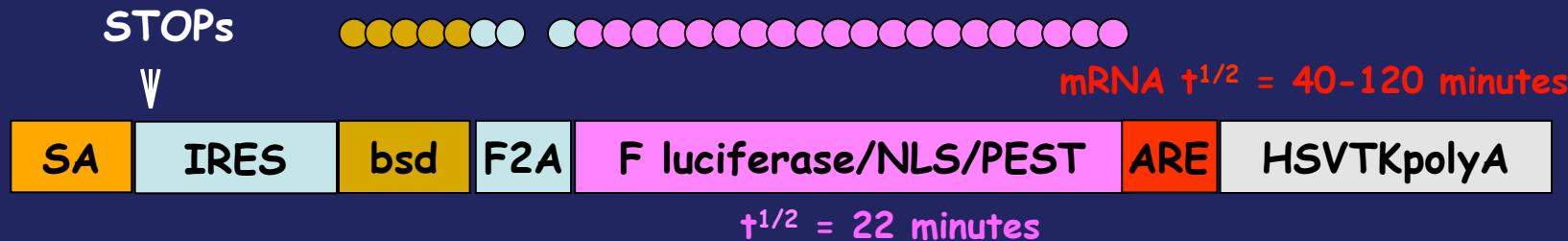
## Protein half-life



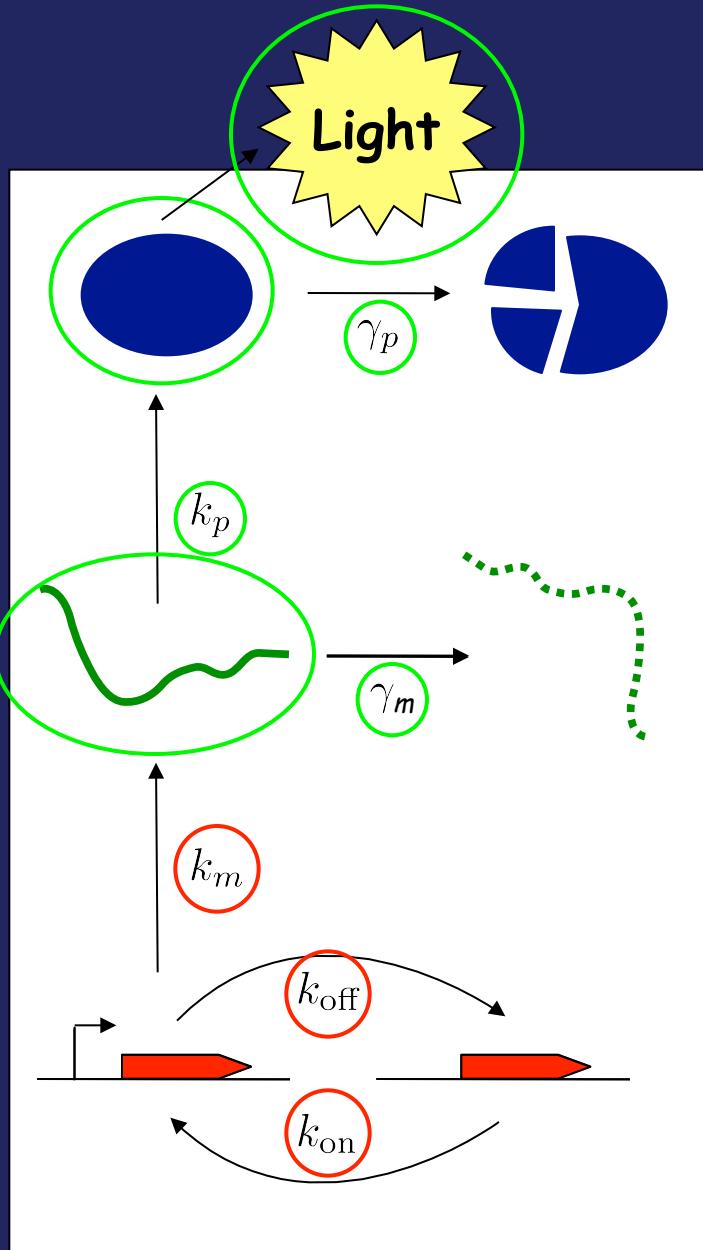
## mRNA half-lives inferred from luminescence traces



# Why are mRNA half-lives different in each cell line ?

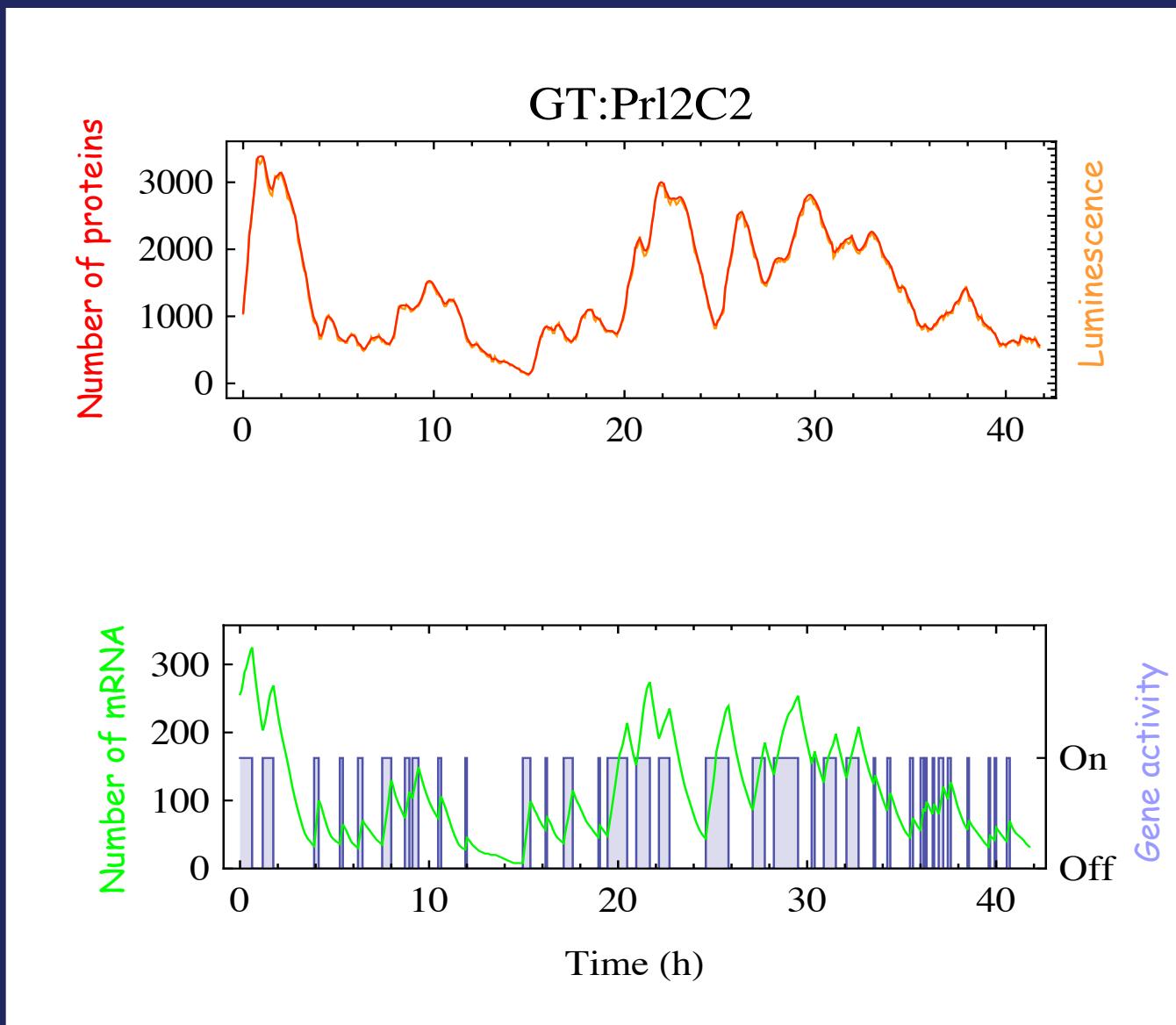


# Back to the gene expression model



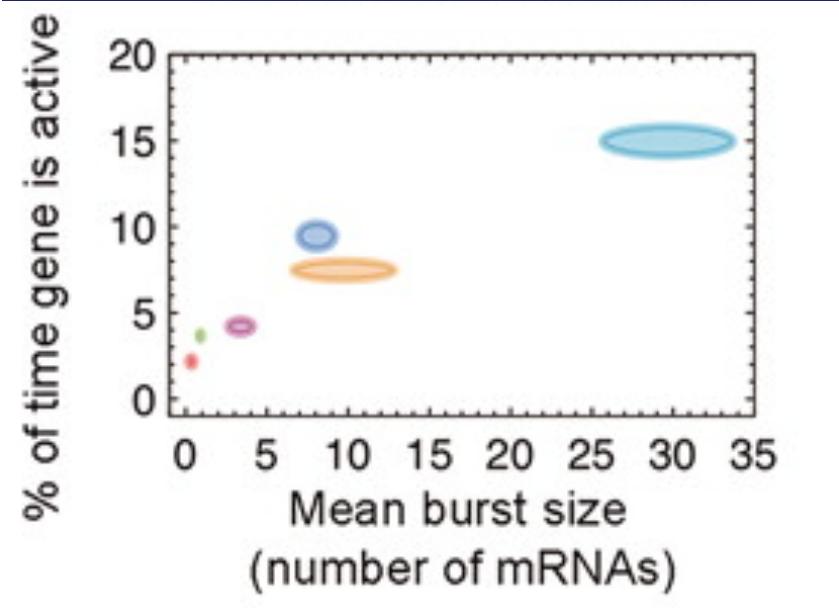
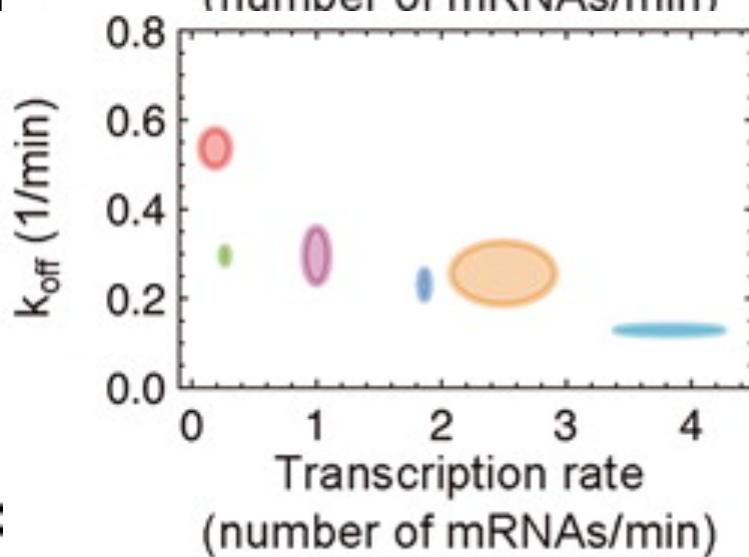
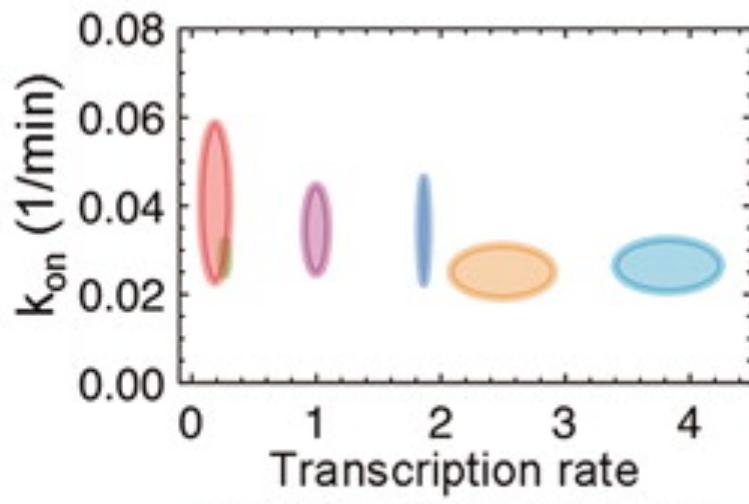
Have to be inferred

# Inferring protein, mRNA and gene activity profiles



# Transcription kinetics is highly gene-specific

Bmal1a, GT:Glutaminase, GT:Prl2C2  
GT:Serpine1, Sh3kbp1, GT:Plectin1



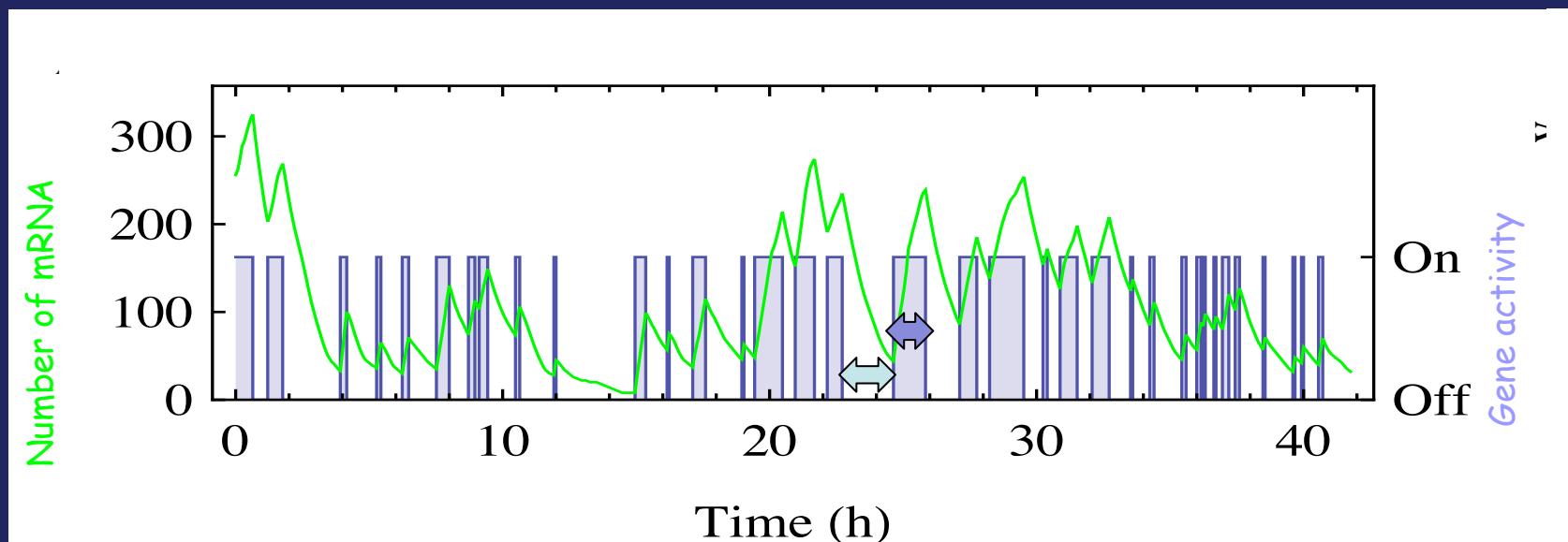
# Distributions of waiting times

« on »

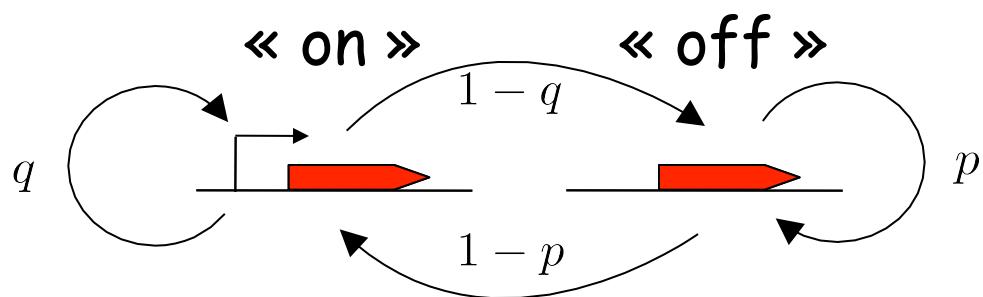
« off »



How long does a gene wait in one state before switching to the other state ?



# Theoretical distribution of waiting times

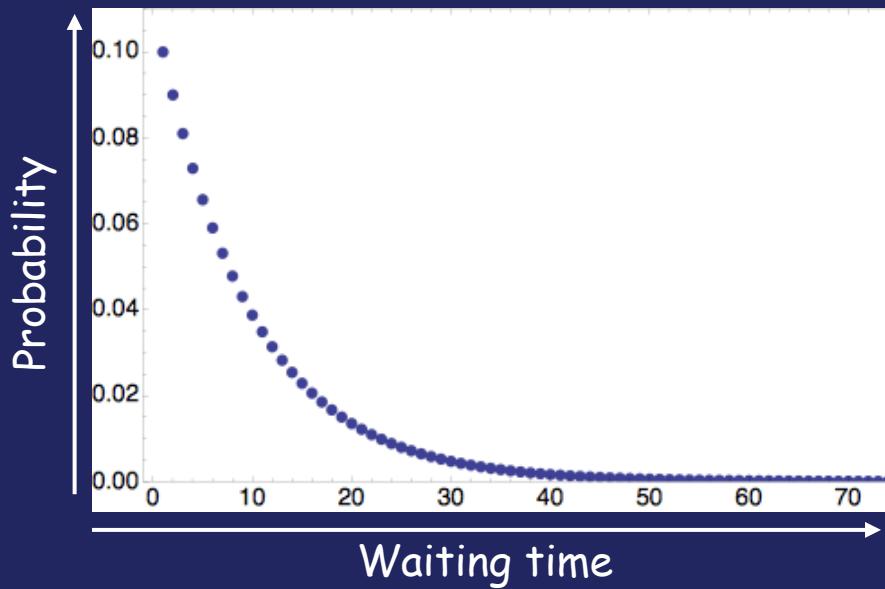


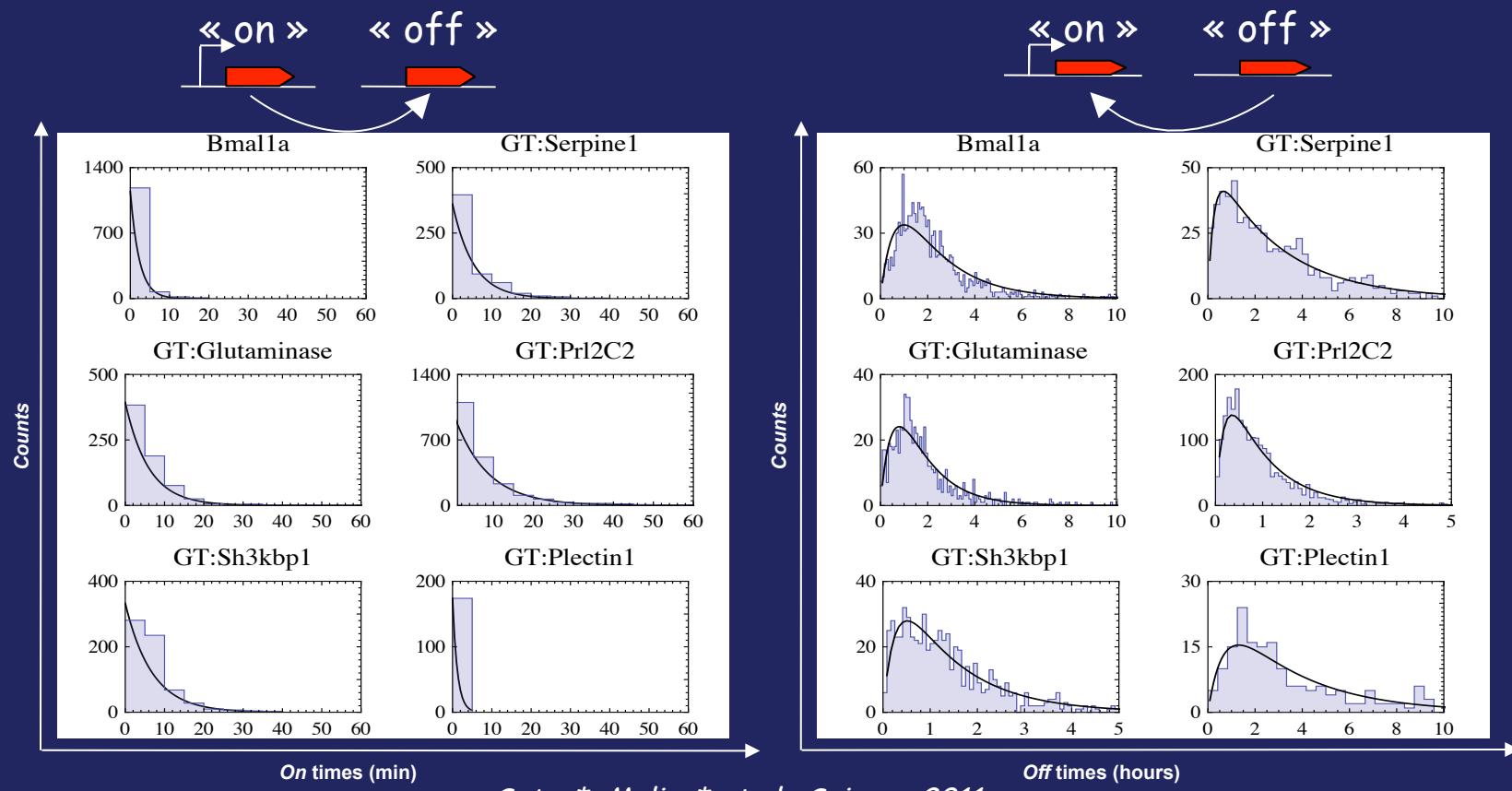
$p$ : prob. that gene stays off after 5'  
 $q$ : prob. that gene stays on after 5'

Probability of having a certain waiting time:

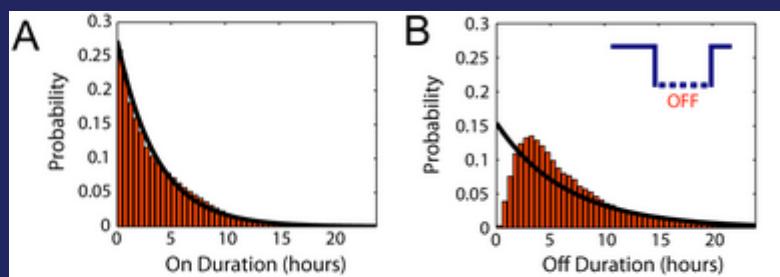
$$P(\text{gene on during } n \text{ intervals before turning off again}) = q^n(1-q)$$

$$P(\text{gene off during } n \text{ intervals before turning on again}) = p^n(1-p)$$

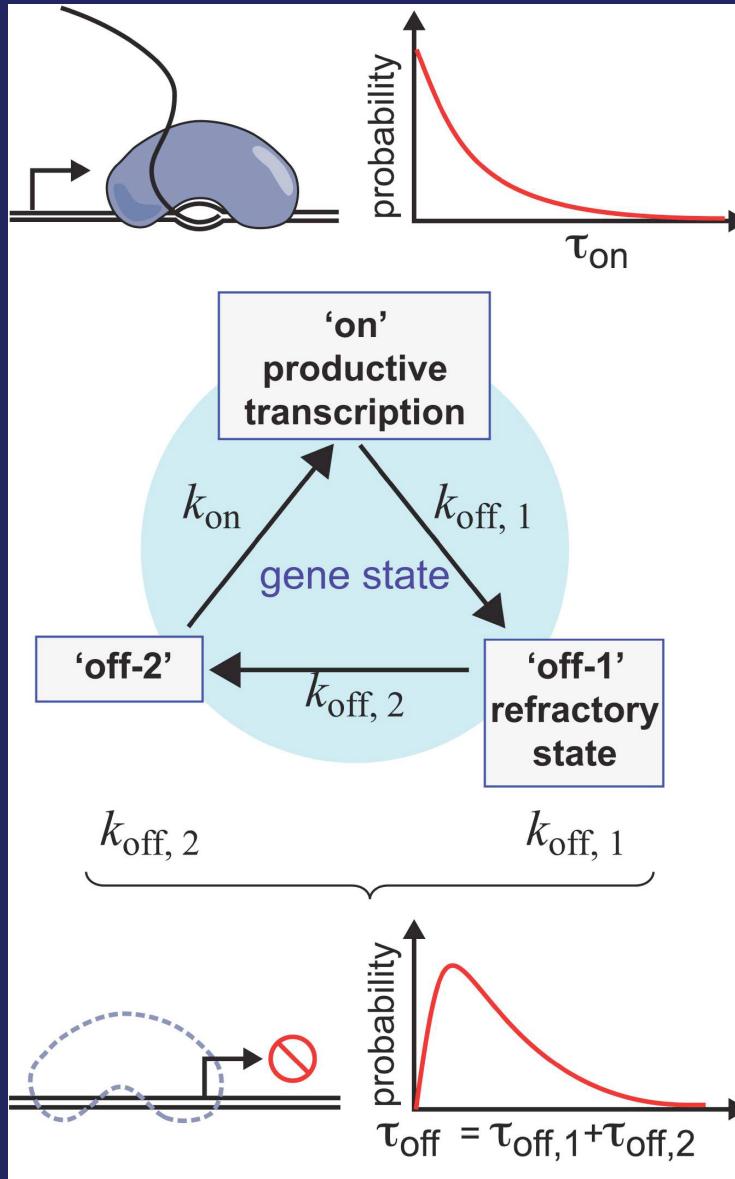




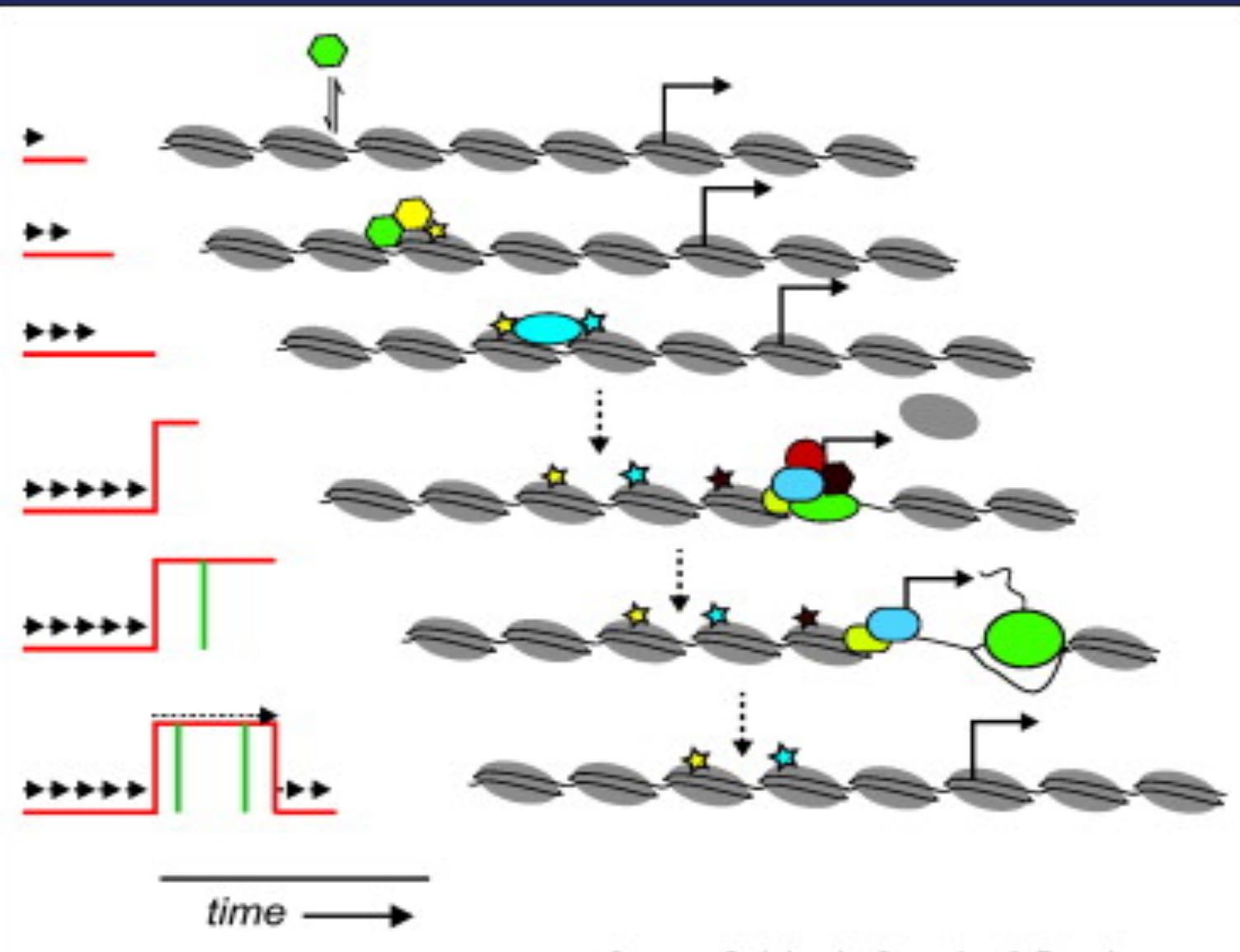
→ There is a refractory period in the « off » state



# Presence of (at least) one additional state



# Promoter progression model



Current Opinion in Genetics & Development

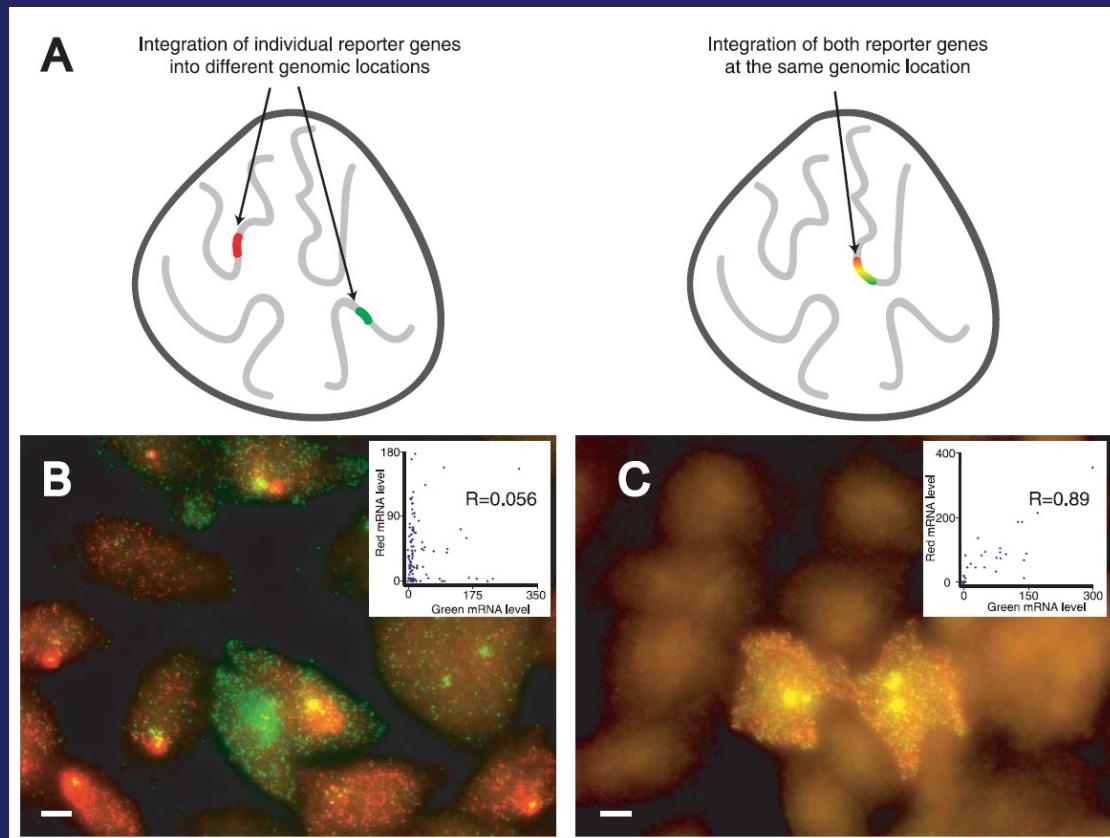
# What is setting the pace of transcription ?

Chromatin « permissivity » (histone acetylation, methylation...)

Transcription machinery assembly

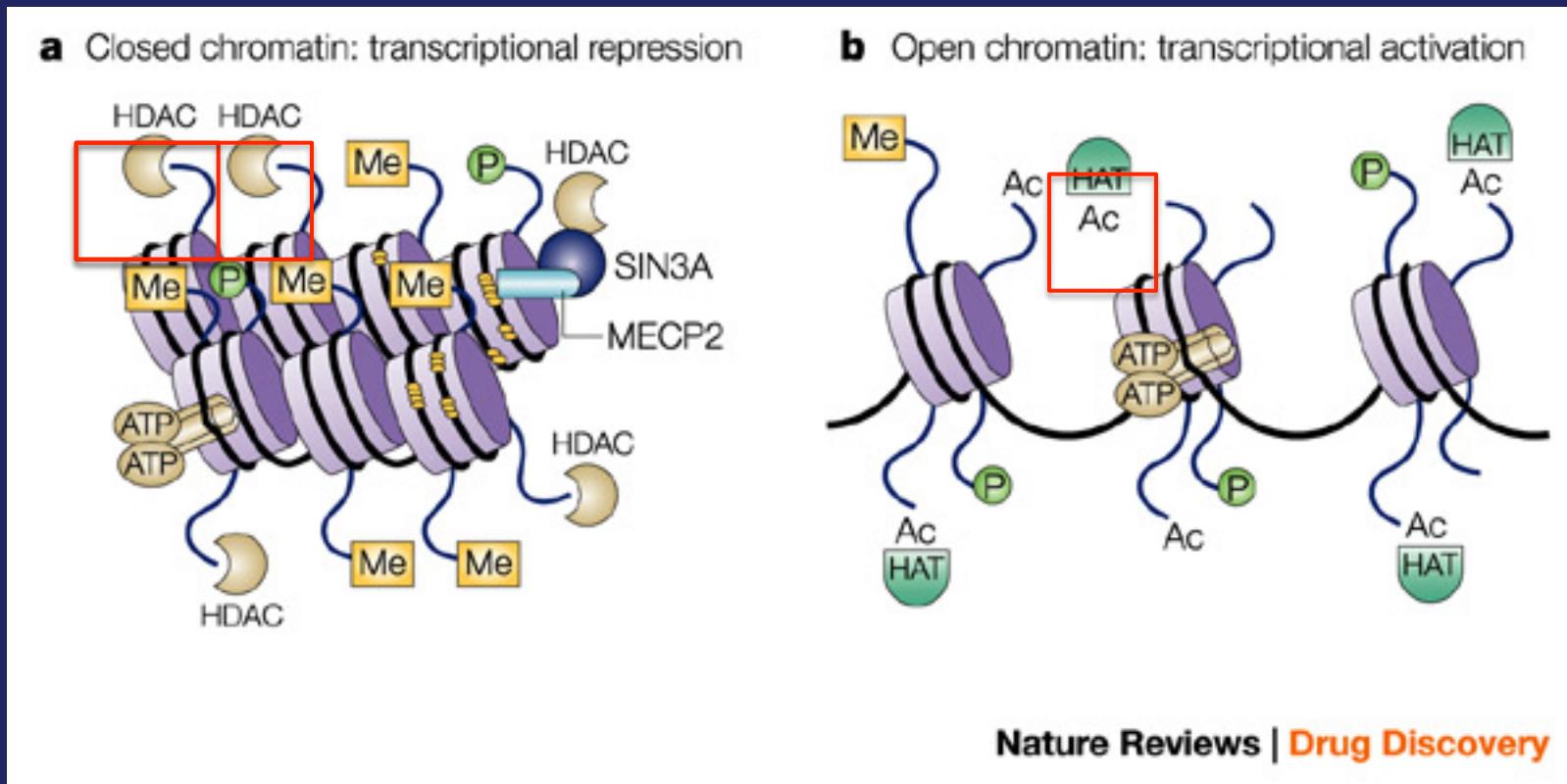
Proximal pausing of transcription machinery

Recruitment to transcription factories



Raj et al, 2006

# Reminder: enzymatic modifications of histones regulate the accessibility of DNA to transcription

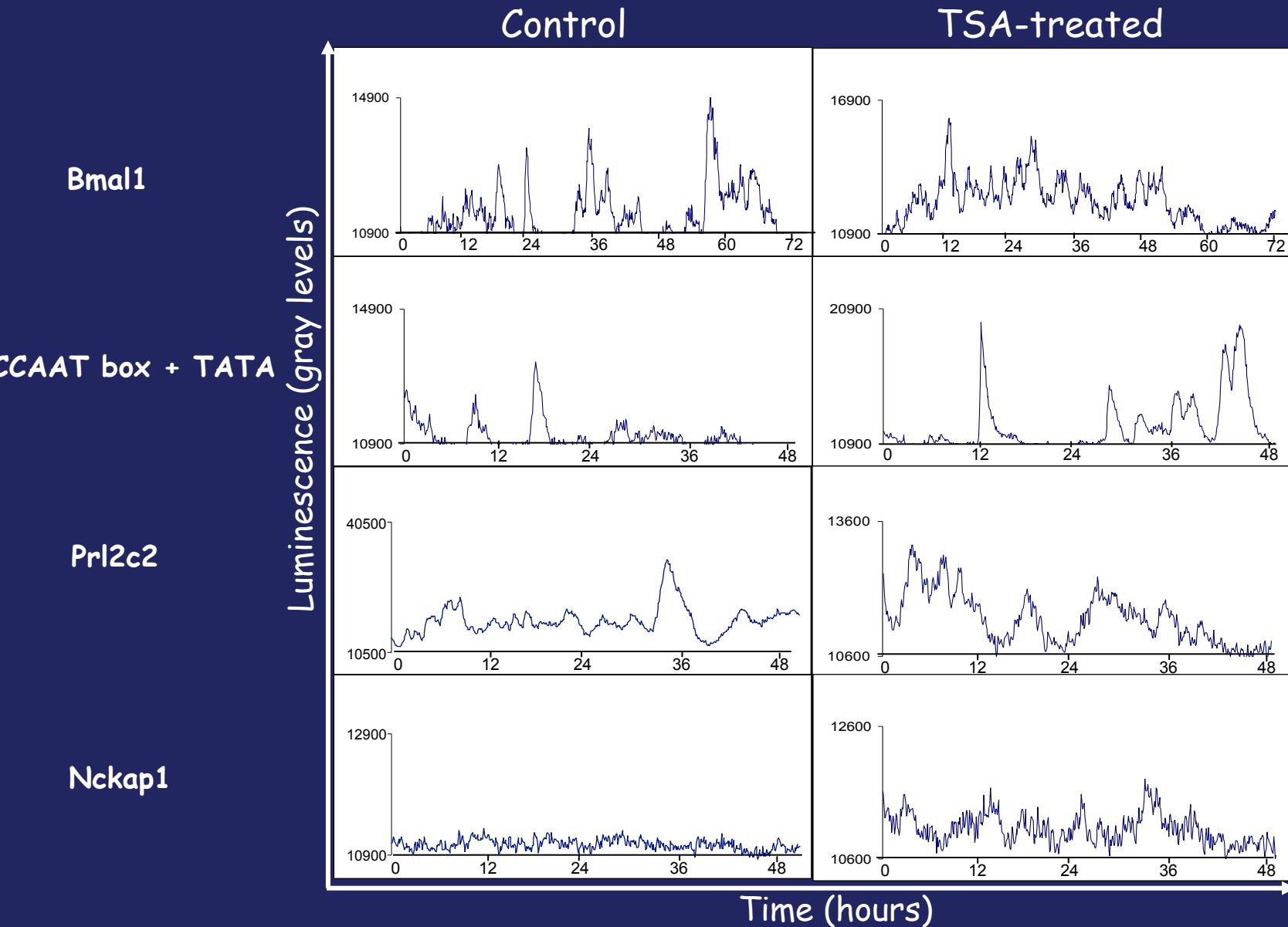


HDAC: Histone deacetylase → remove acetyl group from histones → chromatin is closed  
HAT: Histone acetyl-transferases → Add acetyl group to histones → chromatin is open  
Histones can also be methylated, phosphorylated, etc.

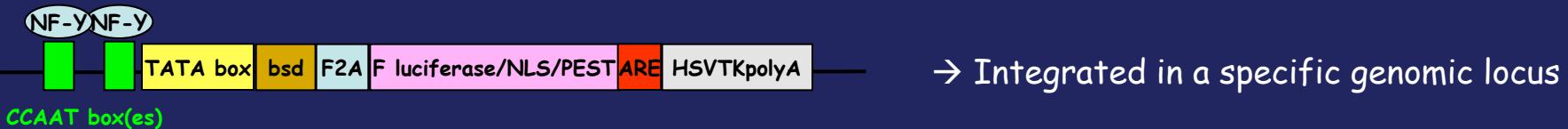
→ "Histone Code": a certain pattern of histone modification tells us about the activity of a region of the chromatin

# Influence of chromatin state

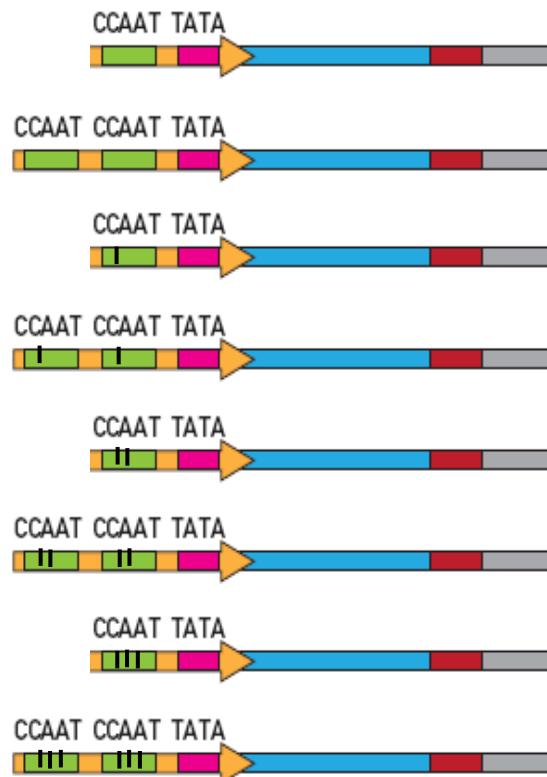
→ Block histone deacetylation (TSA) and look at transcriptional kinetics



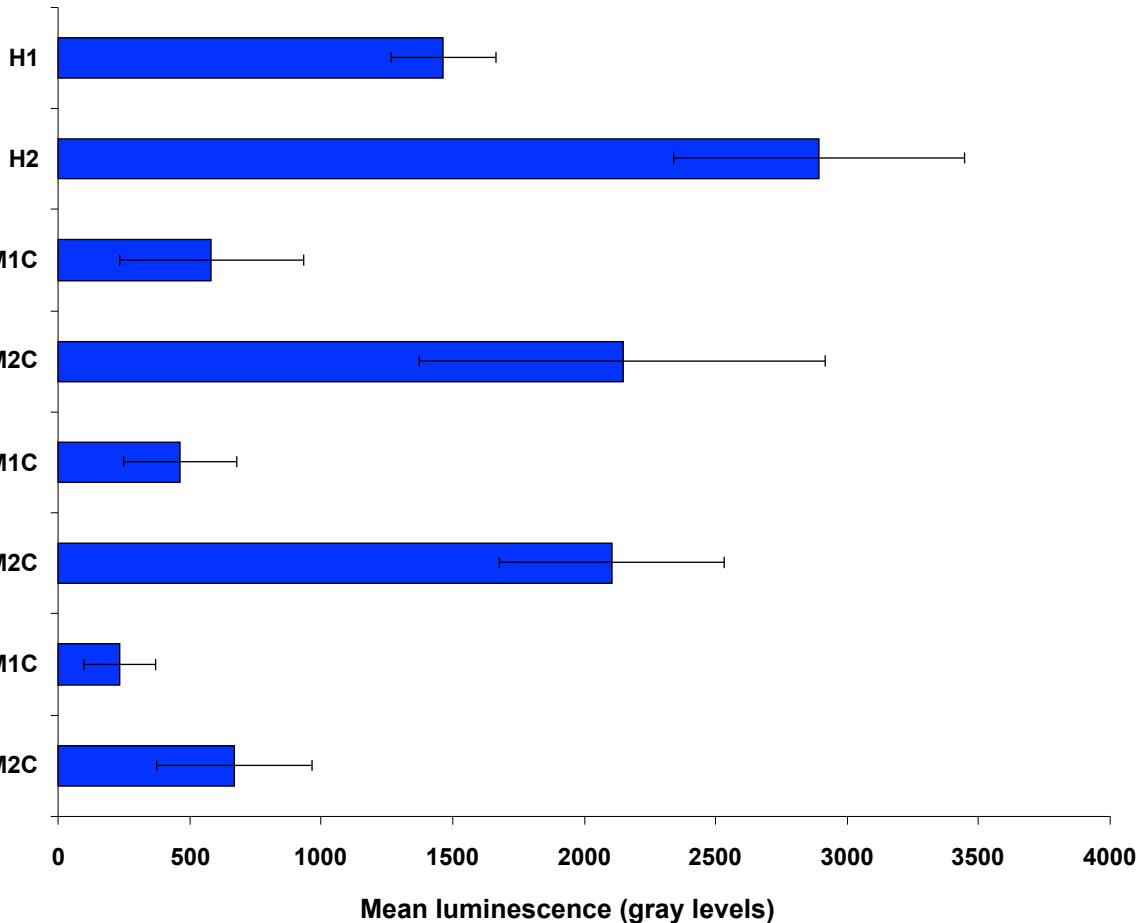
# Influence of promoter sequence on transcription kinetics



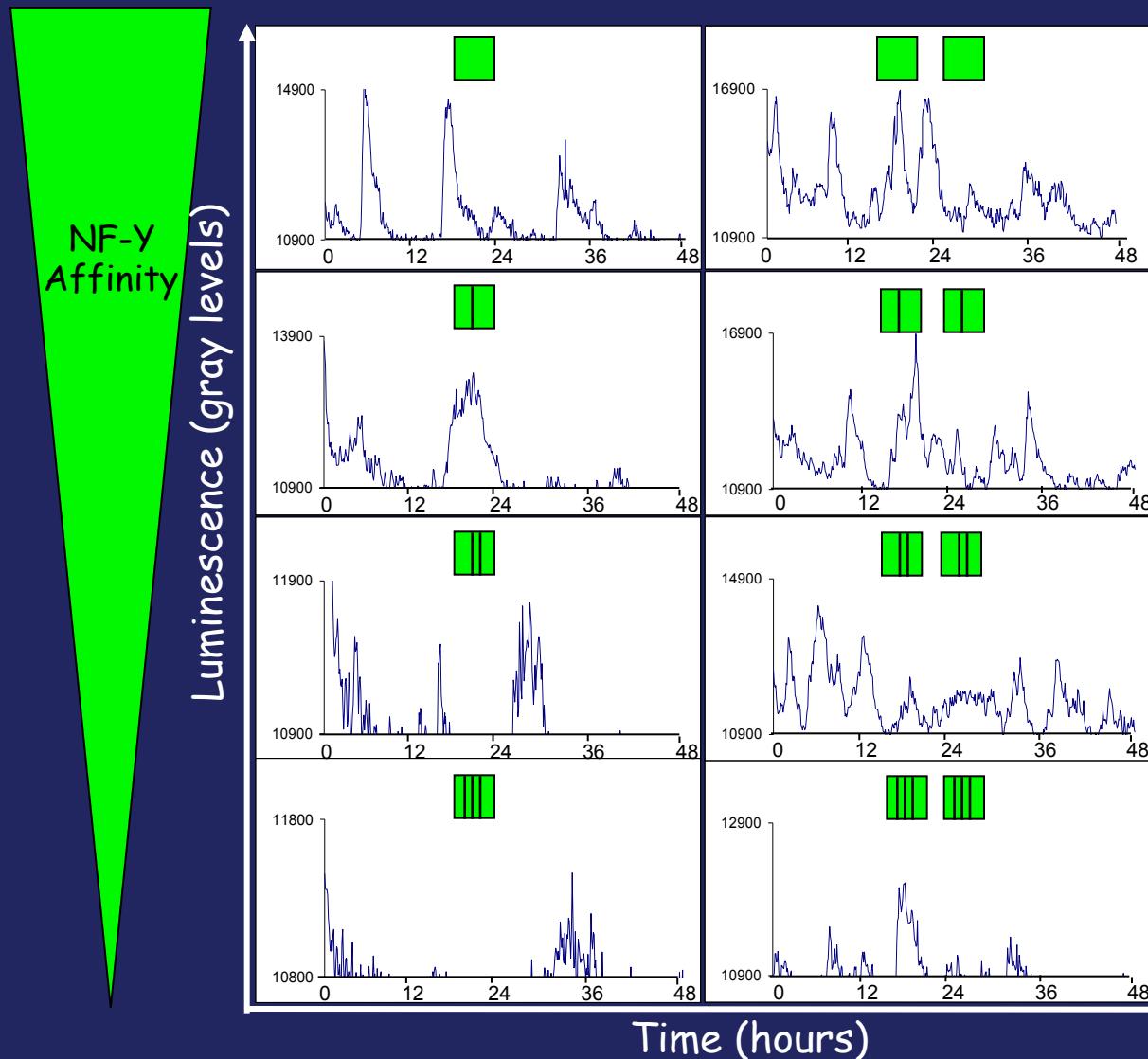
## Whole population luminescence



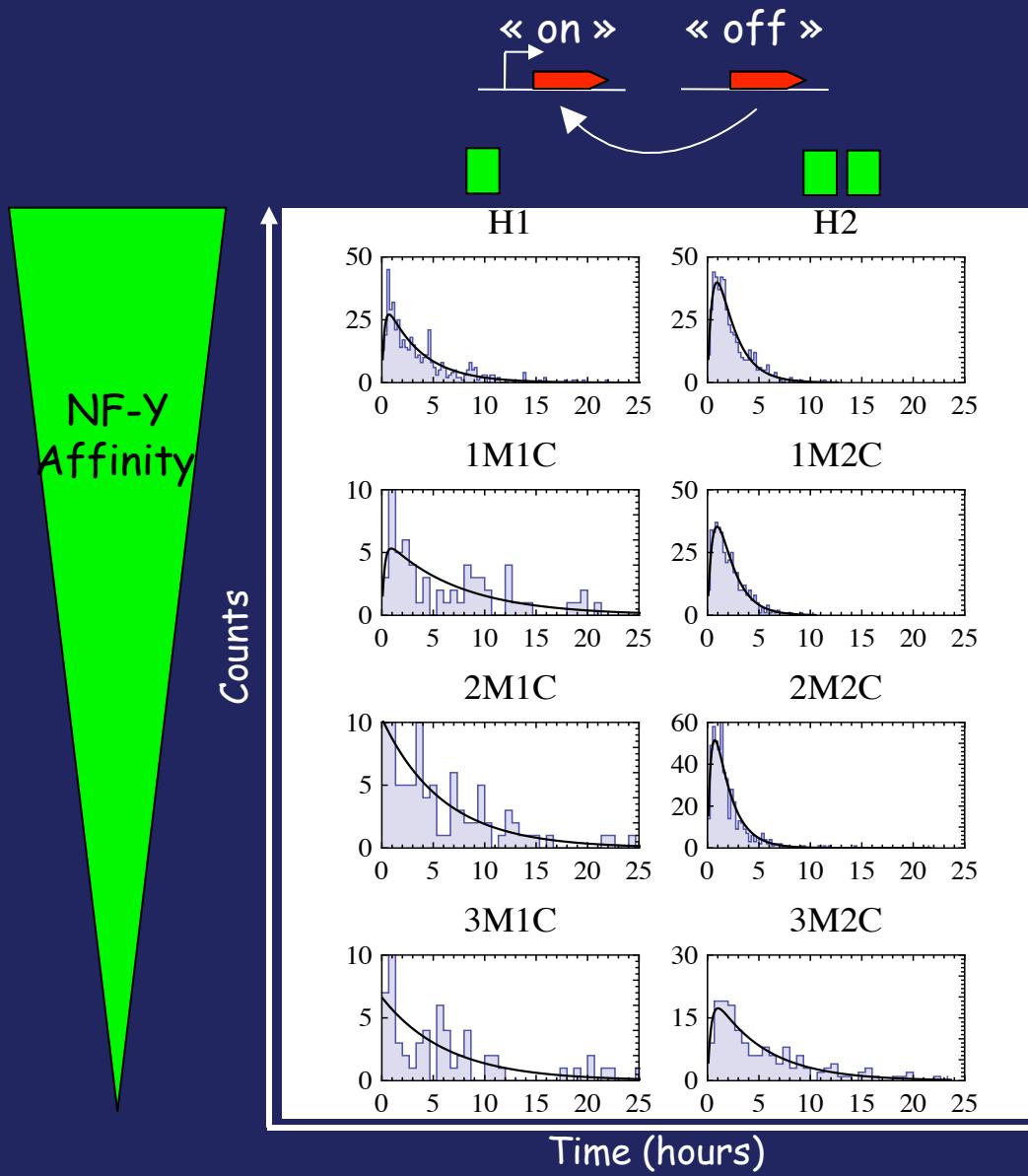
| = point mutation



# Single cell recordings (48 hours)



# Influence of promoter sequence on waiting time distributions



# Conclusions

- ❖ Kinetics of mammalian gene transcription varies widely between different genes
- ❖ When in the « off » state, a gene needs a certain amount of time to be « on » again, suggesting the presence of a refractory period
- ❖ Transcription kinetics is relatively resistant to changes in chromatin acetylation state
- ❖ Simple changes in regulatory sequences can modify transcriptional kinetics, independently of genomic location
- ❖ The mechanisms underlying transcriptional discontinuity remain unclear