Программа «Структурная биология»
Курс « Оптическая спектроскопия»

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Лаборатория оптической микроскопии и спектроскопии биомолекул
ИБХ РАН

Лекция 3

Цель изучения дисциплины: **получение базовых знаний о методах оптической спектроскопии, о возможностях и способах их применения к решению задач структурной биологии**

Методы оптической спектроскопии:

- Молекулярная спектроскопия электронного поглощения света
- Молекулярная флуоресцентная спектроскопия
- Флуоресцентная спектроскопия с временным разрешением
- Поляризационная флуоресцентная спектроскопия
- Флуоресцентная спектроскопия на основе Фёрстеровского резонансного
- переноса энергии
- **Флуоресцентная микроспектроскопия одиночных молекул**
- Спектроскопия кругового дихроизма
- Инфракрасная (ИК) спектроскопия молекул
- Спектроскопия комбинационного рассеяния (КР) света и ее разновидности

Структурные исследования одиночных молекул и их комплексов с использованием FRET- эффекта

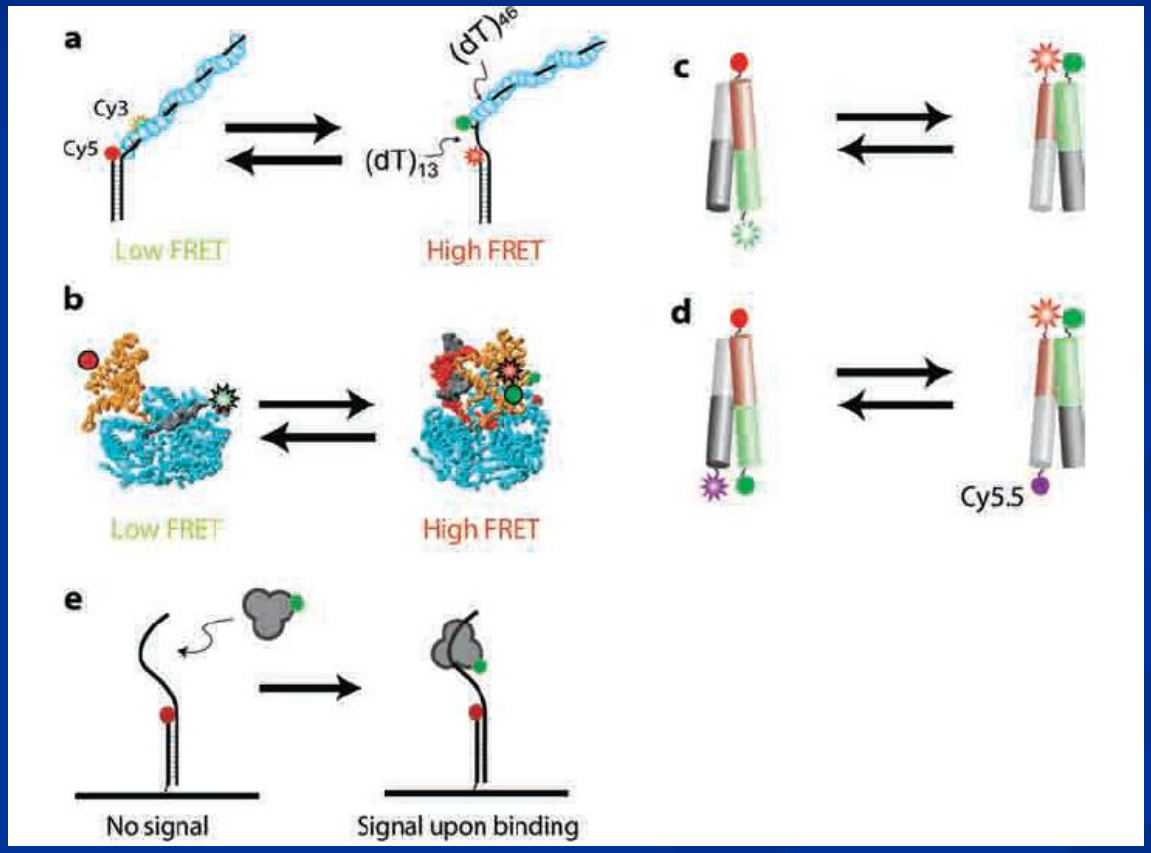
Основная идея –пространственно изолировать, регистрировать и анализировать слабый сигнал от одной молекулы (одного комплекса)

с одновременной регистрацией сигнала в двух и более спектральных диапазонах (конформационные перестройки, взаимодействия молекул)

с разрешением во времени (анализ процессов с участием молекулы)

Исследования на уровне единичных молекул и их комплексов методами флуоресцентной микроскопии

Исследования с применением метода FRET



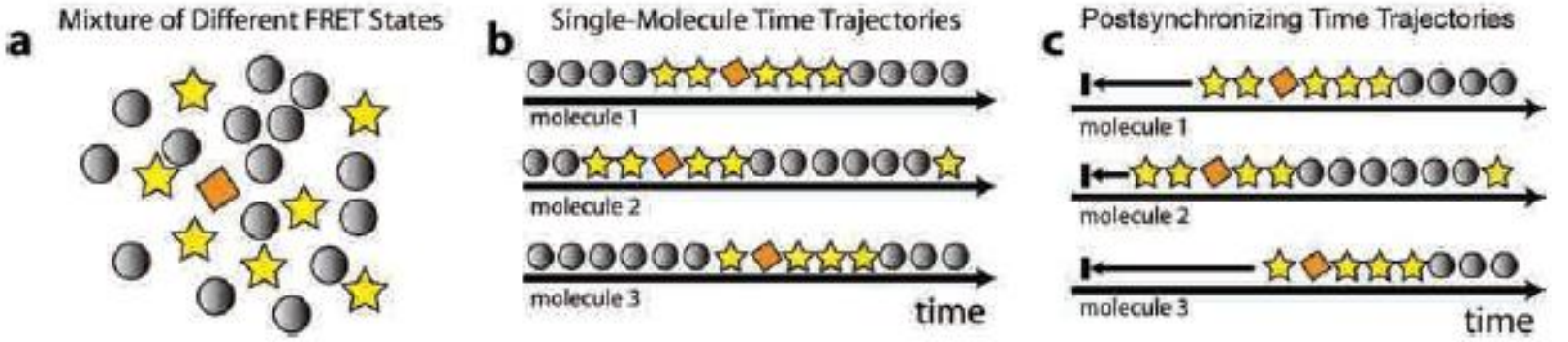
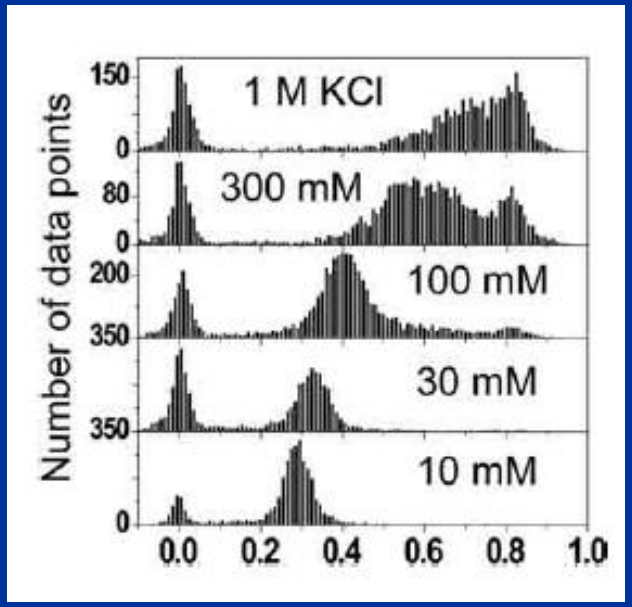
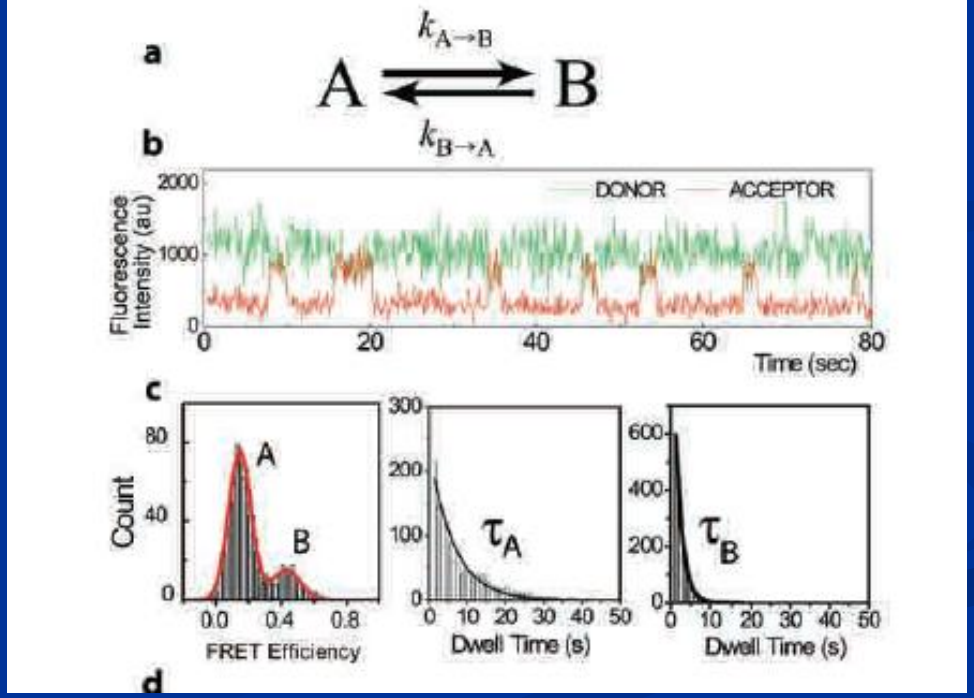
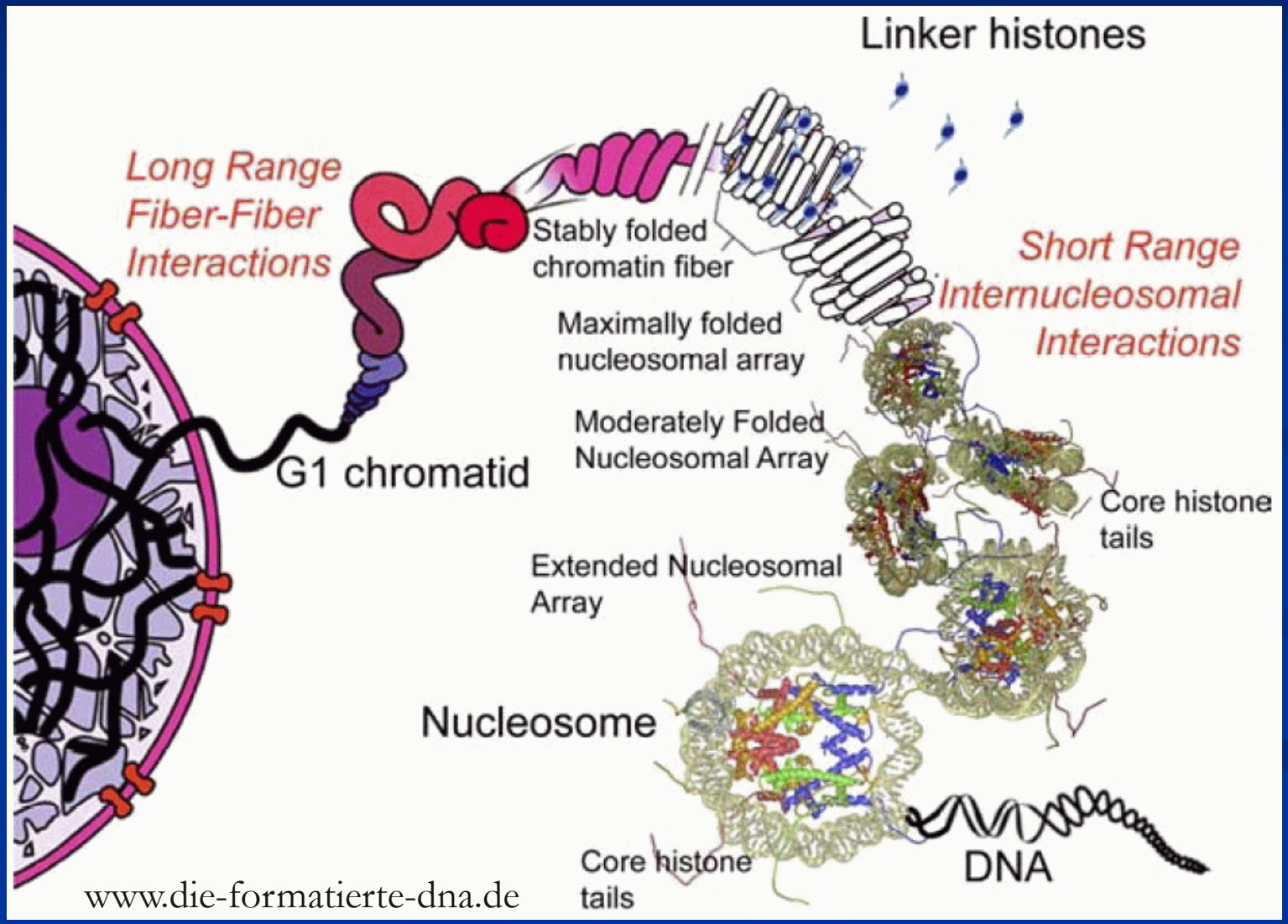


FIGURE 2-2. Single-molecule FRET study. (*a*) FRET in ensemble represents only the average value from many molecules. (*b*) The time traces of individual molecules reveal the true FRET states of different conformations in real time. (*c*) Events under study can be postsynchronized during data analysis.

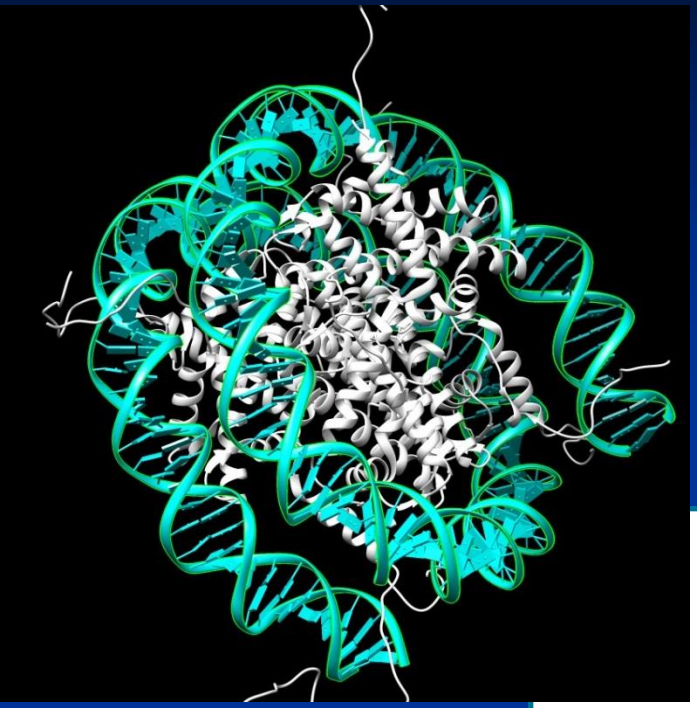


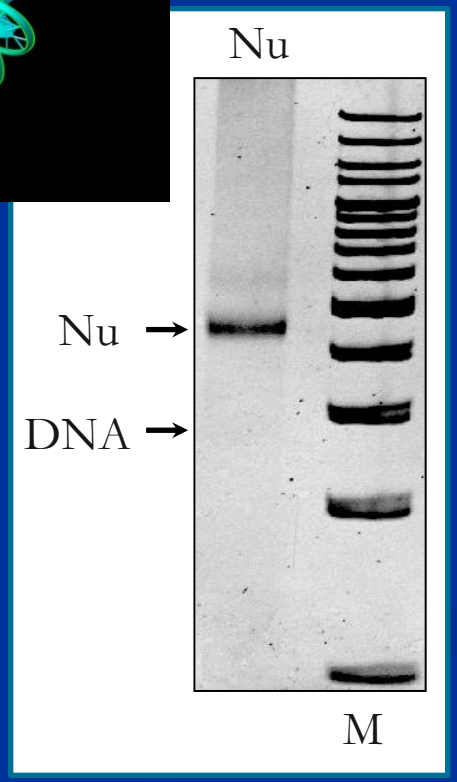


Chromatin: structural and functional complexities



Mononucleosomes are convenient model system to study nucleosome interactions with different protein factors



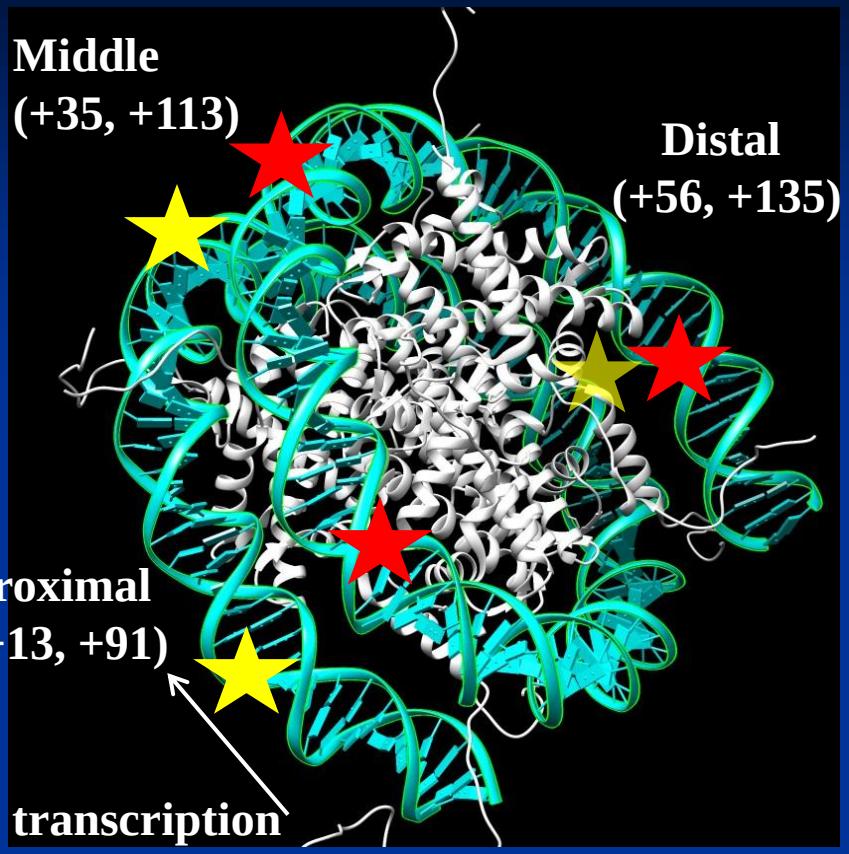


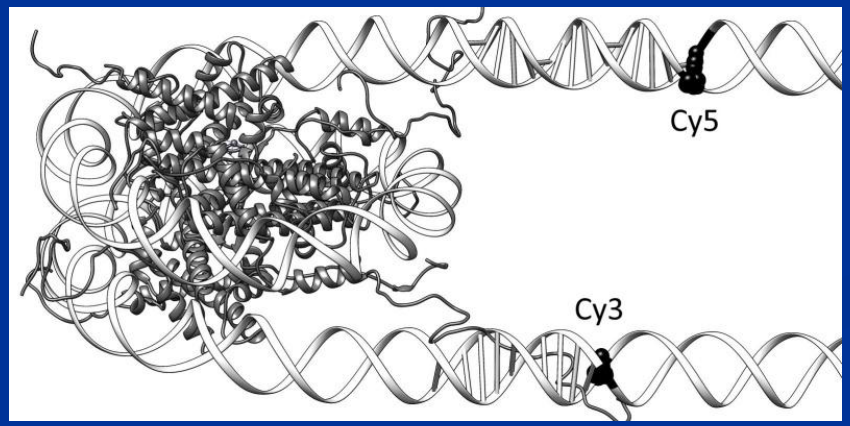
DNA (147 b.p. +20 b.p. linker)
603 strong nucleosome-positioning sequence
+
core histones
(2×H2A, 2×H2B, 2×H3, 2×H4)

nucleosome nanoparticle (10×5 nm size)

Studies with
biochemical and molecular biology techniques

Method: fluorescence microscopy of single particles (complexes)





Contradiction:

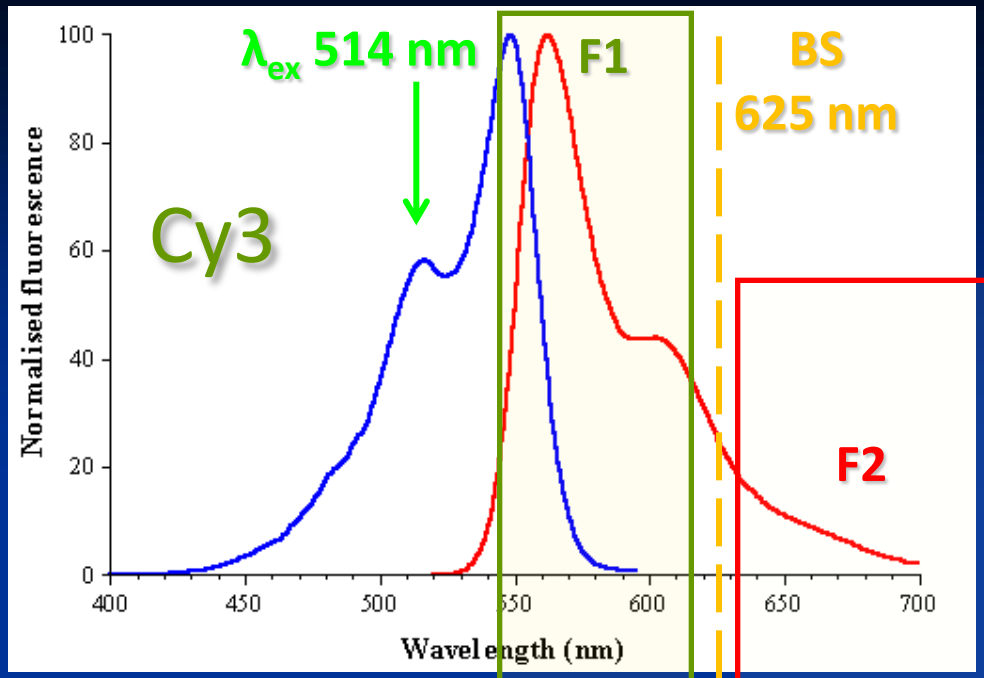
- Resolution of conventional optical microscopy: lateral 200 nm; axial 800 nm.
- Nucleosome size – about 10 nm.

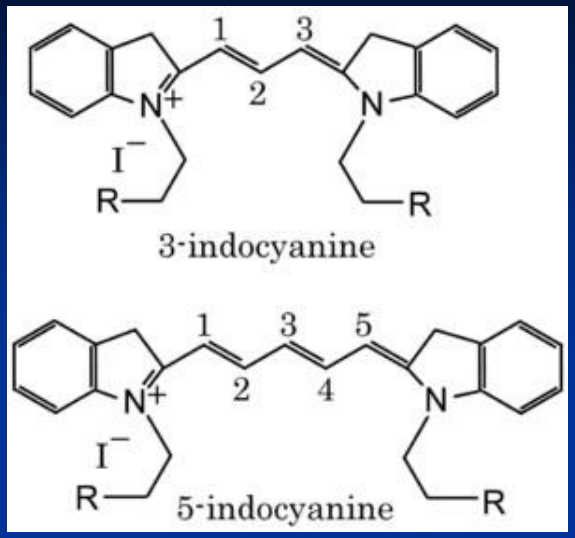
To study structural changes at the level of single nucleosomes it is necessary:

1. To use Forster resonance energy transfer (FRET) effect (a probe of conformational transitions at the scale of 4-9 nm)

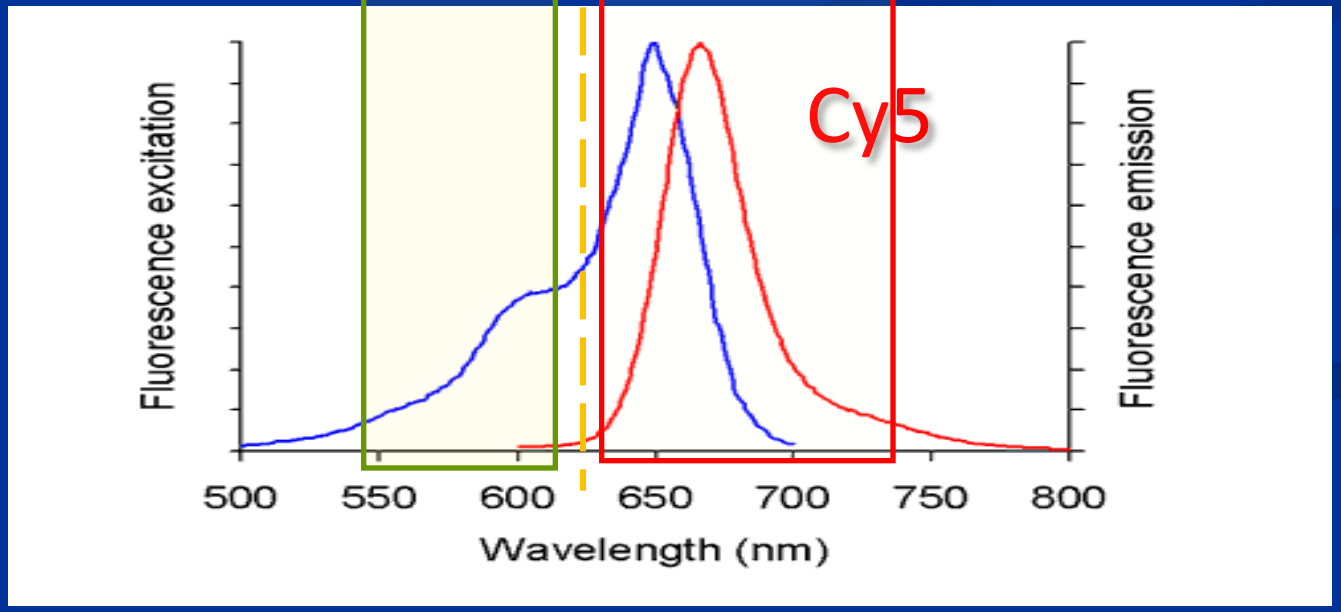
2. To isolate single nucleosomes in space and/or in time.

Kudryashova et al. Methods Mol Biol. 2015, 1288, 395-412

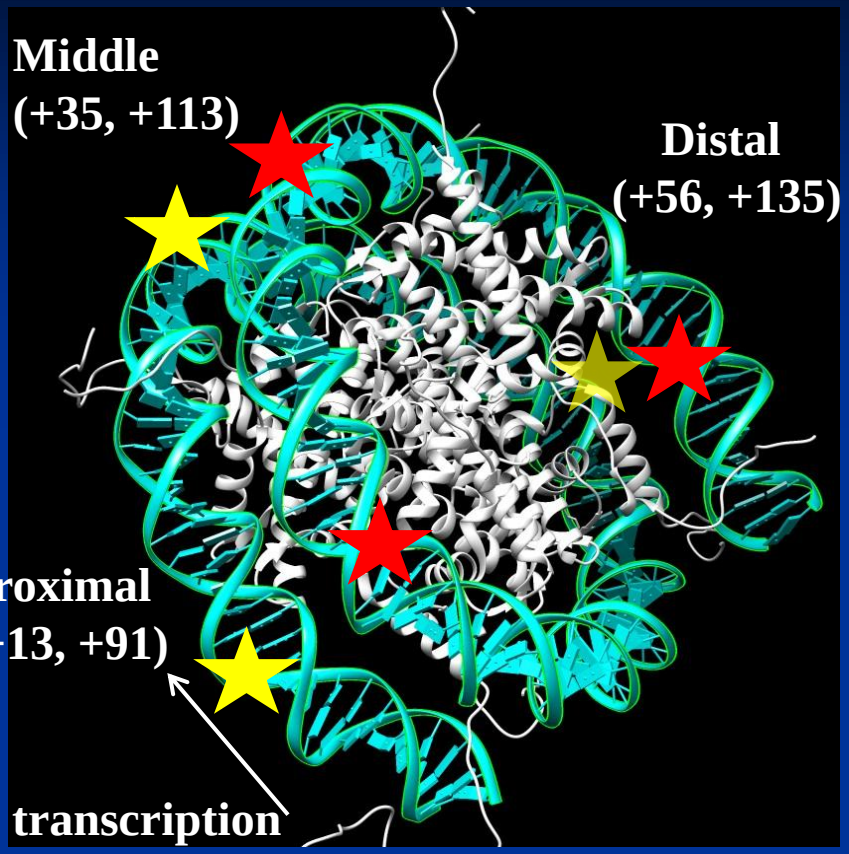


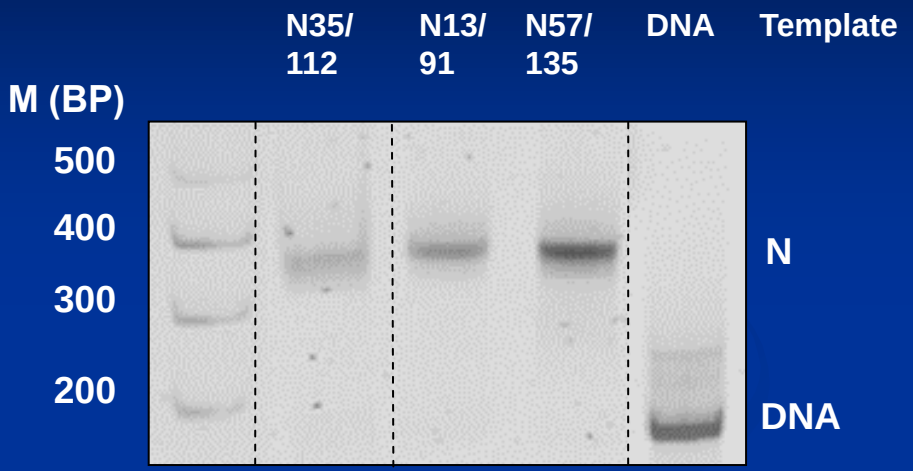


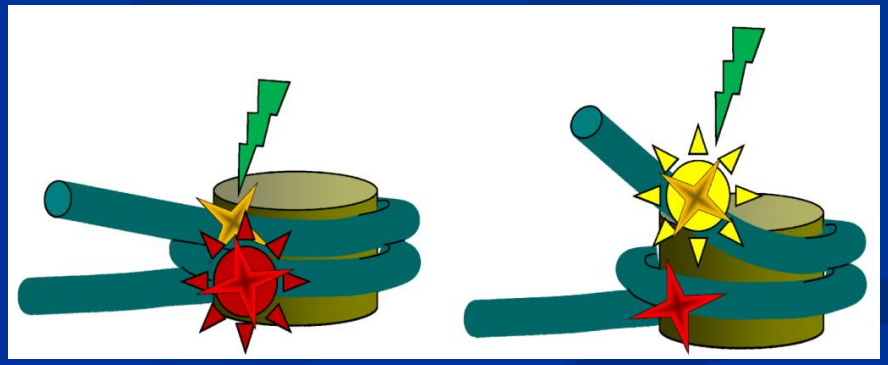
$E \sim 1/[1+(r/R_0)^6]$, $R_0 = 5.6$ nm



Method: fluorescence microscopy of single particles (complexes)



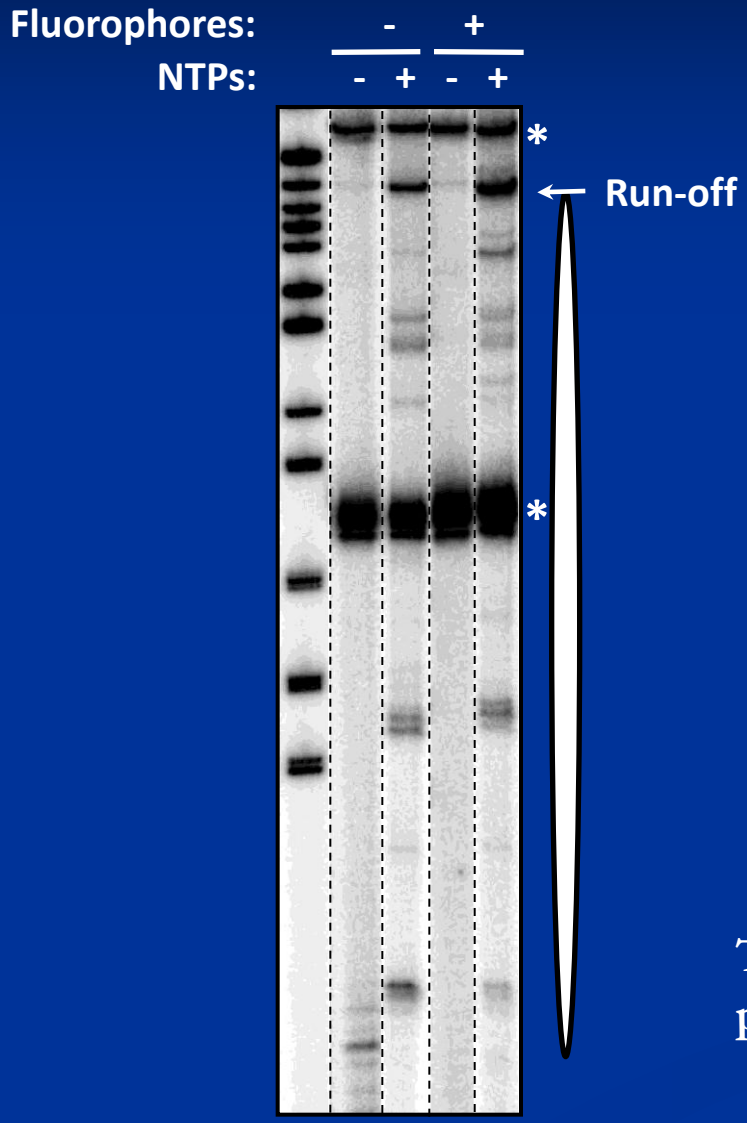




$$E \sim 1/[1+(r/R_0)^6],\ R_0 = 5.6\ \text{nm}$$

$$E = \frac{Ia}{Ia + Id} \qquad E = \frac{Ia - \gamma Id}{Ia + Id(1-\gamma)}$$

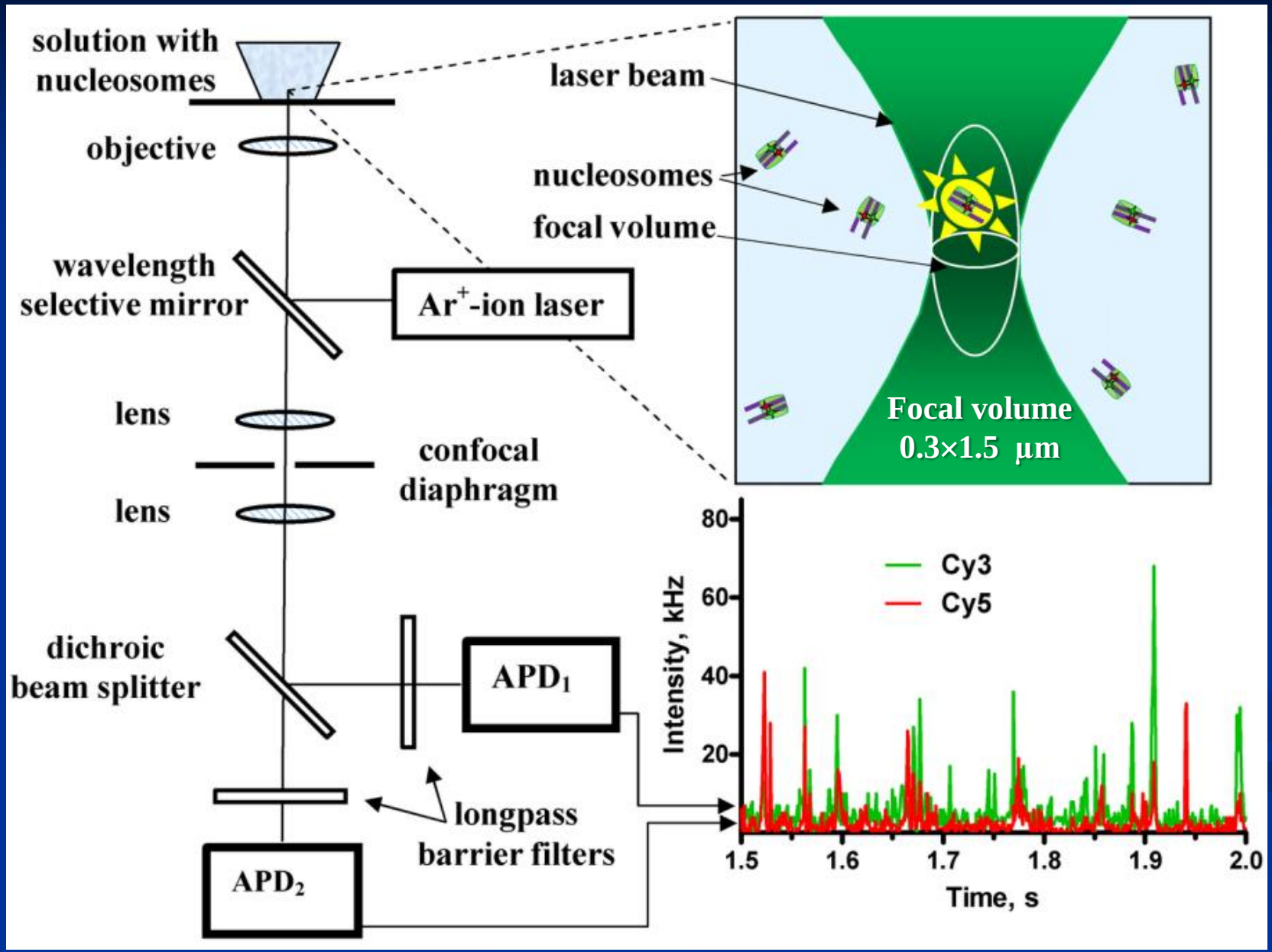
Analysis of transcription through 603 nucleosome containing intact and fluorophore-labeled DNA



No additional pausing was detected on fluorophore-labeled DNA, suggesting that fluorophores do not interfere with progression of the enzyme.

Transcription by RNAP was conducted in the presence of NTPs for 30 s at 150 mM KCl

Study of freely diffusing single nucleosomes and their complexes



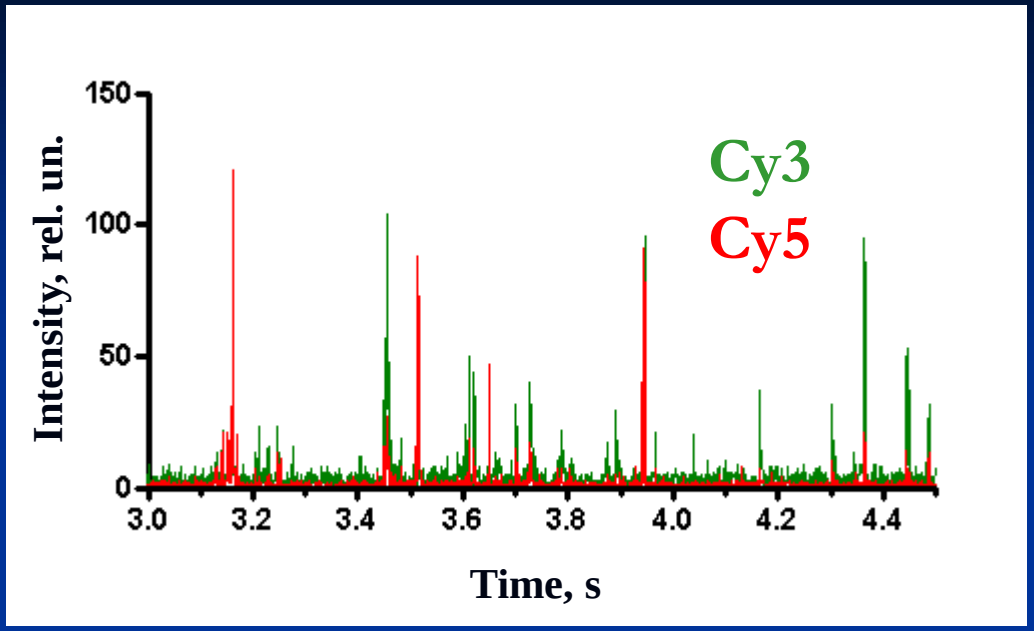
Zeiss: LSM710-Confocor3

1 V/Flex PTC laser ports (405, 440, In *Tune*; ps+cw)
2 IR PTC laser port (tunable Ti:Sa)
3 Vis PTC laser ports & Vis AOTF
4 Monitoring diodes
5 InVis TwinGate beam splitter (upgradable)
6 Vis TwinGate beam splitter (user exchangeable)
7 Scan mirrors (FOV 20, 6k × 6k)
8 Master pinhole
9 Splitter for external channels
10 Spectral separation and recycling loop
11 Spectral beam guides
12 QUASAR PMT spectral channel # 1
13 QUASAR PMT spectral channels # 2–33 (or # 2)
14 QUASAR PMT spectral channel # 34 (or # 3)
15 Ext. channels (# 4 + 5: APDs, FLIM, FCS etc.)

Supported by
M.V. Lomonosov Moscow State University Program of Development

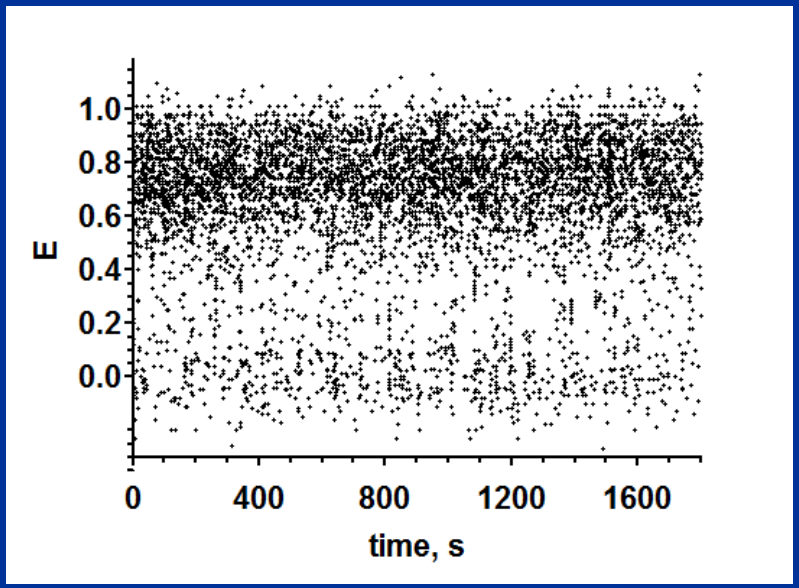
Amount of sample:
volume - 10 µl
concentration – 0.2-1 nM
statistics- 1000-10000 particles/ 10 min

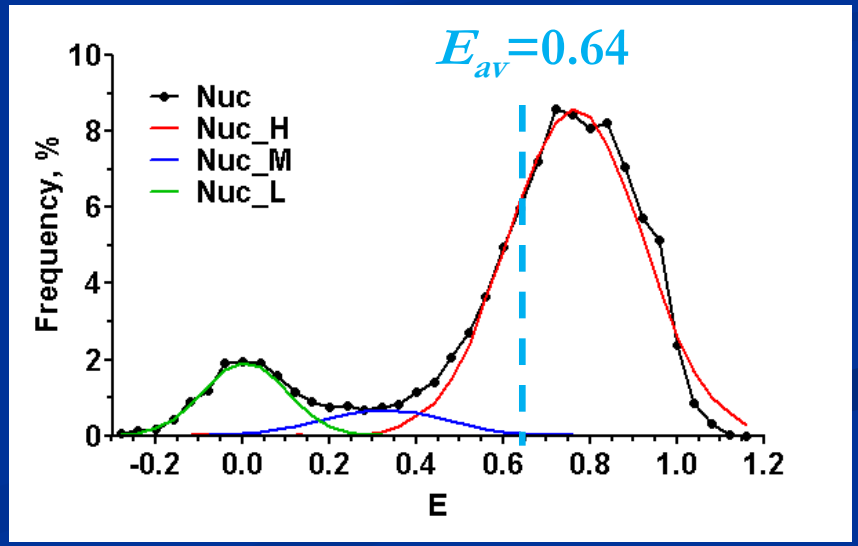
Study of freely diffusing single nucleosomes and their complexes



$$E=\frac{Ia - \gamma Id}{Ia + Id(1-\gamma)}$$

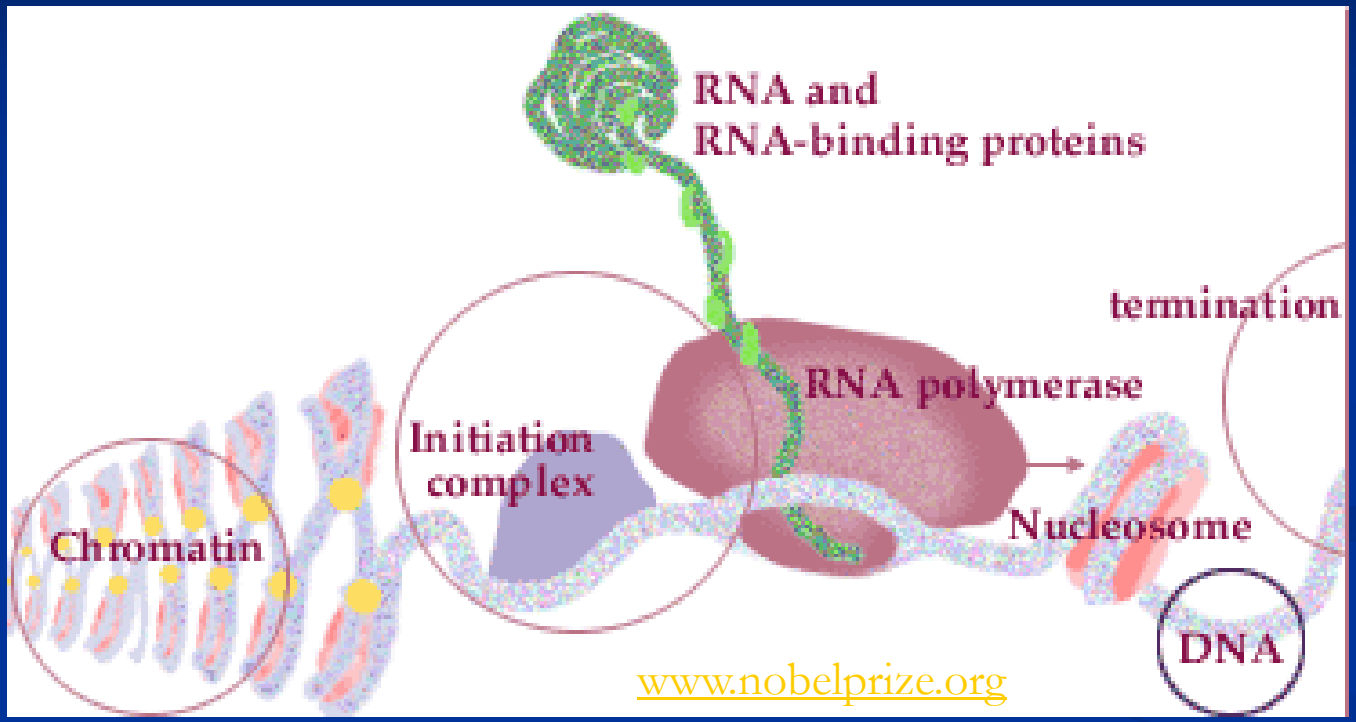
$E \sim 1/[1+(r/R_0)^6]$, $R_0=5.6$ nm



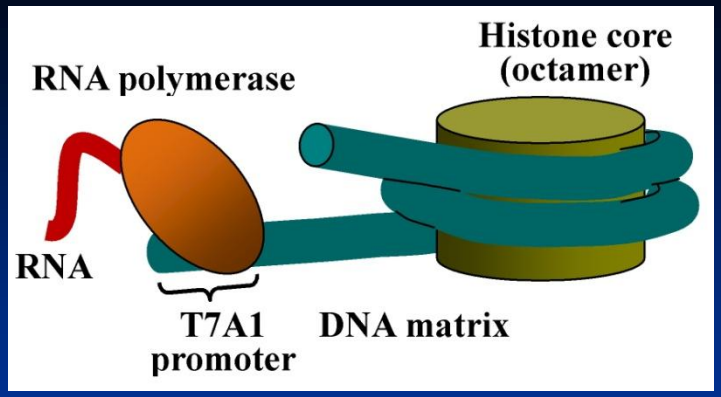


Nucleosomes: distal labeling

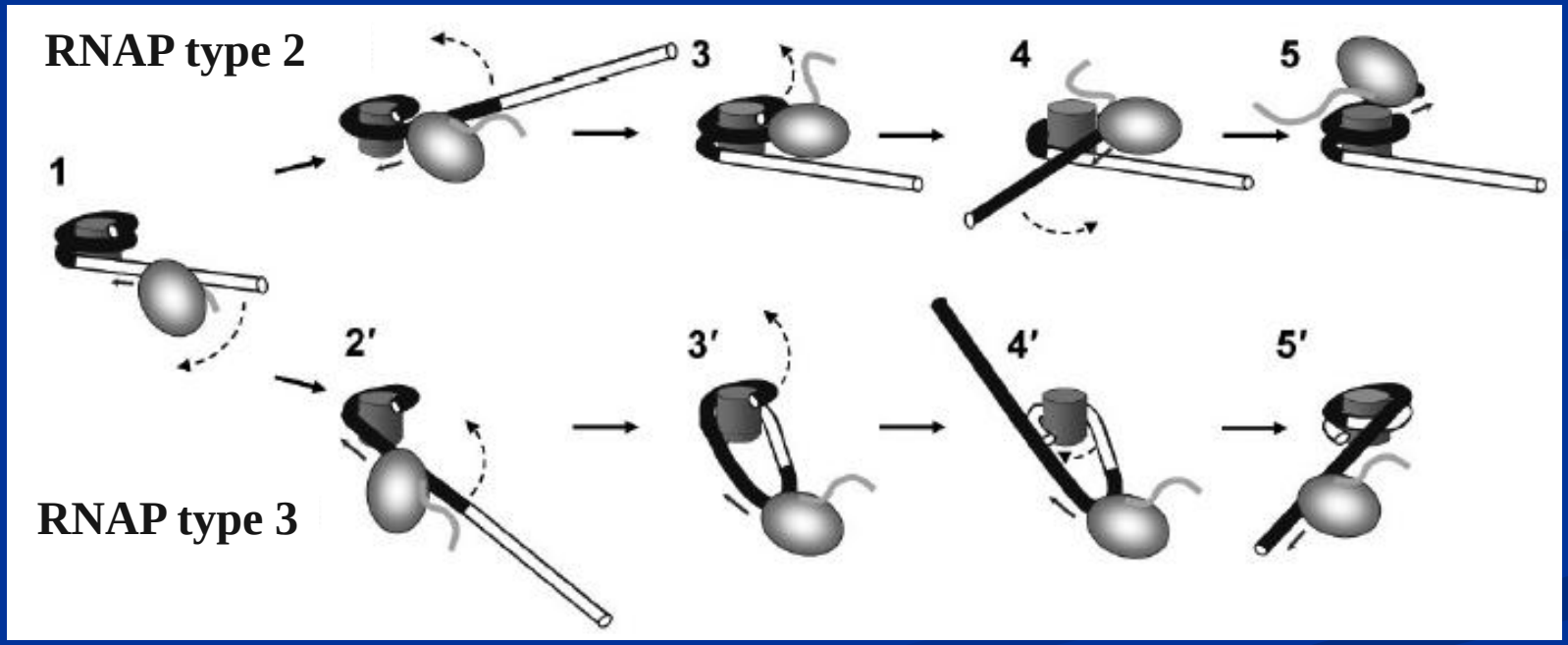
Task1. Study of nucleosome transcription with RNA polymerase



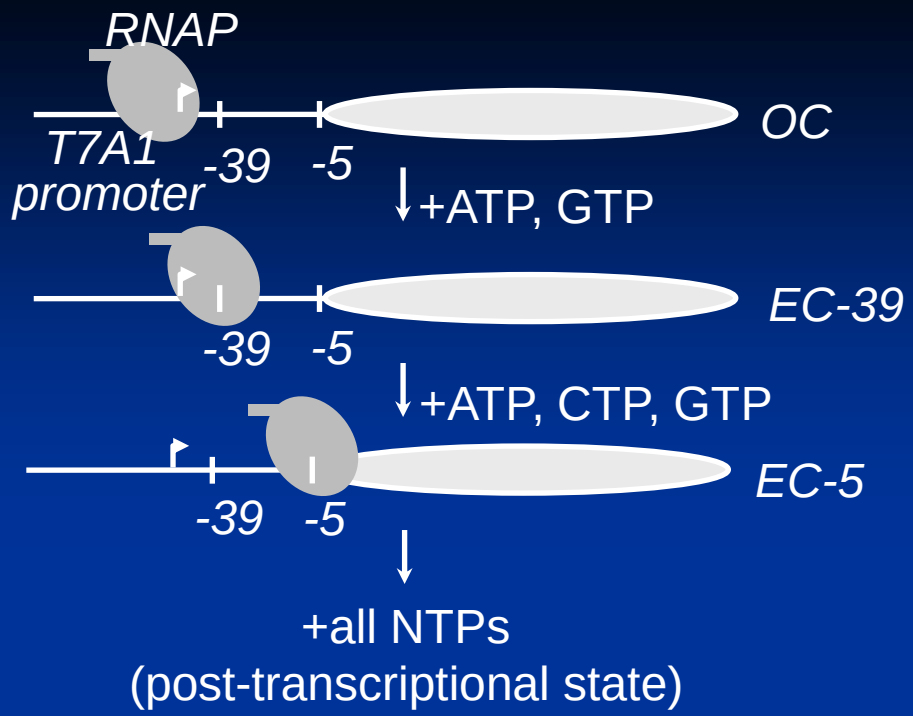
Transcription is the first step of gene expression: a particular DNA region is copied into RNA by the RNA polymerase enzyme.



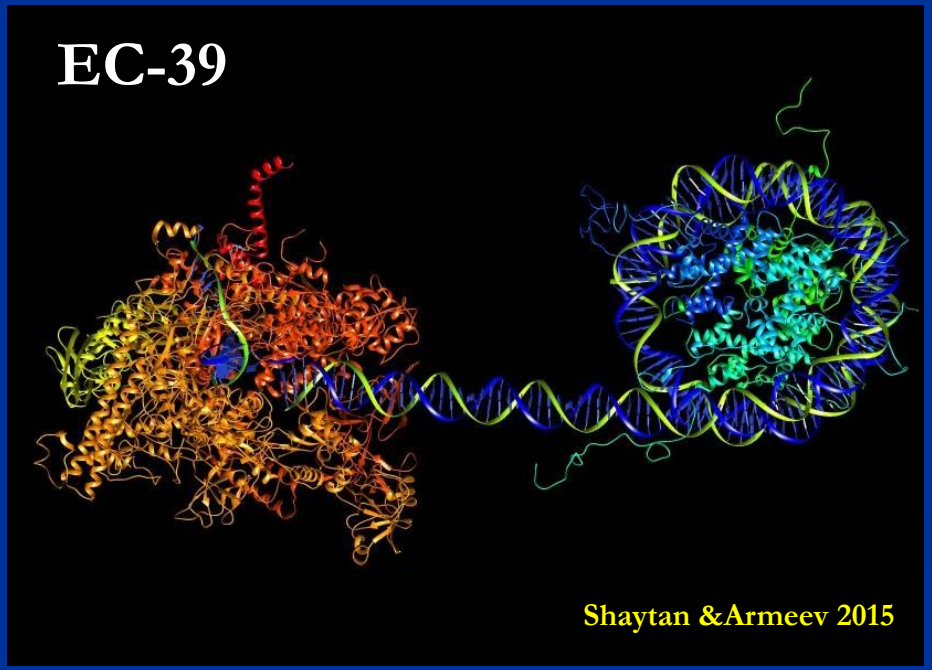
Transcription of chromatin is a functionally important and complex process that occurs with participation of dozens different proteins

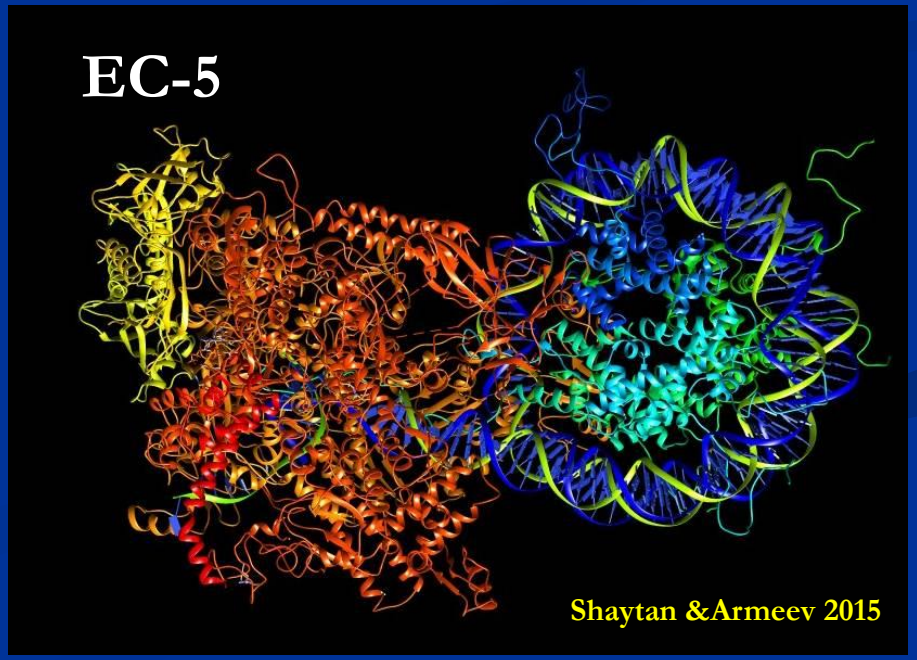


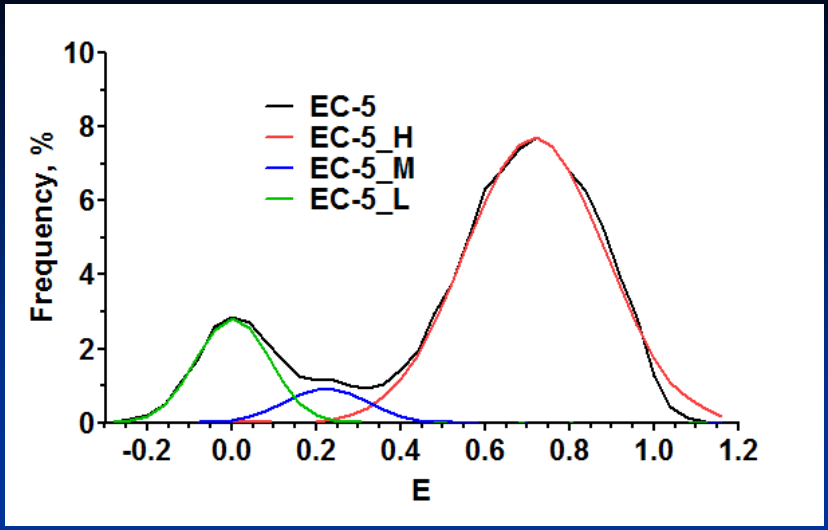
Transcription stages: RNAP binding to promoter, initiation, elongation and termination

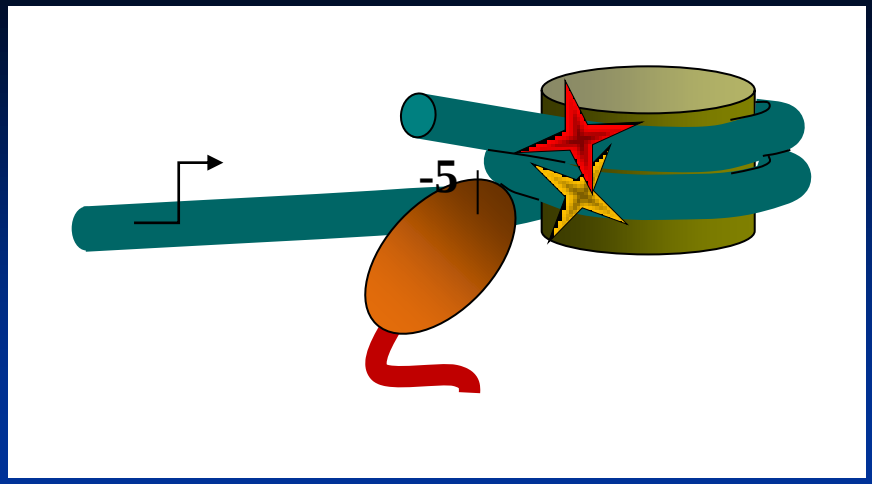


Formation of stalled elongation complexes in a mononucleosome system

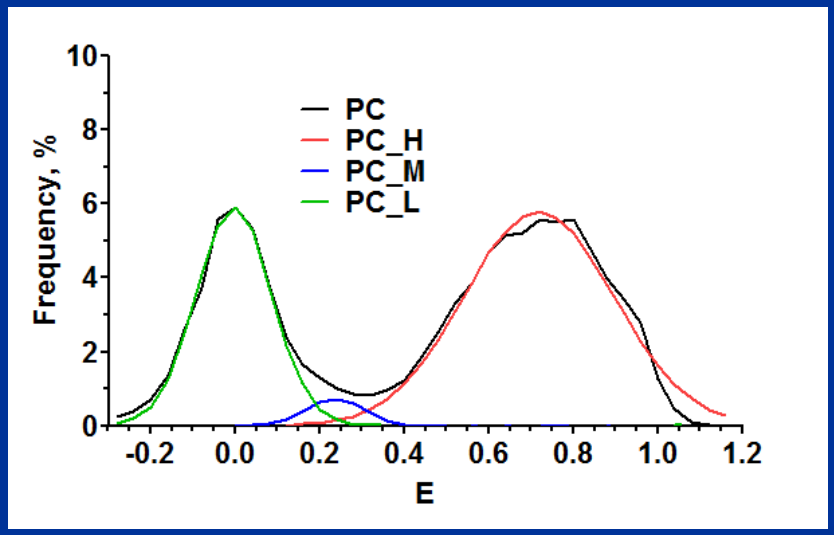


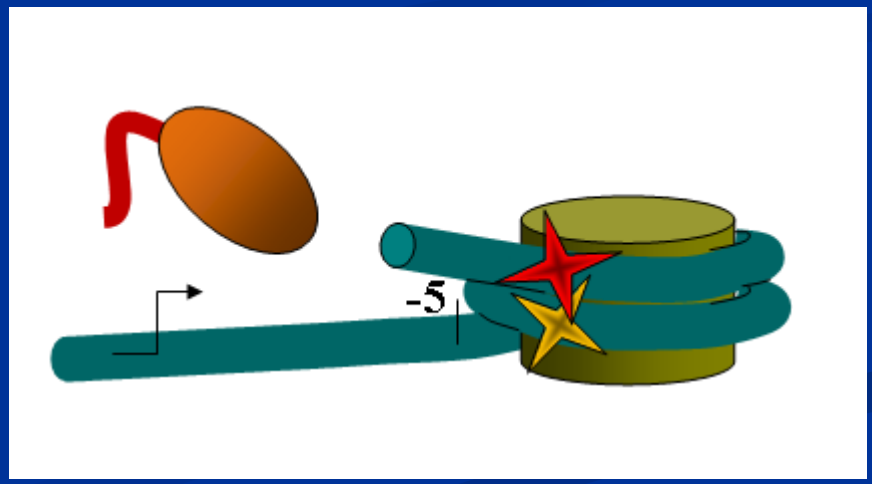




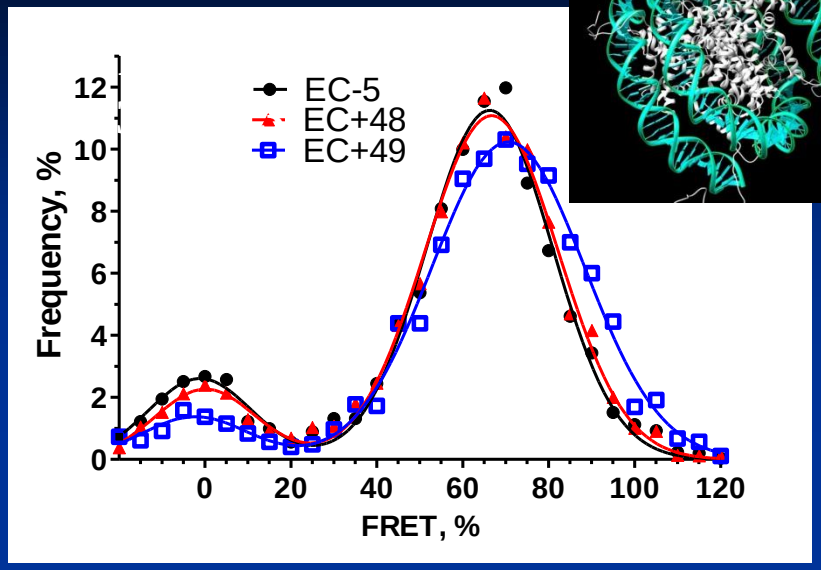


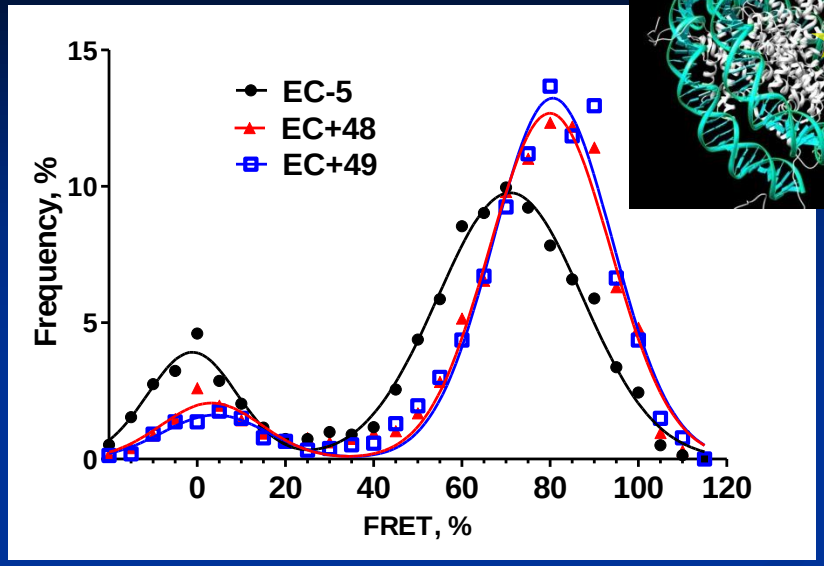
Formation of EC-5 does not disturb nucleosome structure in the distal region

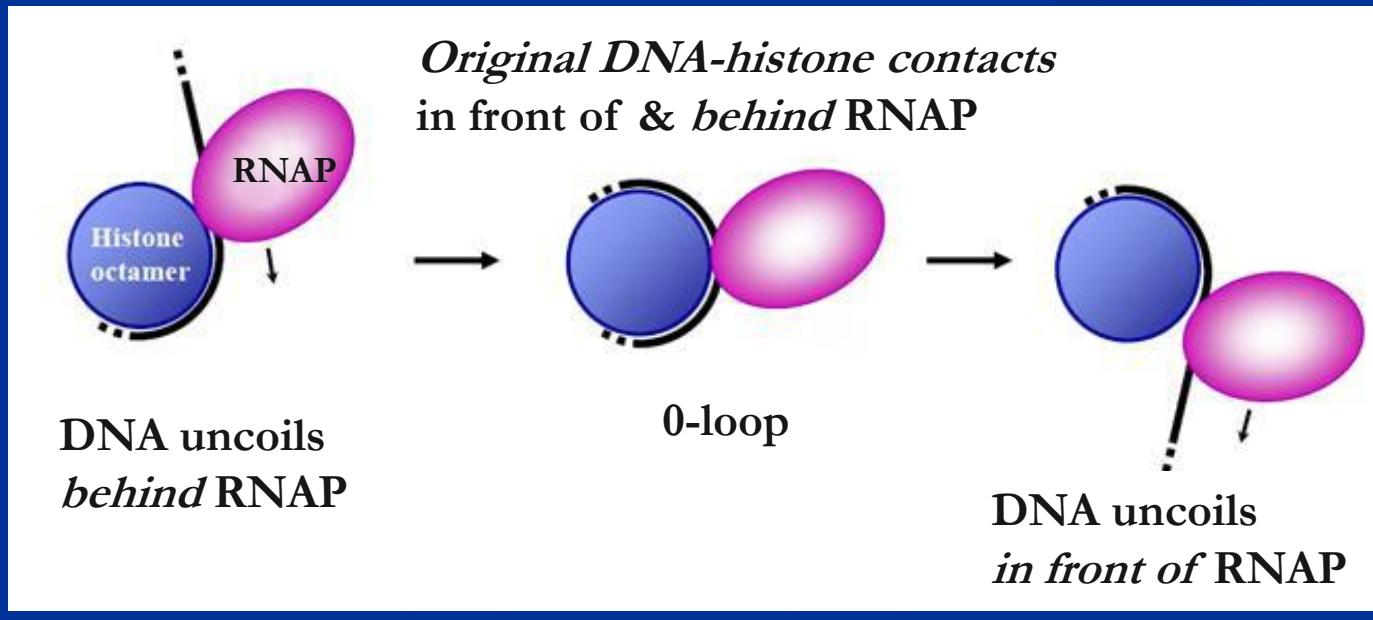




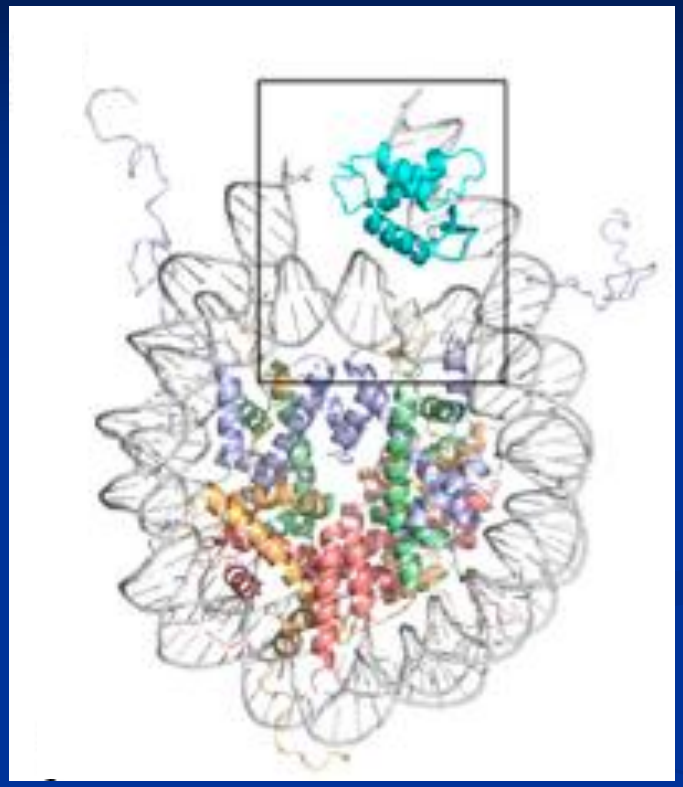
Nucleosome survives after transcription


(+35, +113)
EC+48; EC+49
EC-5
EC+48
EC+49
Frequency, %
FRET, %
0
2
4
6
8
10
12
0
20
40
60
80
100
120


EC+48; EC+49
(+56, +135)
EC-5
EC+48
EC+49
Frequency, %
FRET, %
0
5
10
15
0
20
40
60
80
100
120

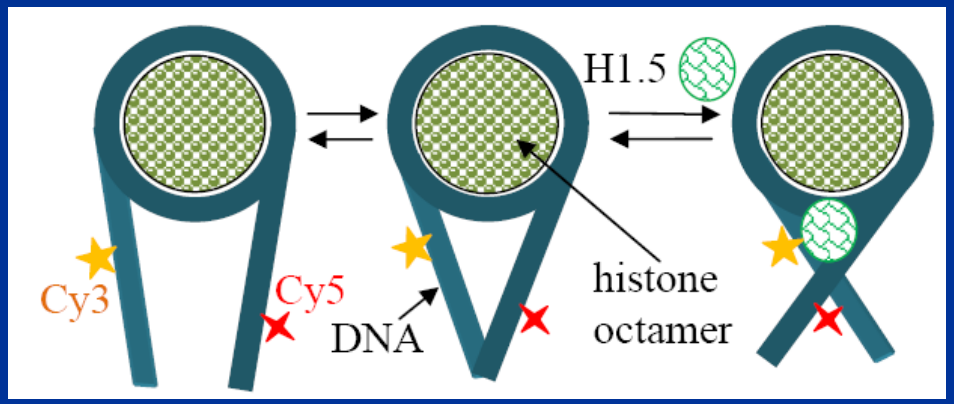

Original DNA-histone contacts
in front of & *behind* RNAP
RNAP
Histone octamer
DNA uncoils
behind RNAP
0-loop
DNA uncoils
in front of RNAP

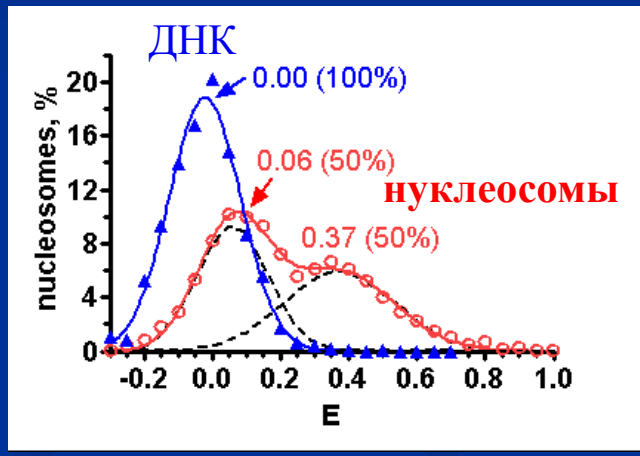
Task 2. Study of interactions of nucleosomes with linker histone H1

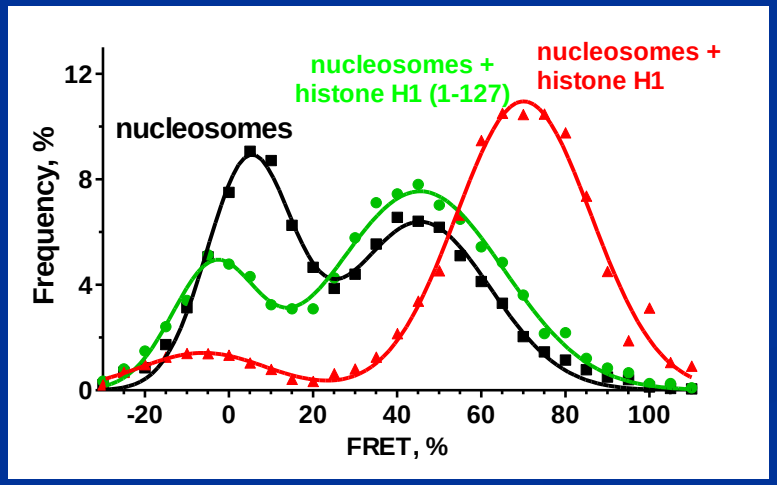


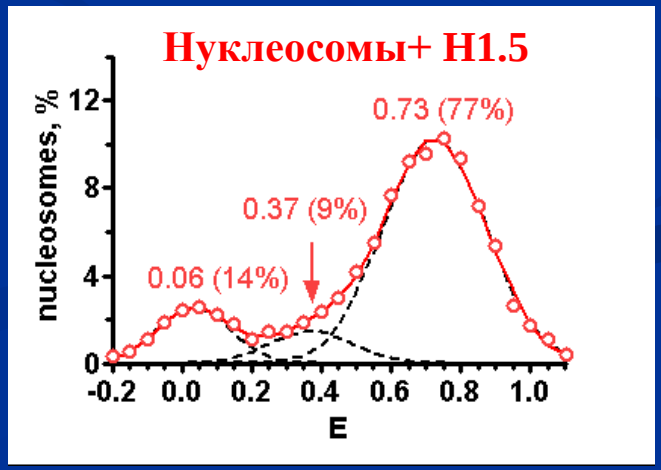
An asymmetrical structural model of the gH1-nucleosome complex. Zhou et al. PNAS (2013), 110, 19390–19395

Interactions of nucleosomes with the linker histone H1.5









Interactions of FACT with nucleosomes

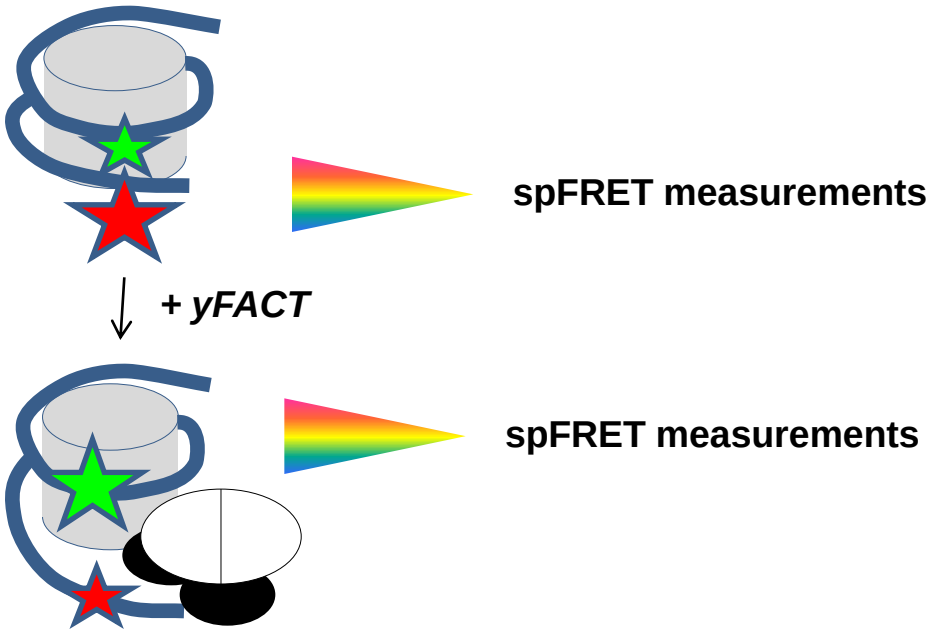
Positions of Cy3/Cy5 on nucleosomal DNA

Yeast FACT consists of

- Spt16/Pob3 subunits

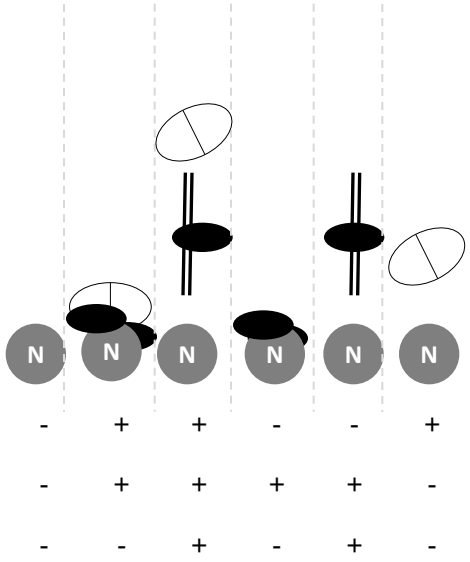
and require

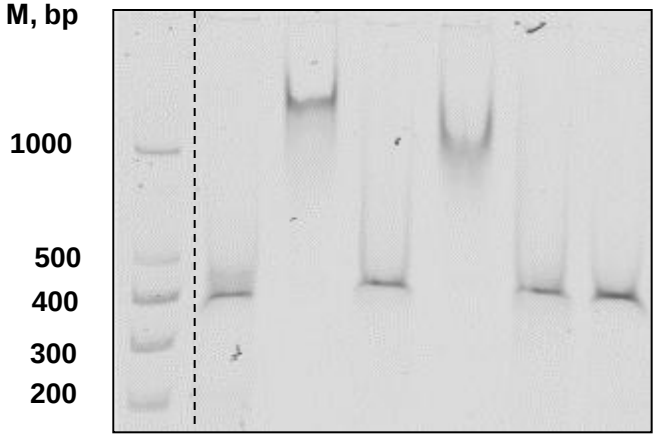
- Nhp6



Valieva et al. Nat Struct Mol Biol. 2016; 23(12), 1111-1116.

A)





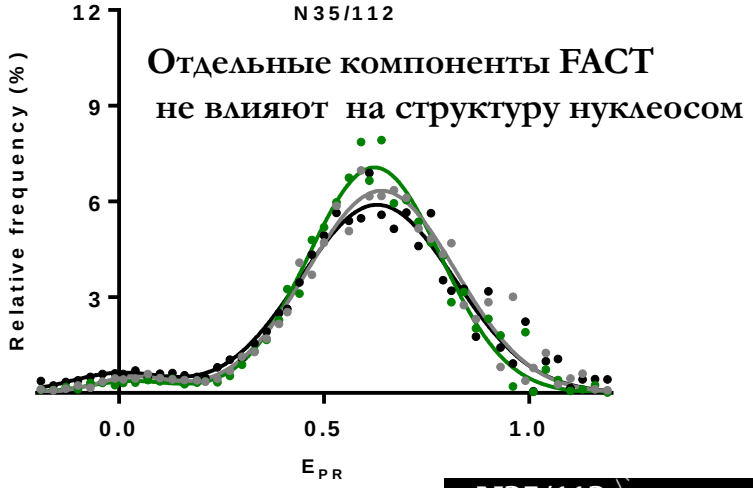
N - Labeled nucleosomes

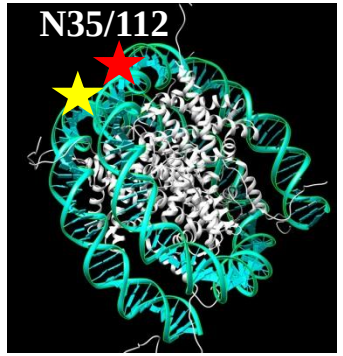
- Spt16/Pob3 FACT subunits

- Nhp6 FACT subunit

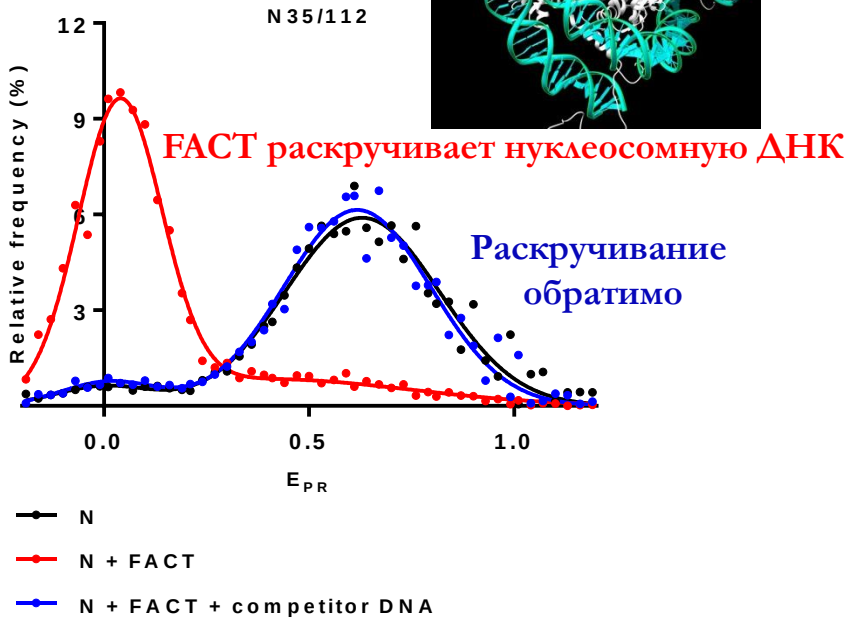
- Competitor DNA

B)

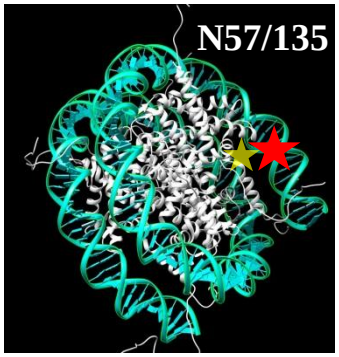


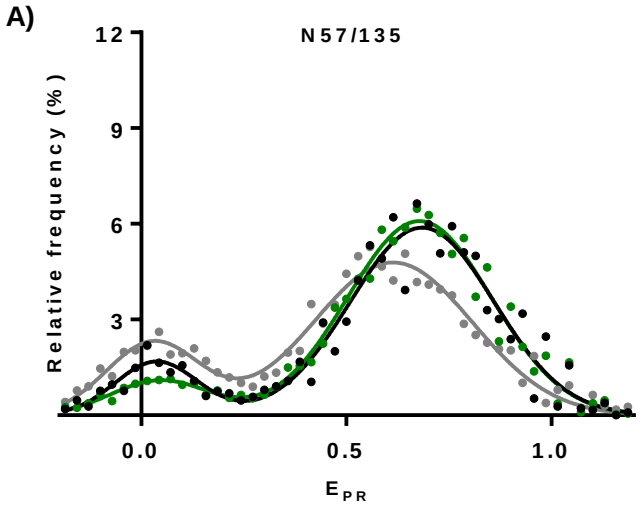


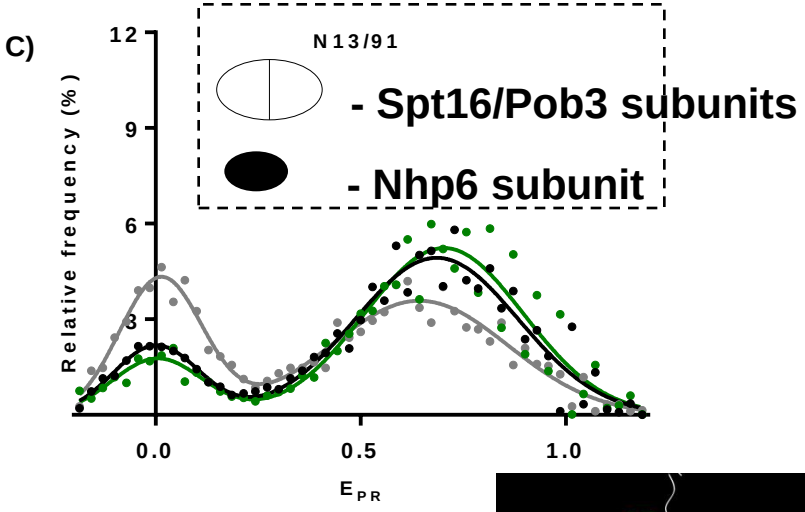
C)

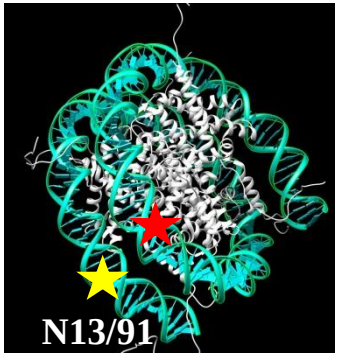


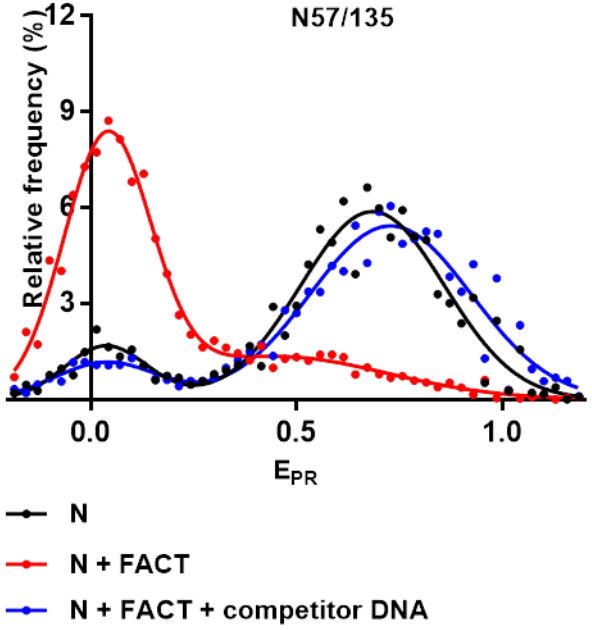
Valieva et al. Nat Struct Mol Biol. 2016; 23(12), 1111-1116.

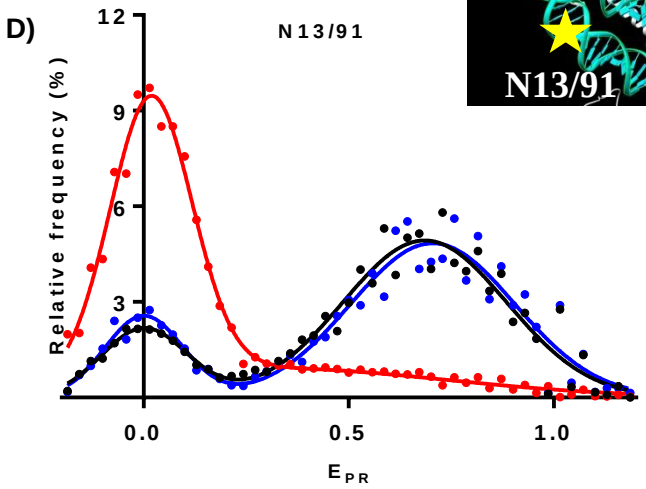




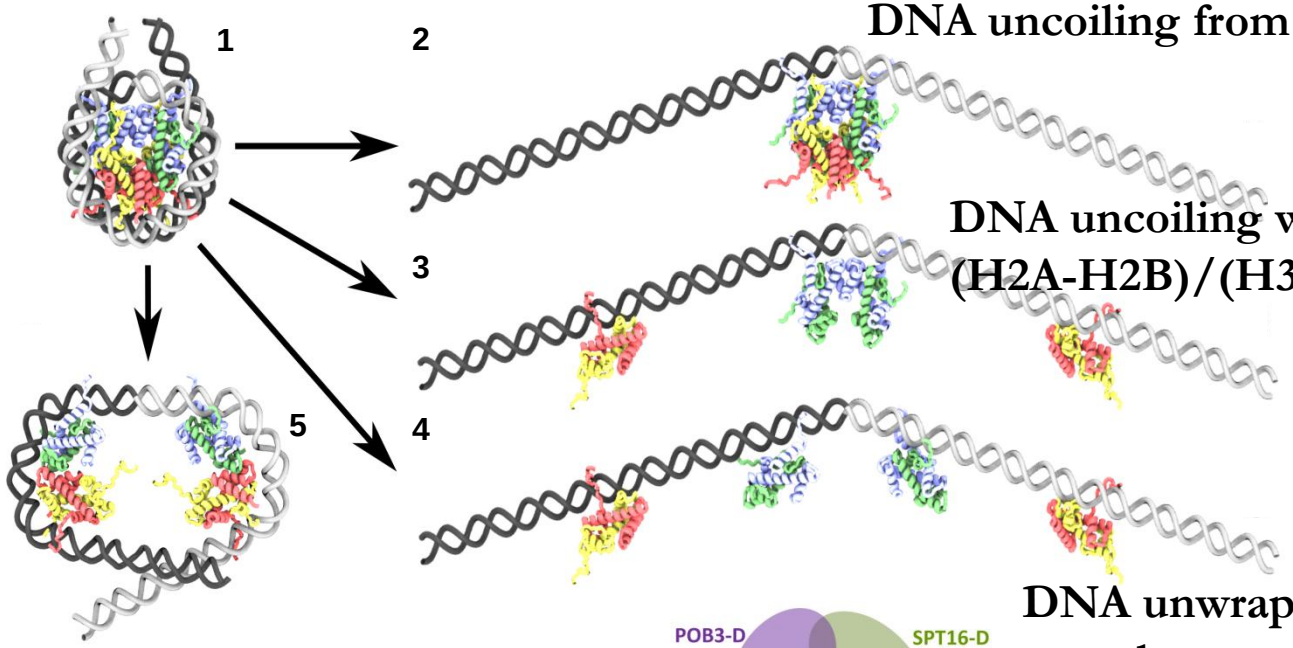








Valieva et al. Nat Struct Mol Biol. 2016; 23(12), 1111-1116.

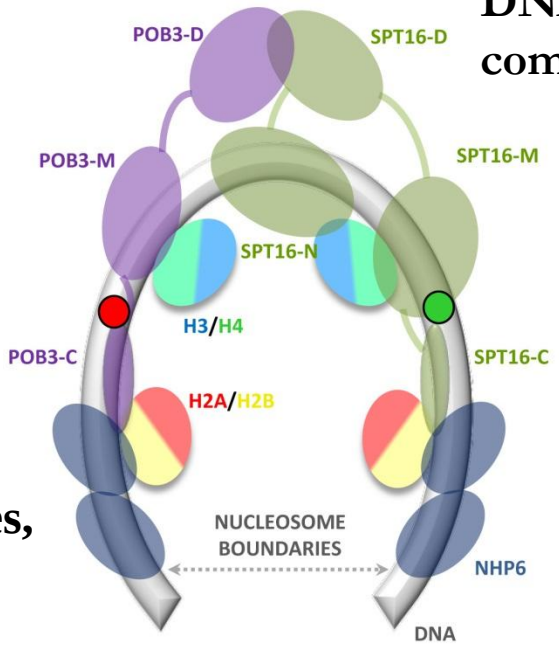


DNA uncoiling from intact octamer

DNA uncoiling with opening of $(H2A\text{-}H2B)/(H3\text{-}H4)_2$ interface

DNA unwrapping with complete octamer disassembly

Opening of (H3-H4) dimer-dimer interface; no further DNA uncoiling



FACT is likely to interact both with uncoiled DNA and with core histones, replacing some of DNA-histone and histone-histone interactions

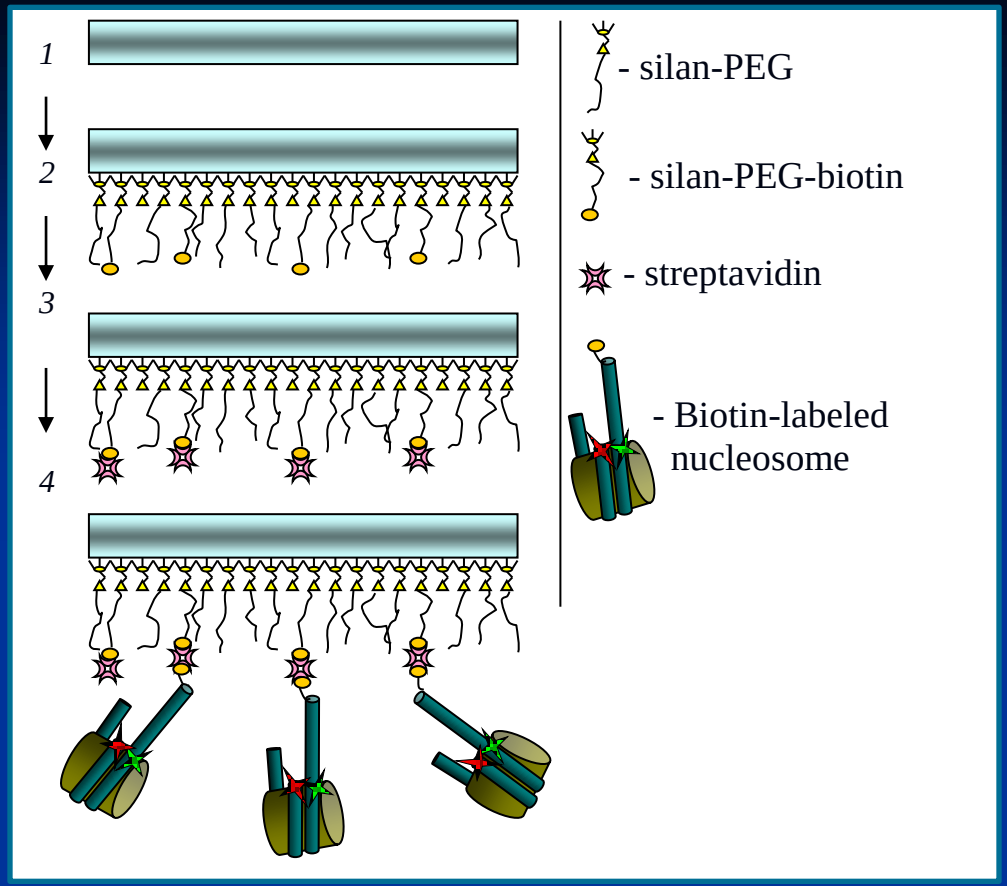
Valieva et al. Nat Struct Mol Biol. 2016; 23(12), 1111-1116.

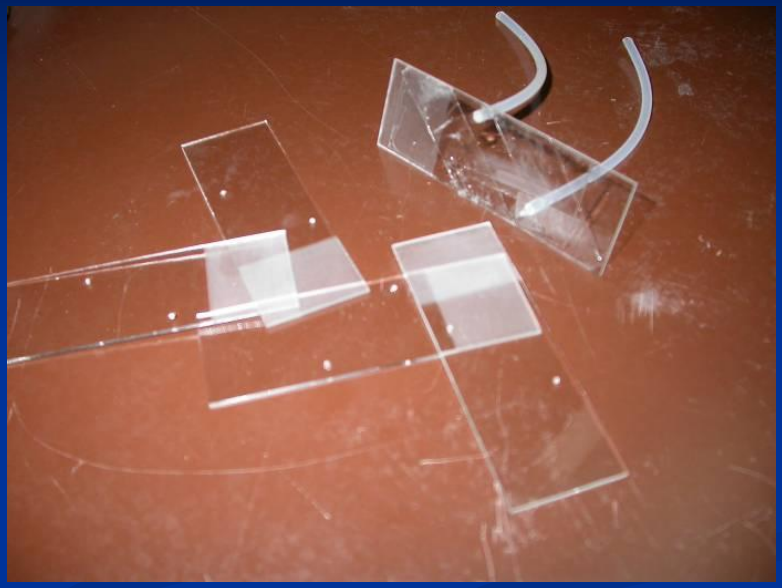
FACT binding results in
a dramatic,
ATP-independent,
symmetrical and
reversible uncoiling of DNA

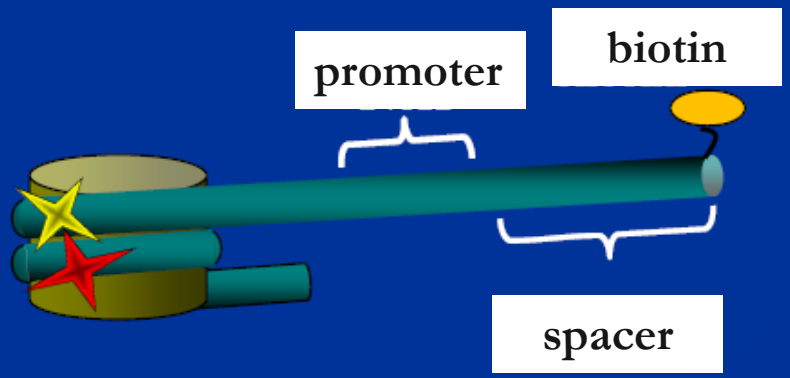
This uncoiling affects at least 70% of DNA in a nucleosome,
occurs without apparent loss of histones and
proceeds *via* an all-or-none mechanism.

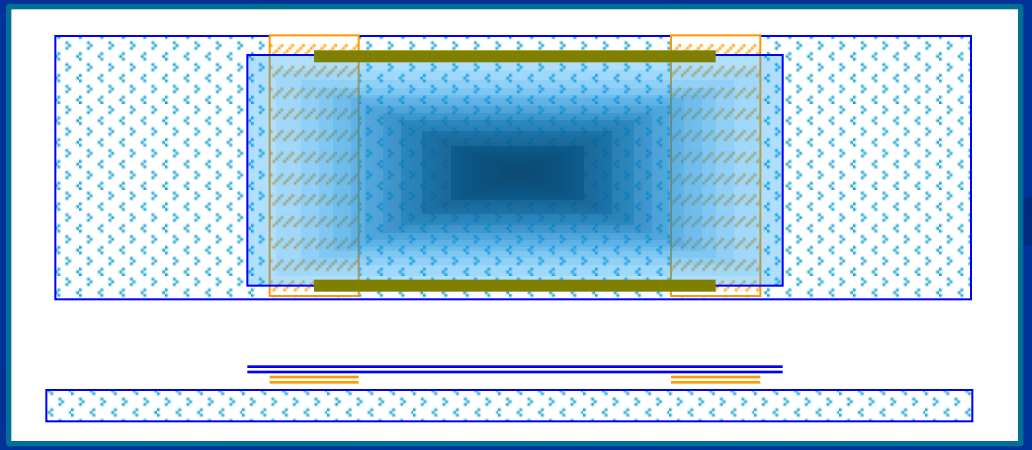
FACT-dependent nucleosome unfolding modulates the accessibility of nucleosomal DNA, and this is an important function of FACT *in vivo*.

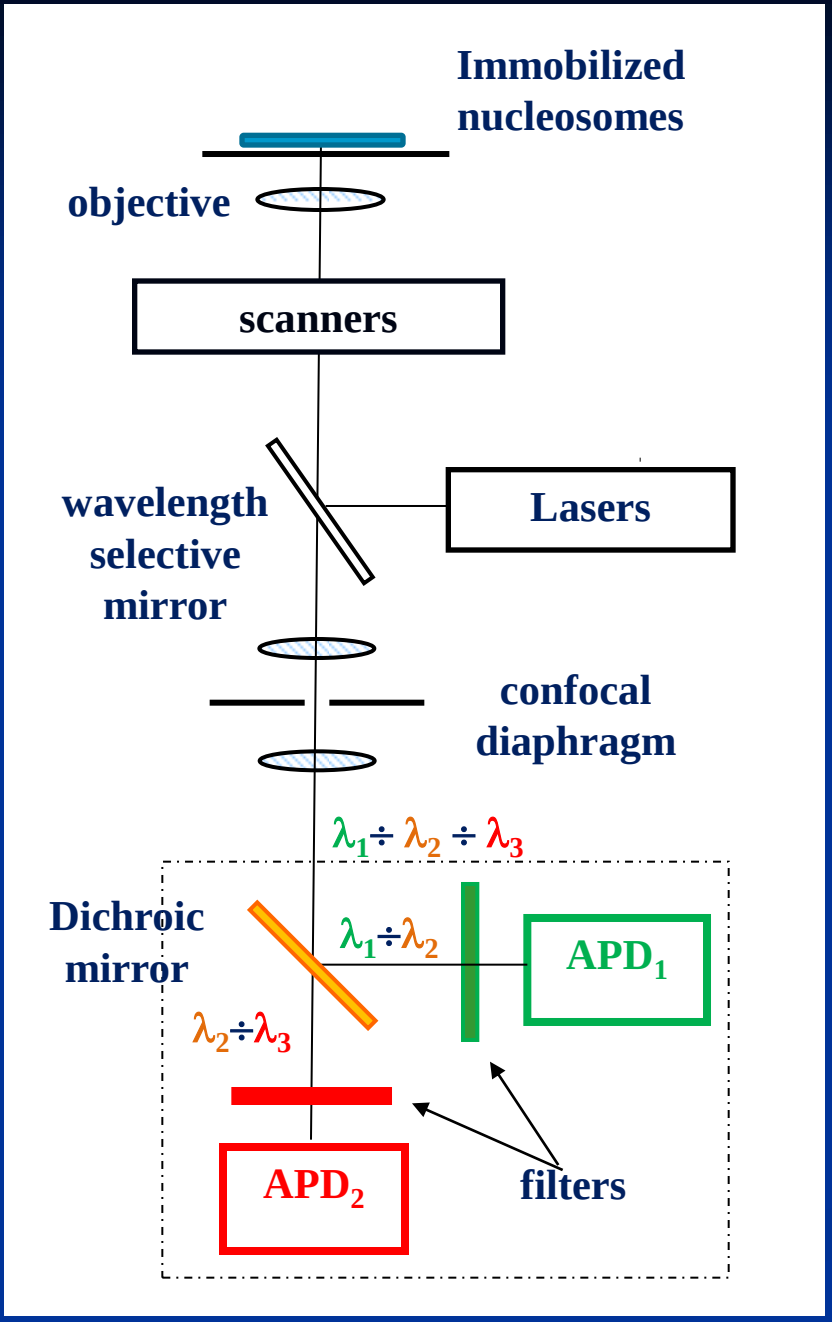
Study of immobilized single nucleosomes







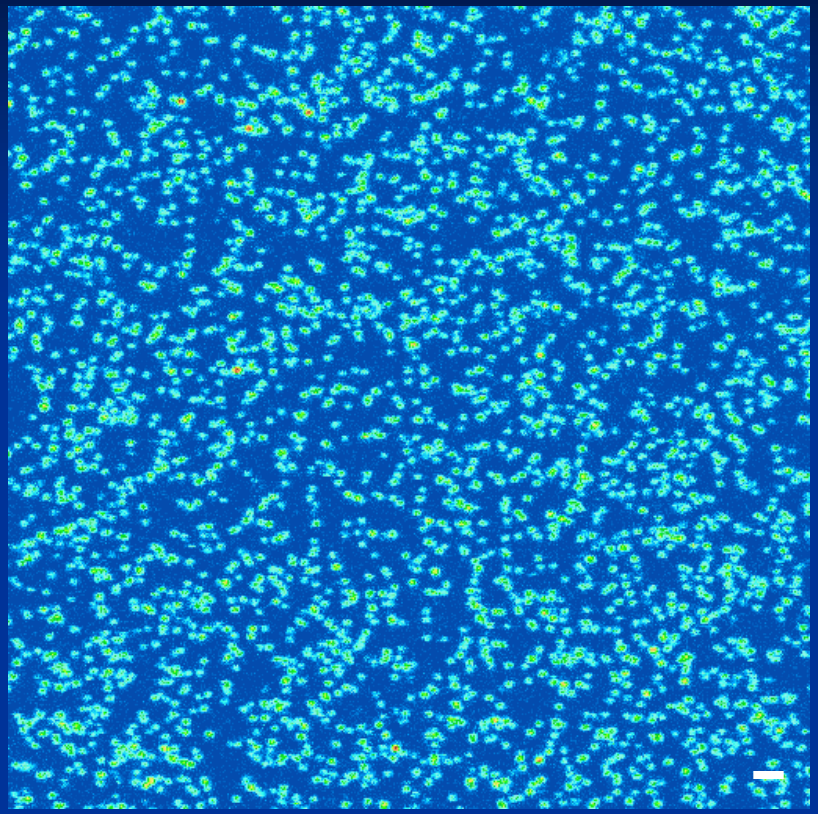



Immobilized nucleosomes
objective
scanners
wavelength selective mirror
Lasers
confocal diaphragm
λ1÷ λ2 ÷ λ3
Dichroic mirror
λ1÷λ2
APD1
λ2÷λ3
APD2
filters

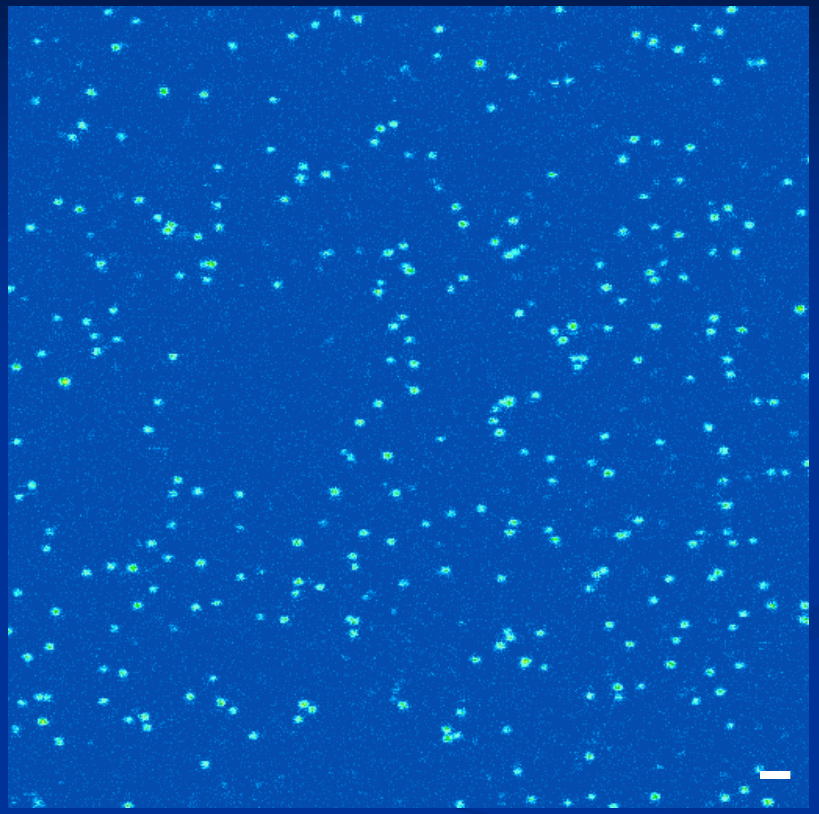

Leica: SP2-APD


Zeiss: LSM710-Confocor3

Immobilization of Cy3-avidin



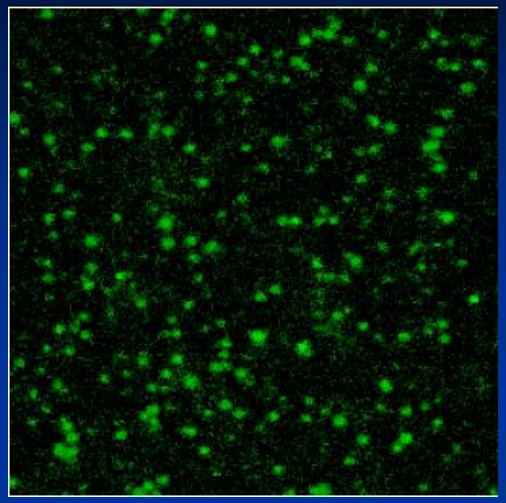
100 ng/ml



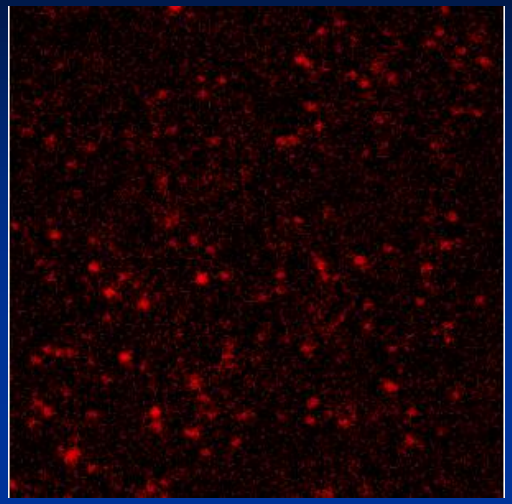
10 ng/ml

Bar– 1 µm. Field: 37.5 × 37.5 µm

Immobilized nucleosomes (distal labeling)

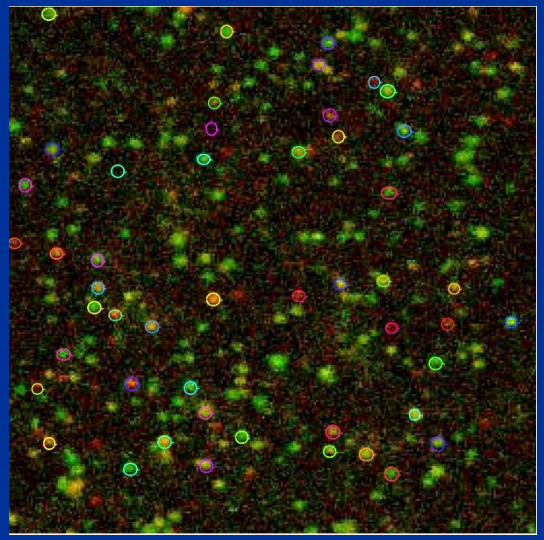


Cy3

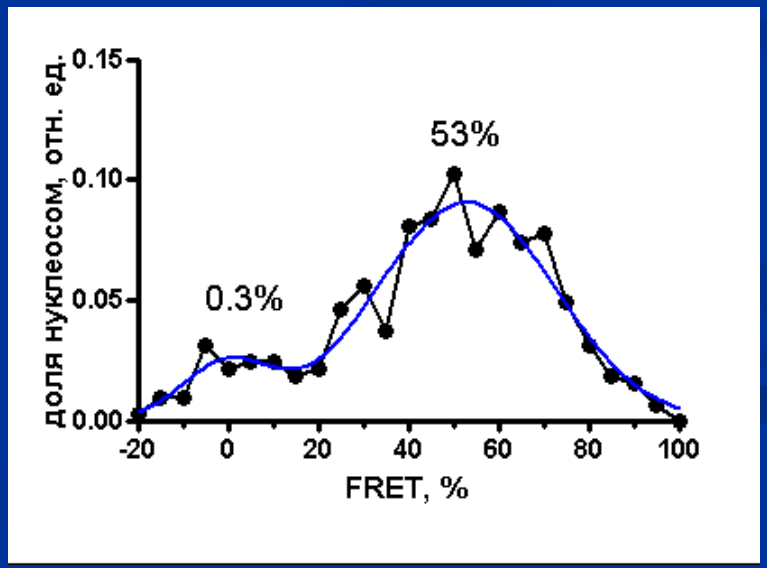


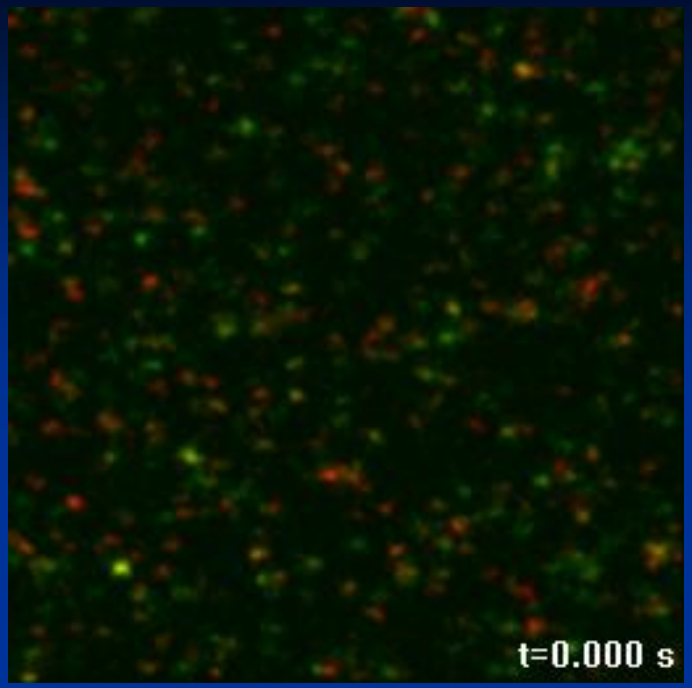
Cy5

$$E = \frac{Ia - \gamma I_Д}{Ia + I_Д(1-\gamma)}$$



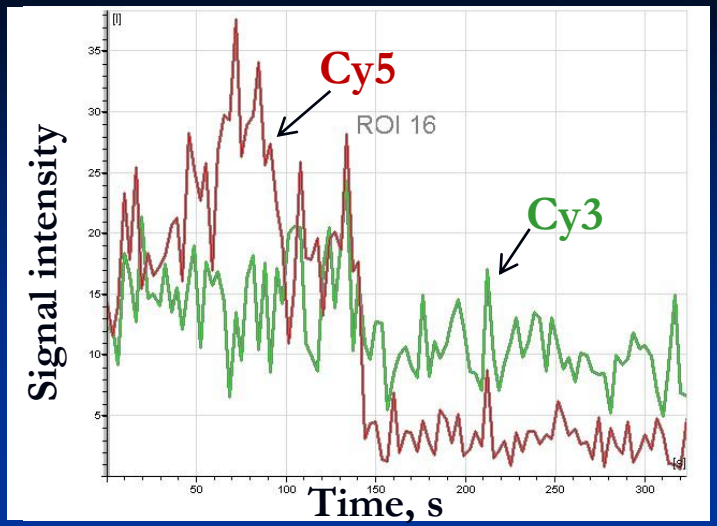
overlapping



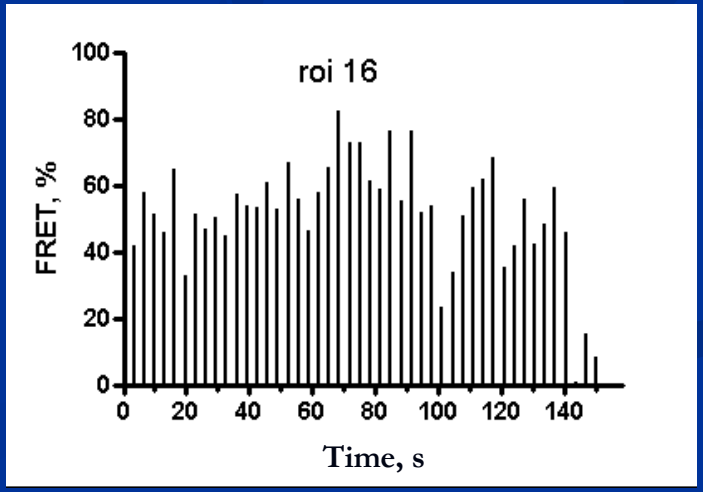


Immobilized EC-5 complex

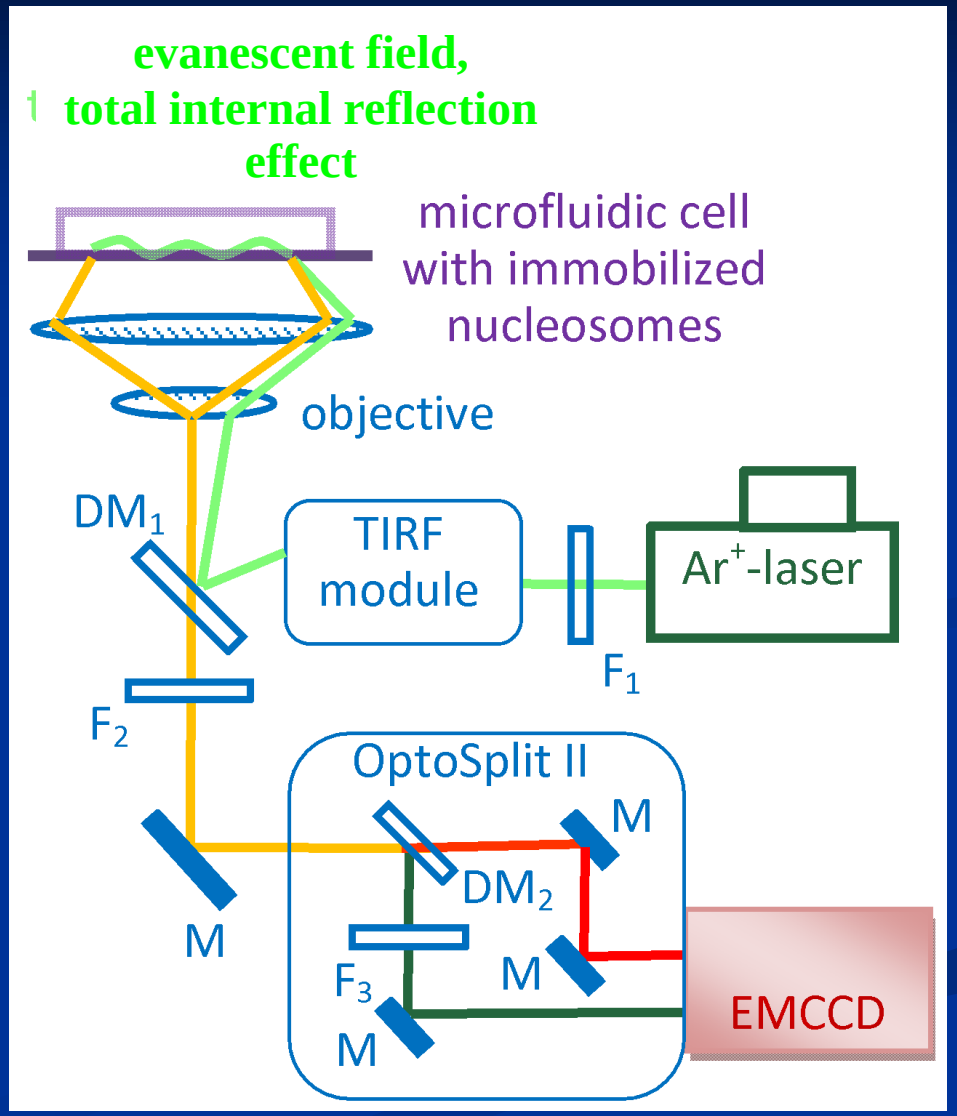
3 s/frame



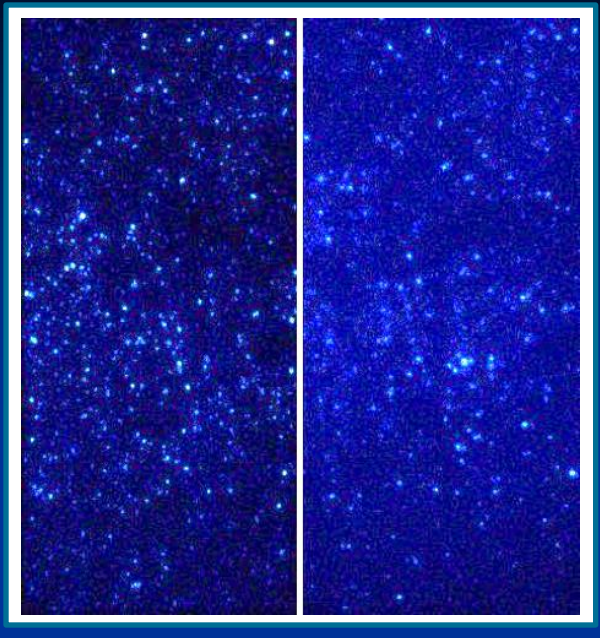
$$E = \frac{Ia - \gamma Id}{Ia + Id(1-\gamma)}$$

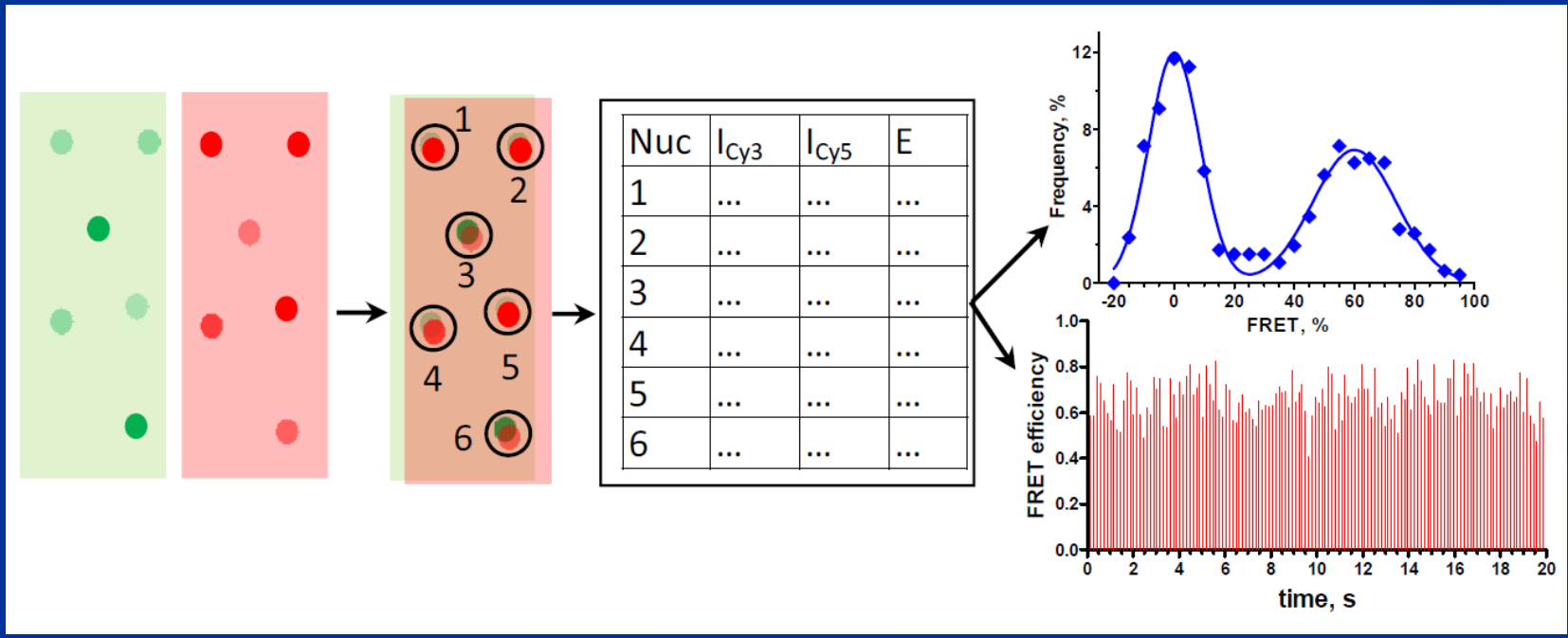


Experimental setup for the study of immobilized nucleosomes using Total Internal Reflection Fluorescence (TIRF) microscopy

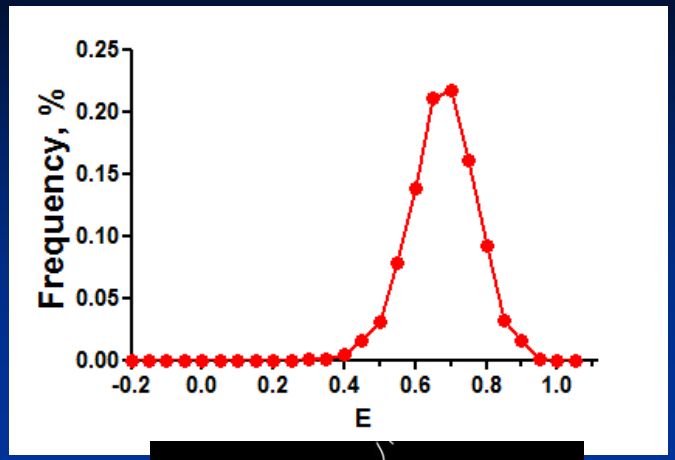


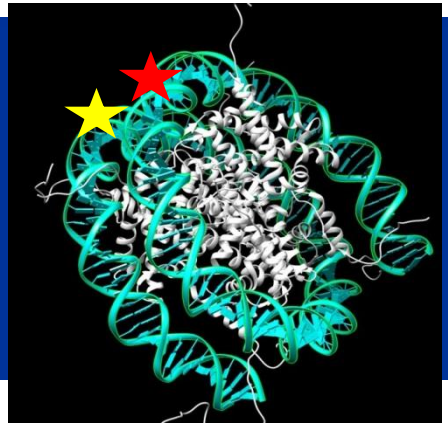
Time resolution is ca. 100 ms

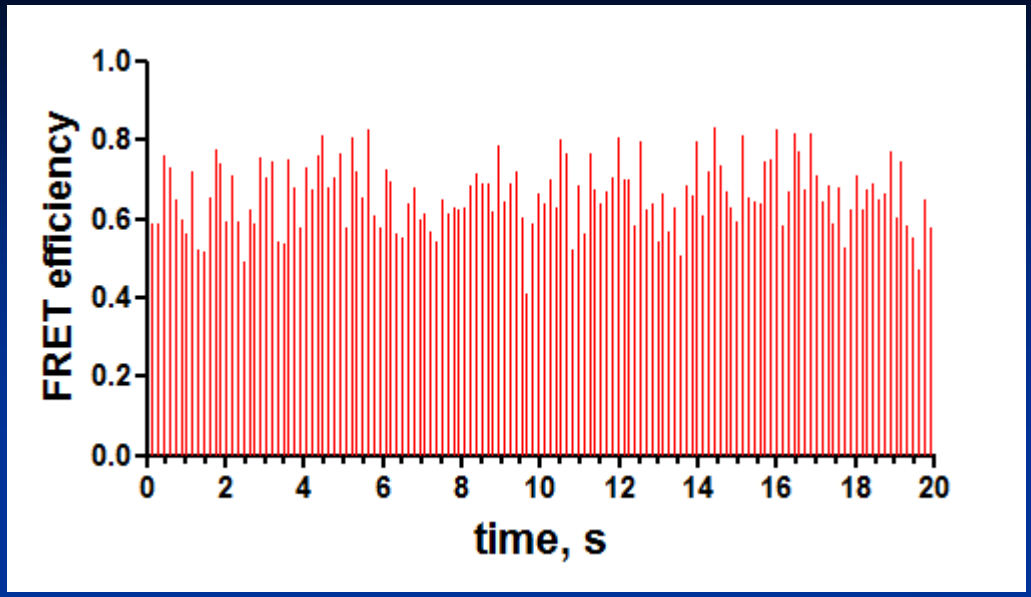



1
2
3
4
5
6
Nuc | I_{Cy3} | I_{Cy5} | E
1 | … | … | …
2 | … | … | …
3 | … | … | …
4 | … | … | …
5 | … | … | …
6 | … | … | …
Frequency, %
FRET, %
FRET efficiency
time, s

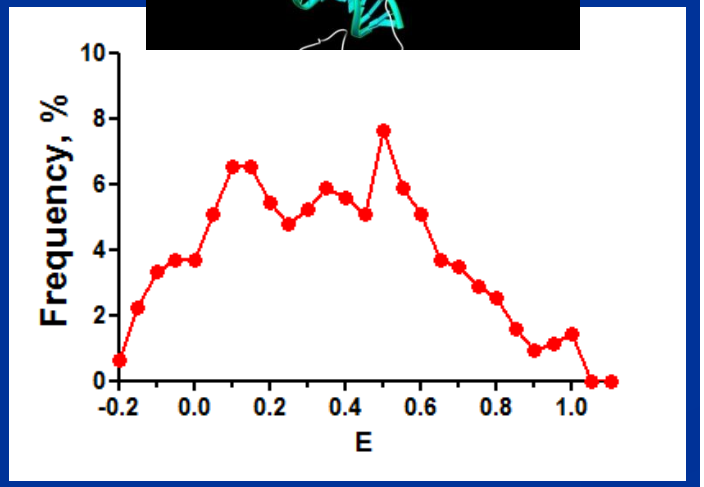
FRET kinetics of an immobilized nucleosome with 140 ms step

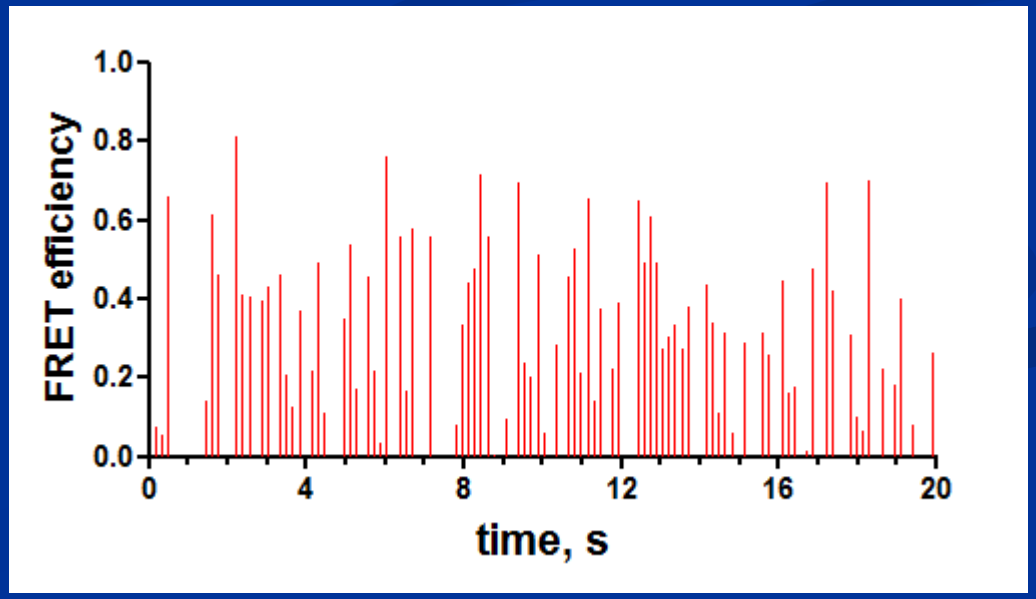






FRET kinetics of an immobilized elongation complex with 140 ms step





Tasks
that can be solved with immobilized nucleosomes

Structure in dynamics (DNA “breathing”)

Lifetime of conformational states

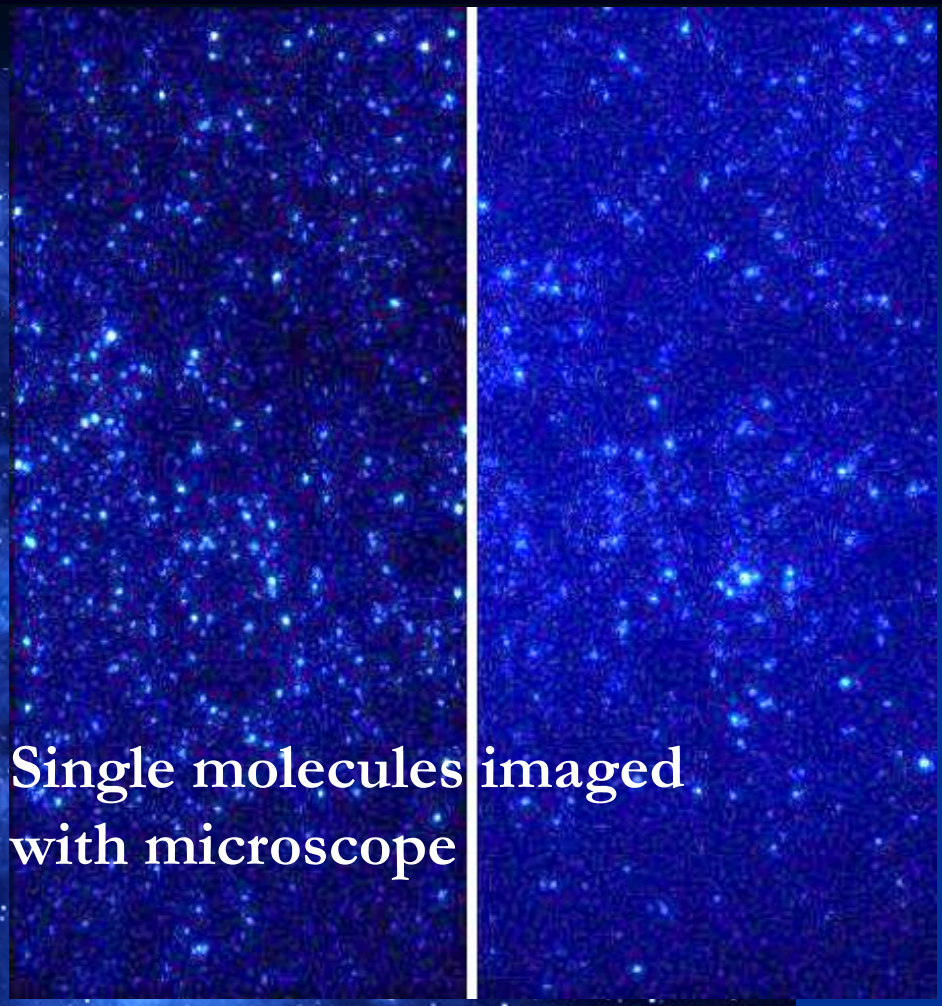
Kinetics of complex formation and dissociation (dissociation constant)

Titration of complexes (dissociation constant)

Formation of an extended set of stalled elongation complexes with RNAP

Transcription in kinetics

Thank you for attention !



Single molecules imaged with microscope

Stars imaged with Hubble

Photo: Getty images