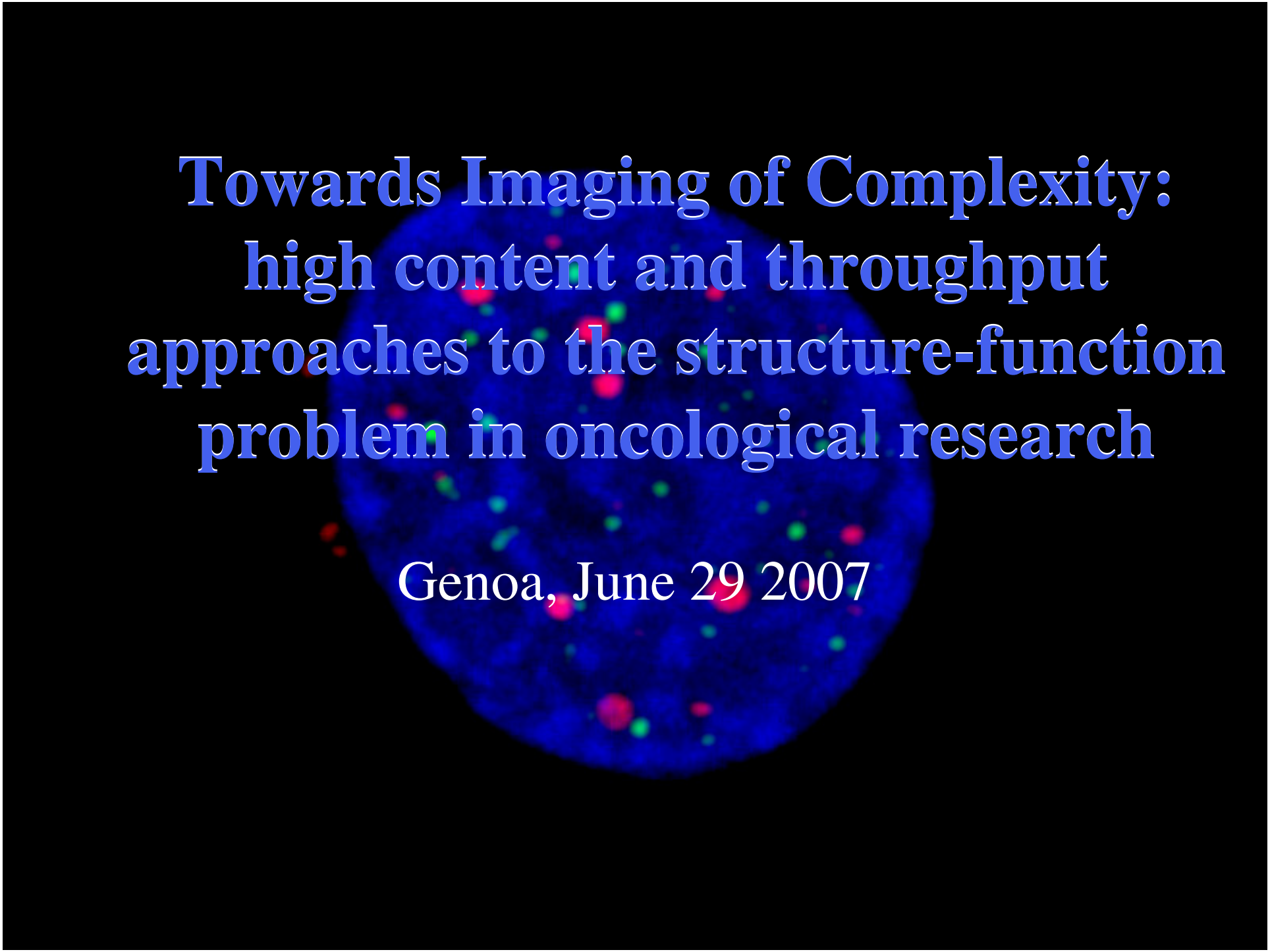


Towards Imaging of Complexity: high content and throughput approaches to the structure-function problem in oncological research

Genoa, June 29 2007

The "OMICS" era…

- The post-genomic era is signed by the efforts to identify and functionally characterize the products of the genes screened by the human genome sequencing (genomics).
- The goal is to design a map of the biochemical network regulating cell physiology and to understand how gene mutations can alter this equilibrium in disease
- Technological development favored the birth of "proteomics" (large scale study of protein structure and function) and "cytomics" (study of the protein expression pattern in (living) cells and tissues) together with the evolution of system for high throughput and high content screening


The key question: what
does a gene (protein) do?

Why fluorescence microscopy?

- WHERE: subcellular localization of structures and bio-molecules.

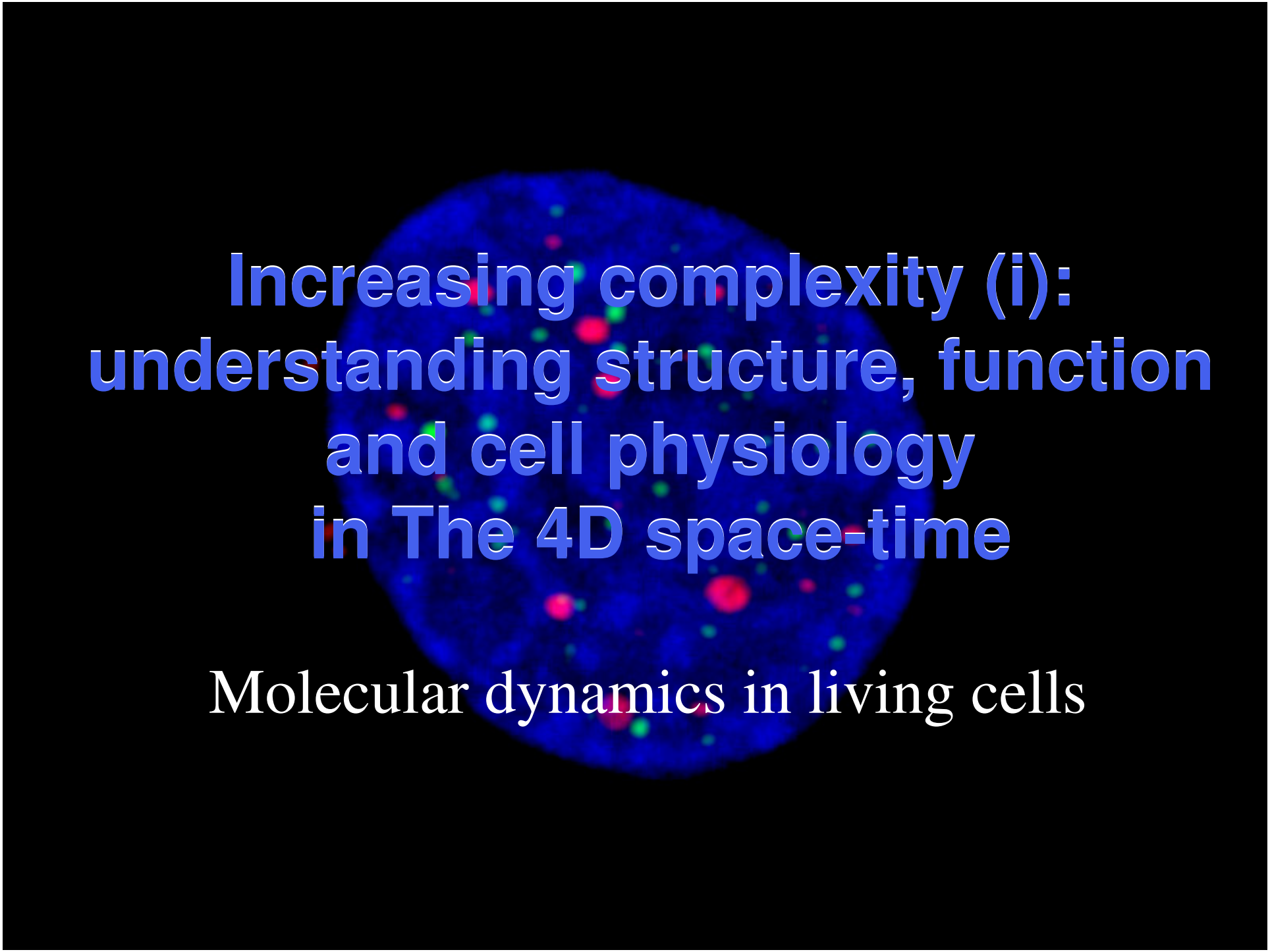
  Spatial compartmentilazion is a fundamental tool in regulation of cellular activities.

  3D: Non invasive 200 nm Resolution, 3D, high sensitivity
- WHEN: temporal definition of biologically relevant events.

  4D: Extremely high temporal resolution with 2D-3D imaging >video frame rate
- WHY: investigation of molecular function for design of biochemical networks.

  Dissection of molecular interactions allows modeling of basic cellular mechanisms

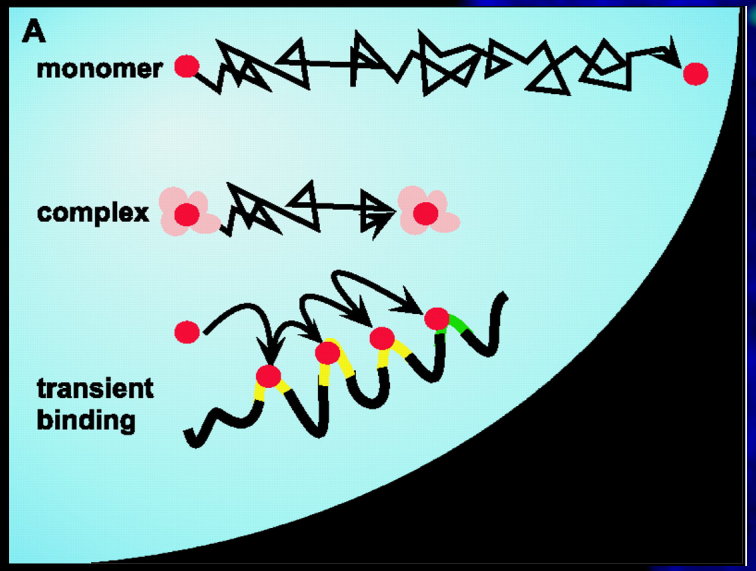
  5D: functional microscopy. “F” Techniques



Increasing complexity (i): understanding structure, function and cell physiology in The 4D space-time

Molecular dynamics in living cells

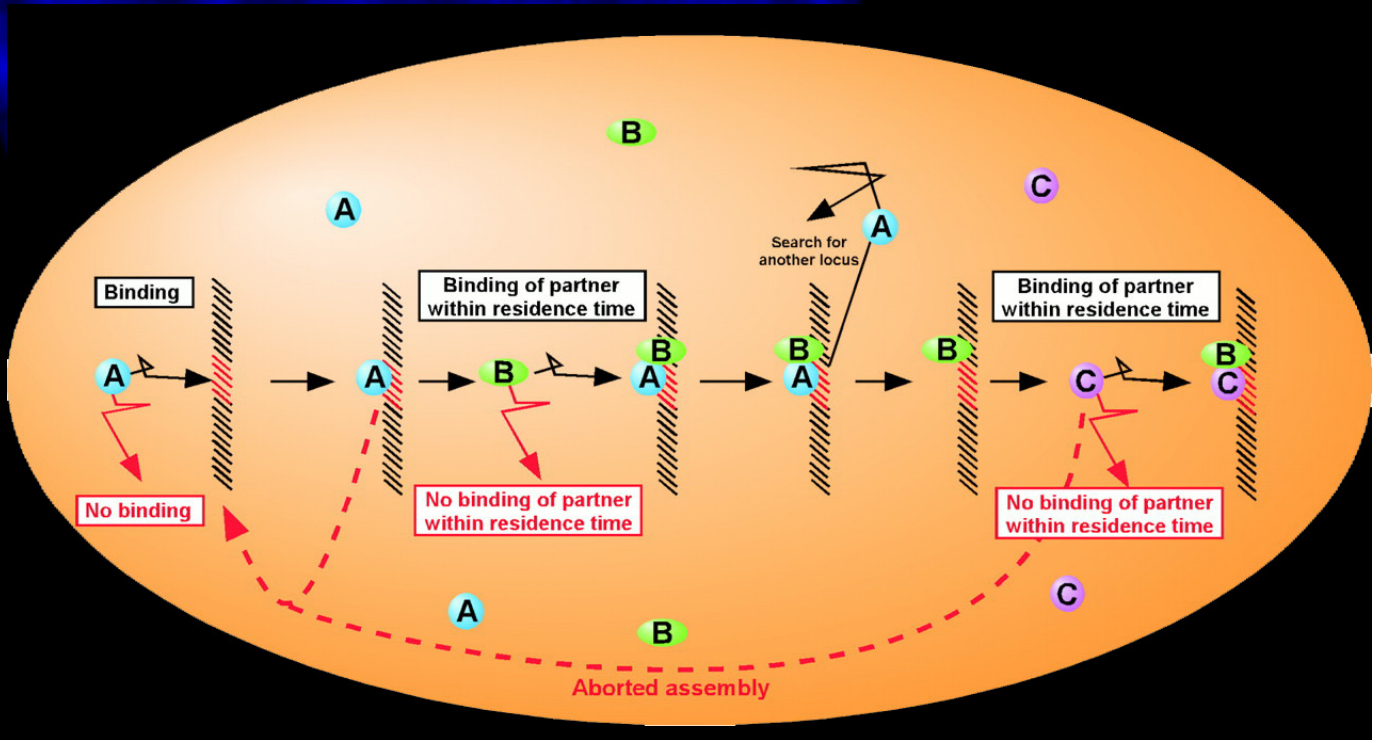
Regulating function: “Eppur si muove”



- Functional regulation requires continuous local recruitment of factors
- Protein diffusion coefficients were measured in the cell nucleus: roaming of the whole space by small molecules requires few seconds
- No energy requirements
- Diffusion coefficients are determined by size (aggregation), viscosity and charge

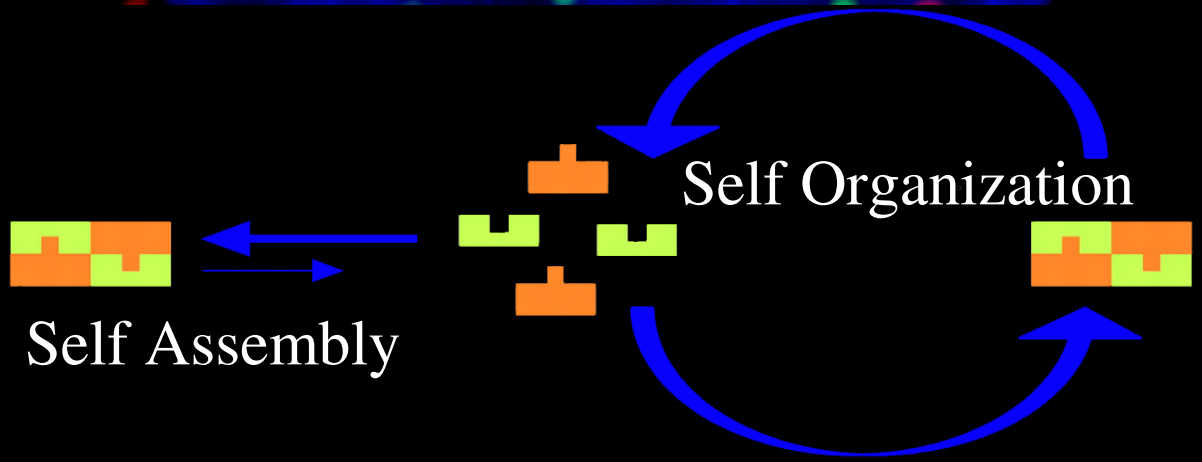
Could you please stop here?

- Local interactions determines a mean trapping (residence) time in each “compartment” (Continuos Time Random Walk)
- Function regulation accomplished by modification of molecular interactions: affinity modifications to create protein domains

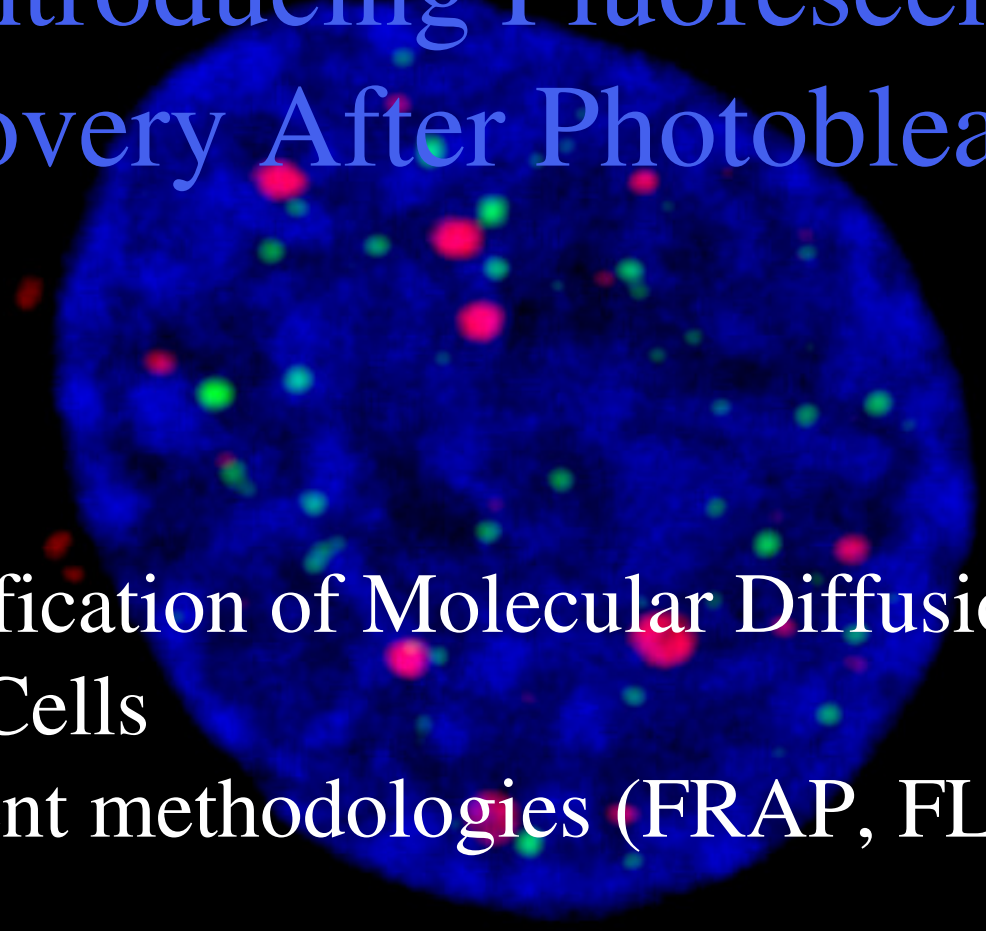


Assembling or organizing: putting the bricks together…

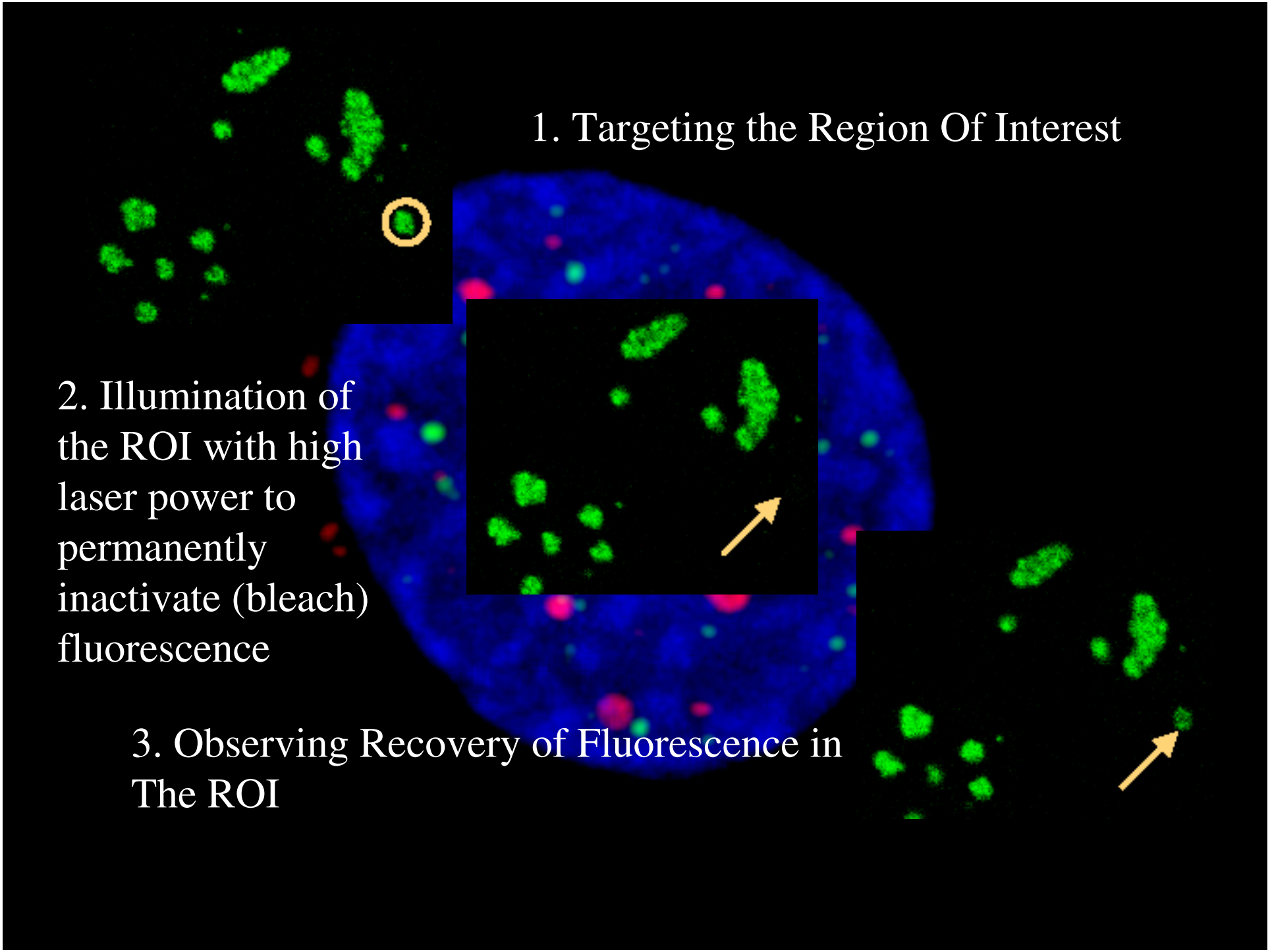
- Self organization allows for highly dynamic control of structures

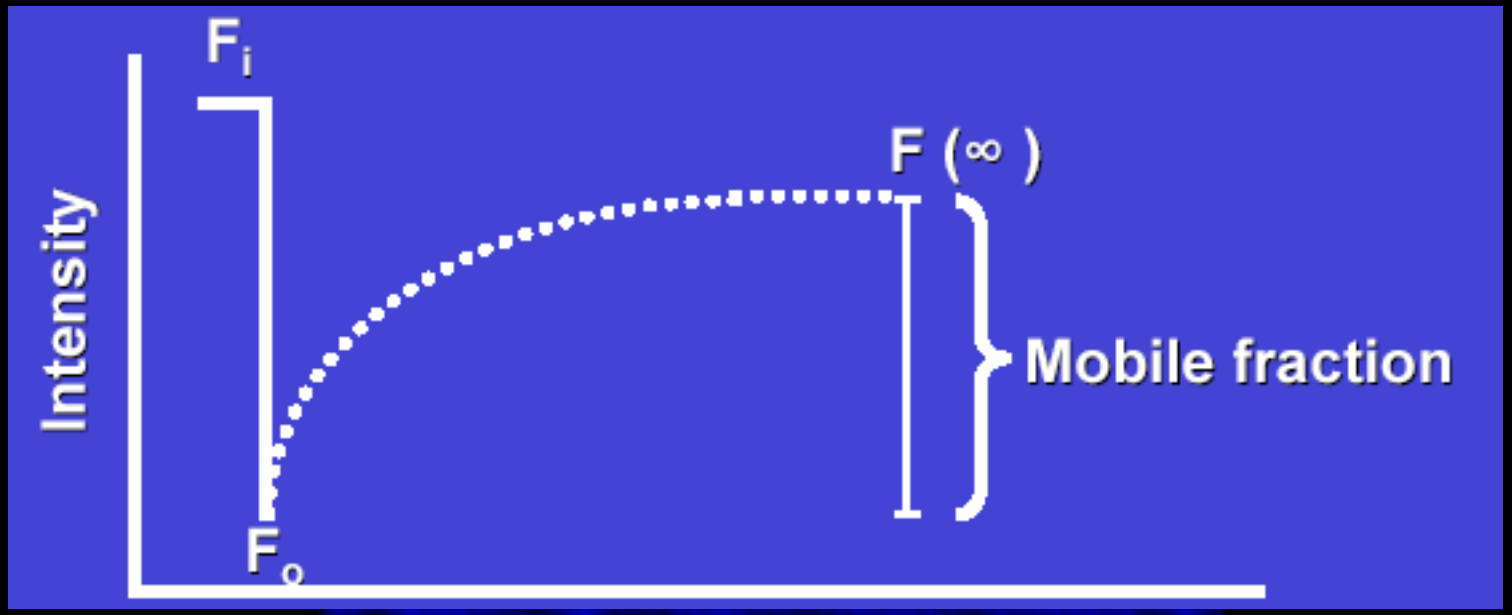


Introducing Fluorescence Recovery After Photobleaching



- Quantification of Molecular Diffusion in Living Cells
- Different methodologies (FRAP, FLIP)


1. Targeting the Region Of Interest
2. Illumination of the ROI with high laser power to permanently inactivate (bleach) fluorescence
3. Observing Recovery of Fluorescence in The ROI



Recovery of fluorescence is only due to the replacement of bleached molecules with fluorescent ones

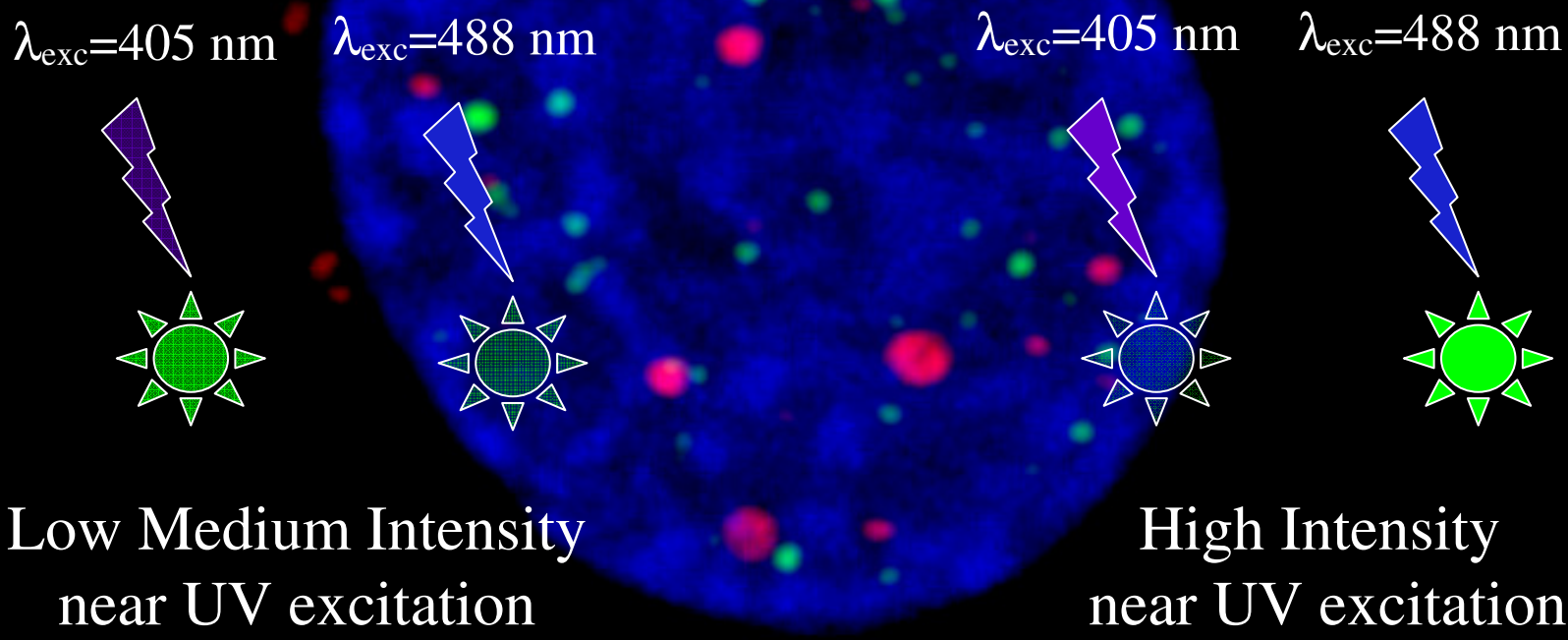
Recovery speed is proportional to diffusion coefficient of the tagged molecular species

Recovery curve:

$$F(t) = F_{\infty}\left(1 - e^{-\frac{t}{\tau}}\right)$$

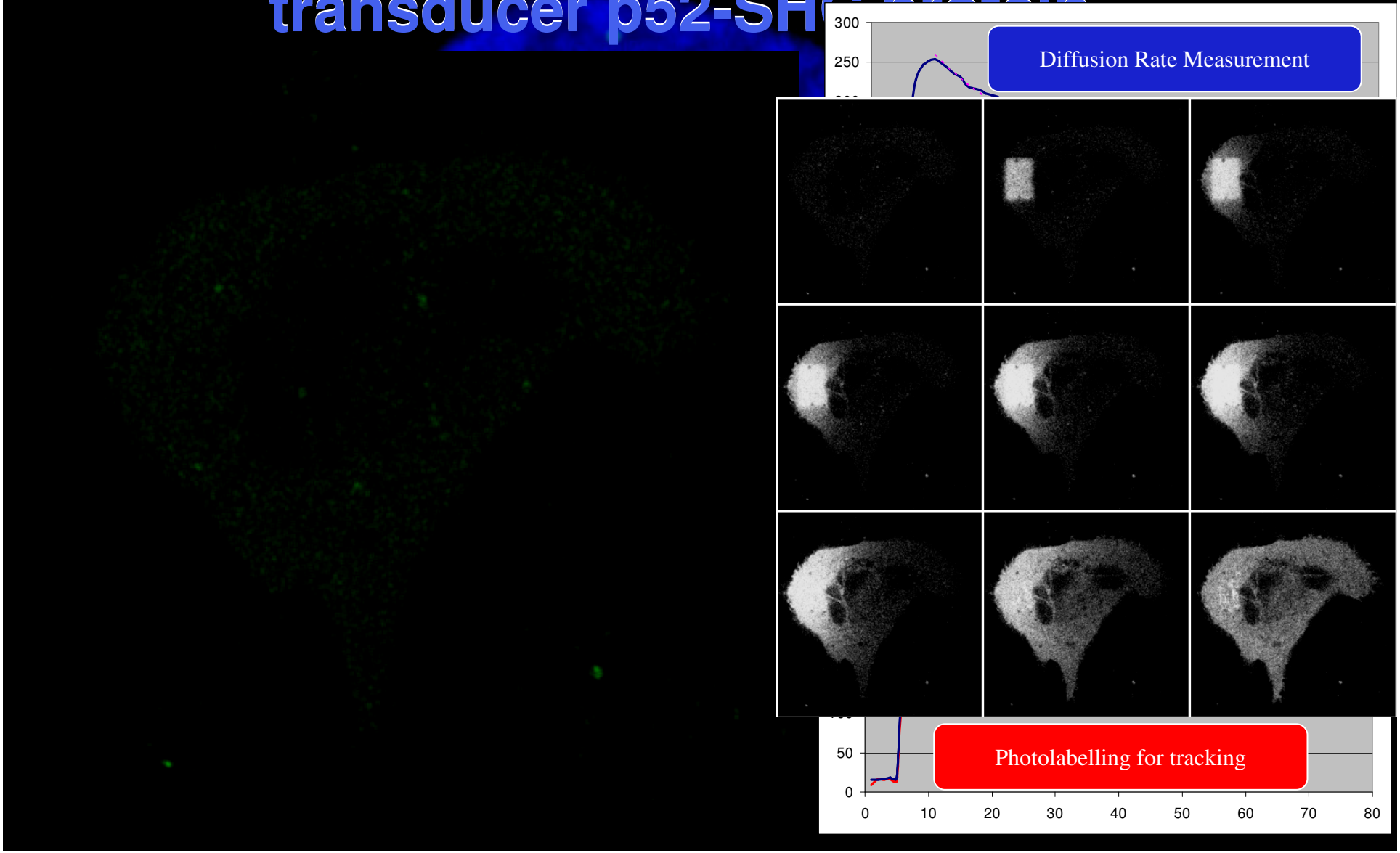
The 4D space-time: phototracking

- paGFP (GFP point mutant) changes photophysical properties according to illumination wavelength

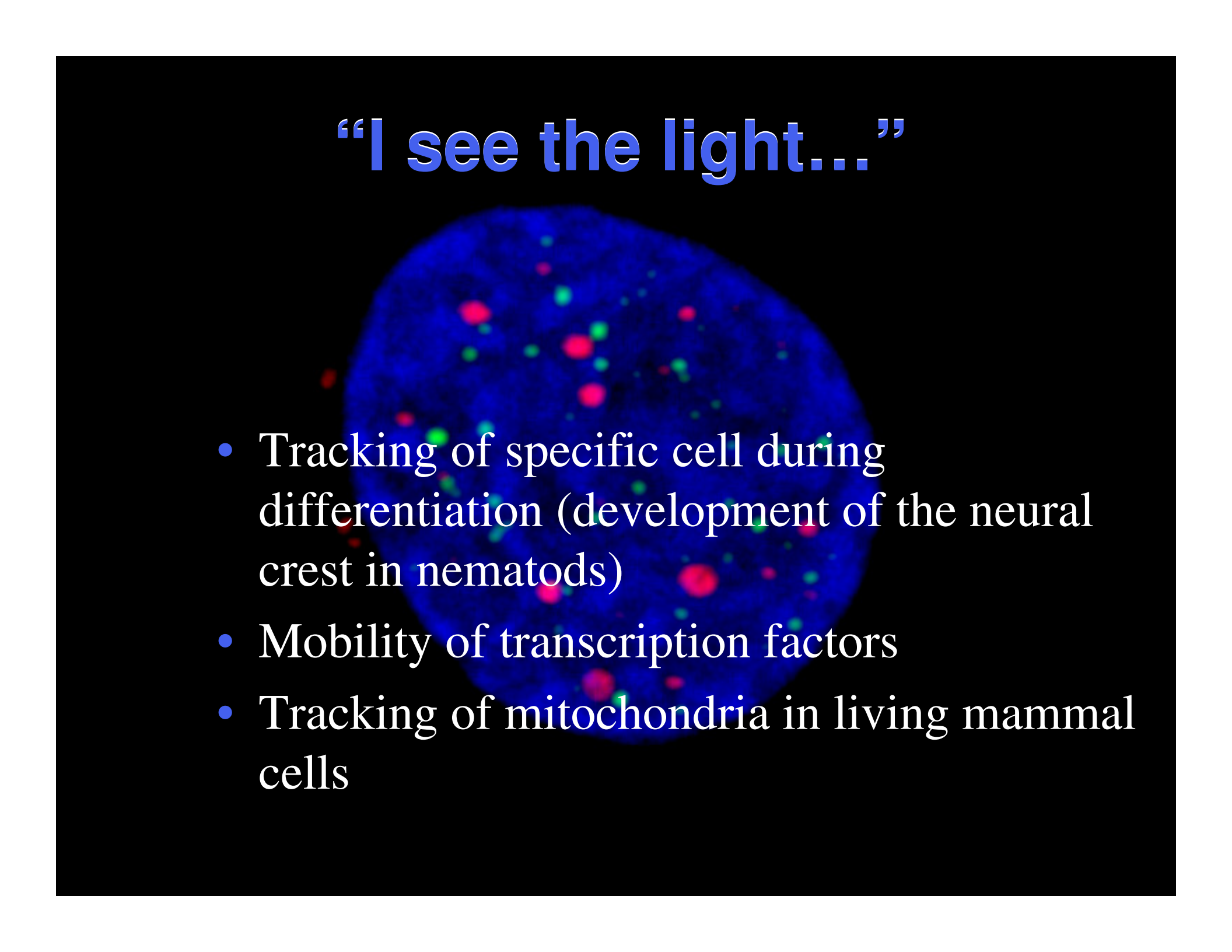


Up to 100 fold increase in fluorescence excited by λ_{exc}=488 nm

Photoactivation for diffusion measurements and tracking of signal transducer p52-SHC protein



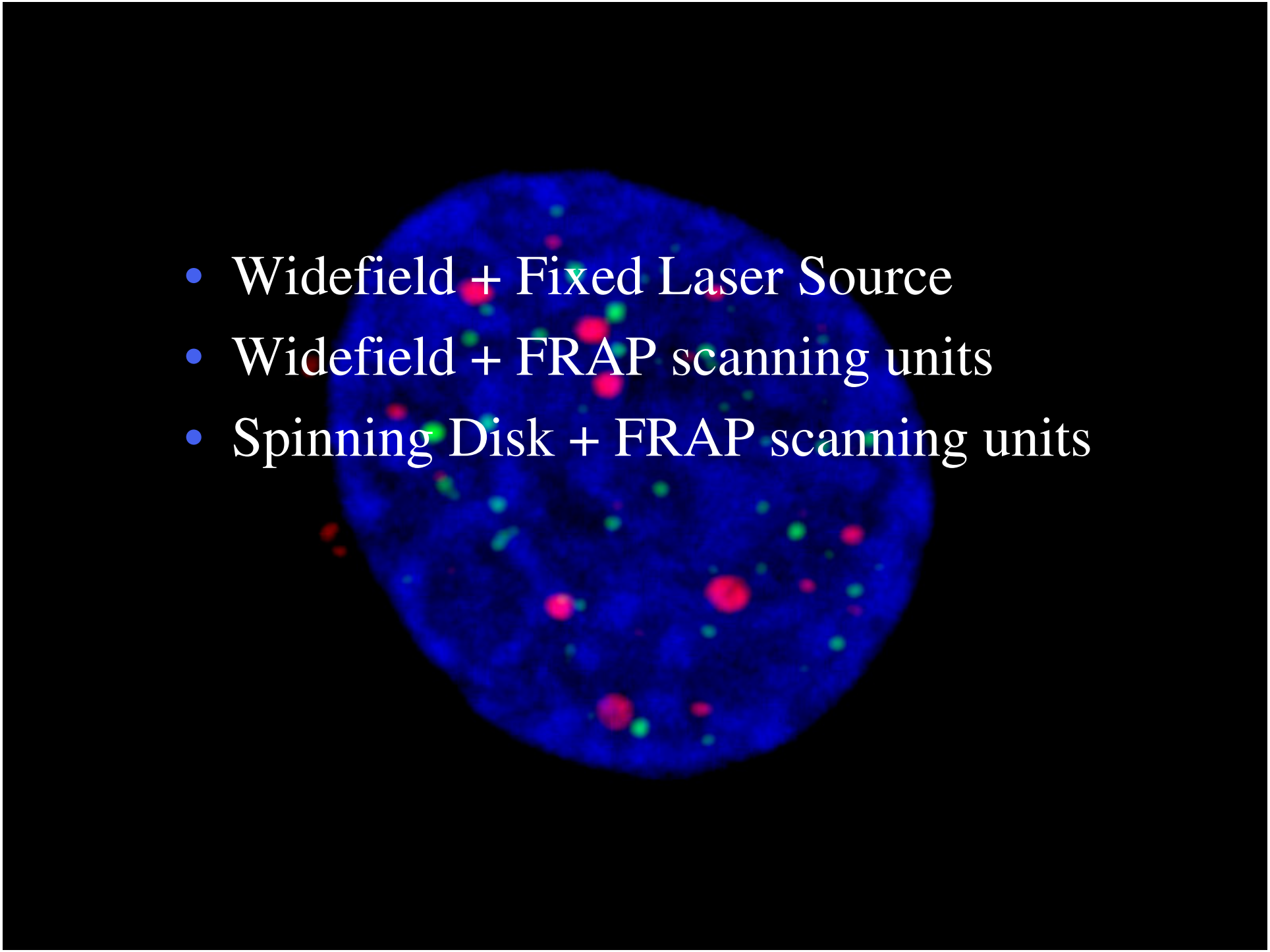
“I see the light…”

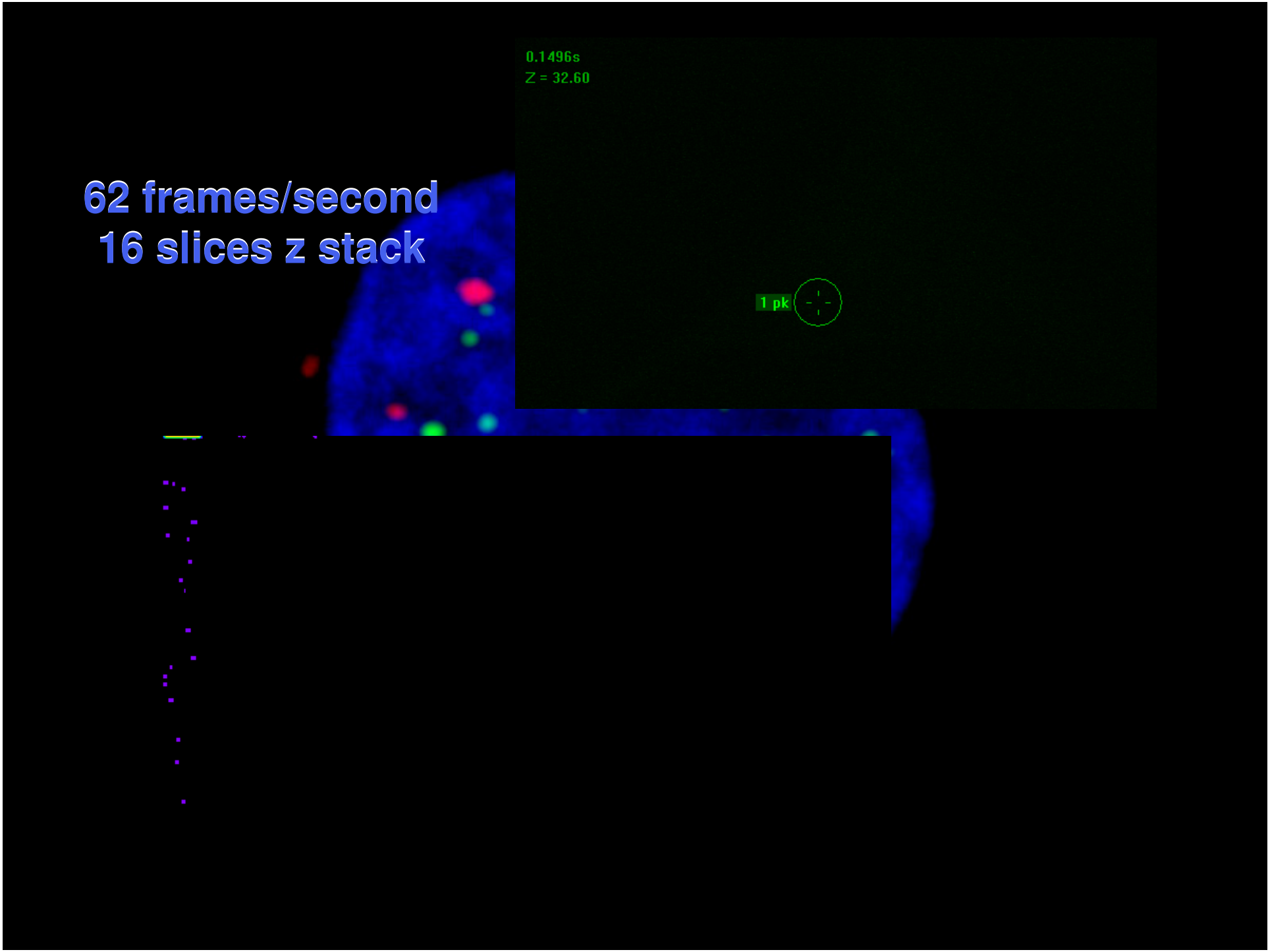


- Tracking of specific cell during differentiation (development of the neural crest in nematods)
- Mobility of transcription factors
- Tracking of mitochondria in living mammal cells

Fast and Furious: FRAP beyond Laser Scanning Confocal Microscoopy

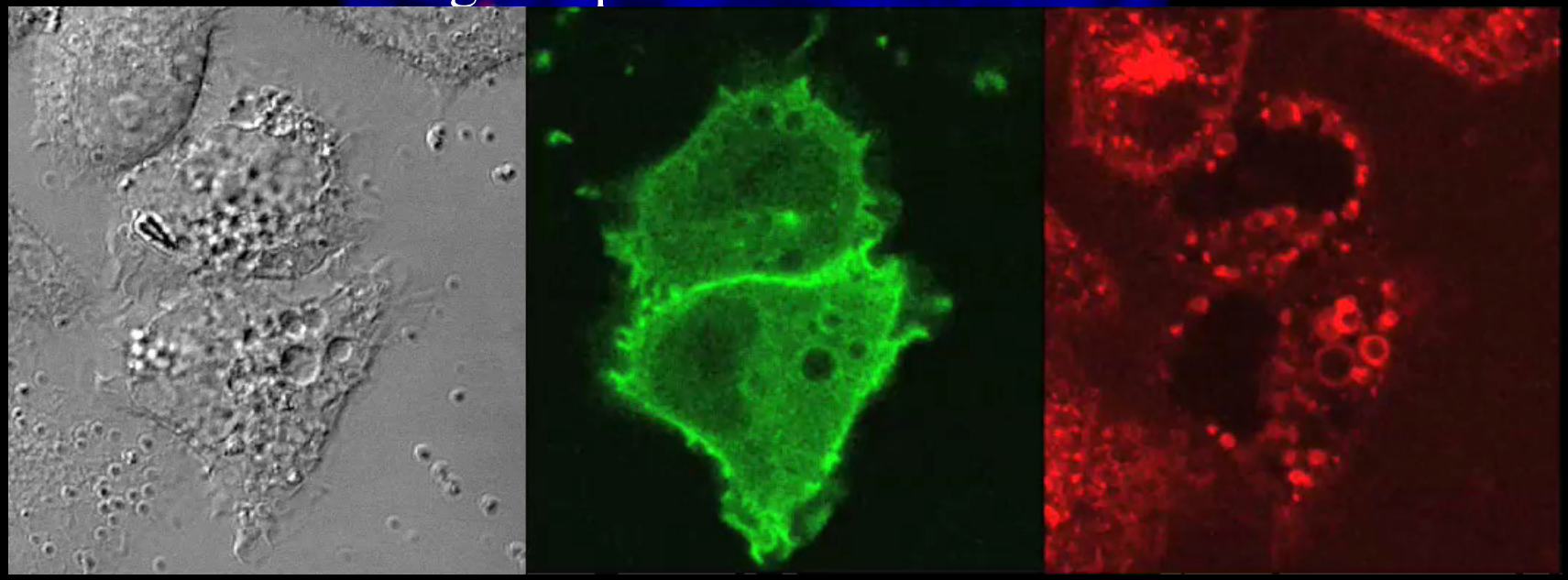
- LSCM allows for precise xy control of bleached regions but suffers of:
  - Low temporal resolution (except for resonant scanning systems)
  - Reduced sensitivity of PMTs and of the collection scheme with respect to CCD based detection systems


• Widefield + Fixed Laser Source
• Widefield + FRAP scanning units
• Spinning Disk + FRAP scanning units


62 frames/second
16 slices z stack
0.1496s
Z = 32.60
1 pk

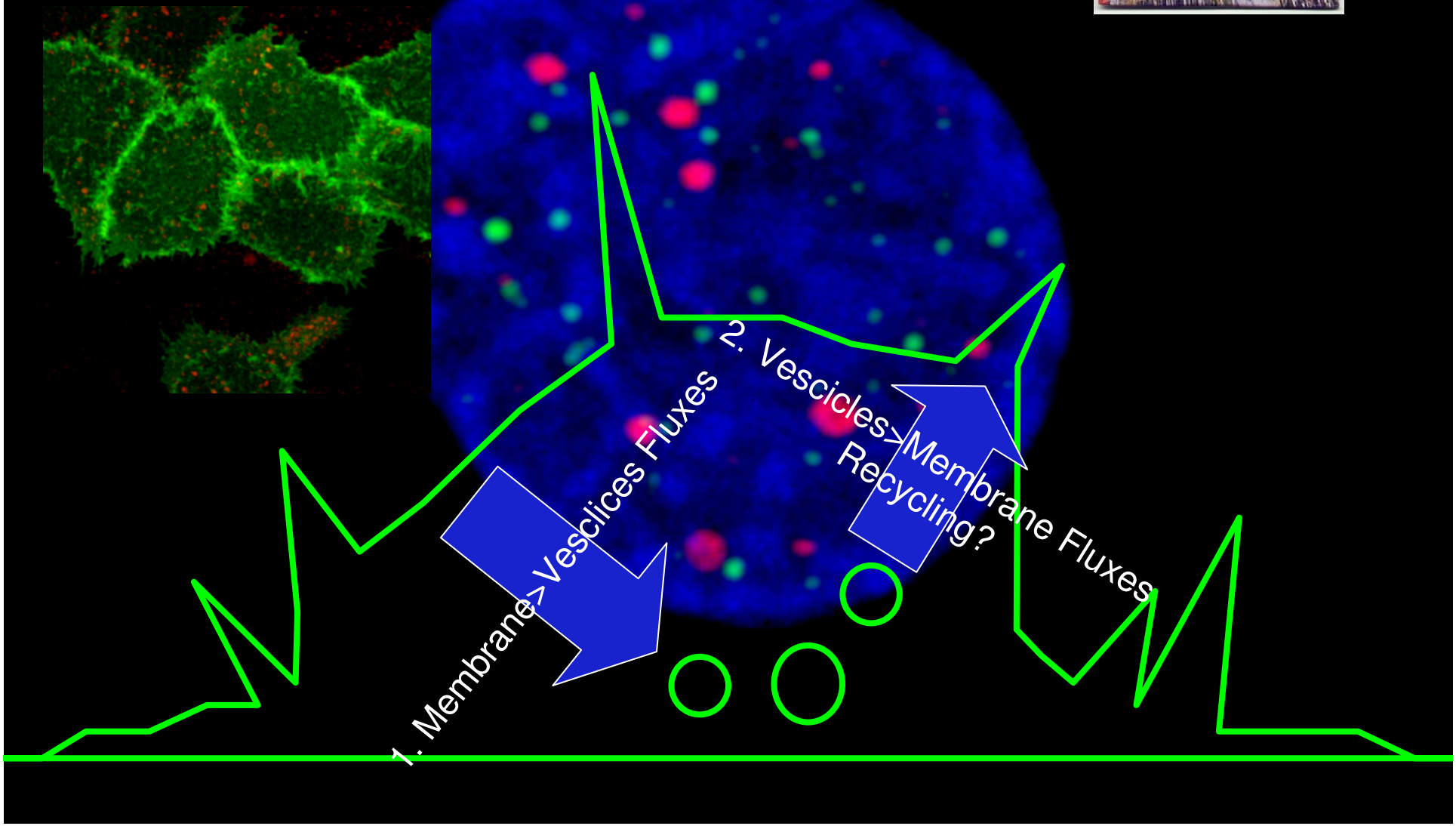
Linking actin cytoskeleton remodelling and endocytosis:

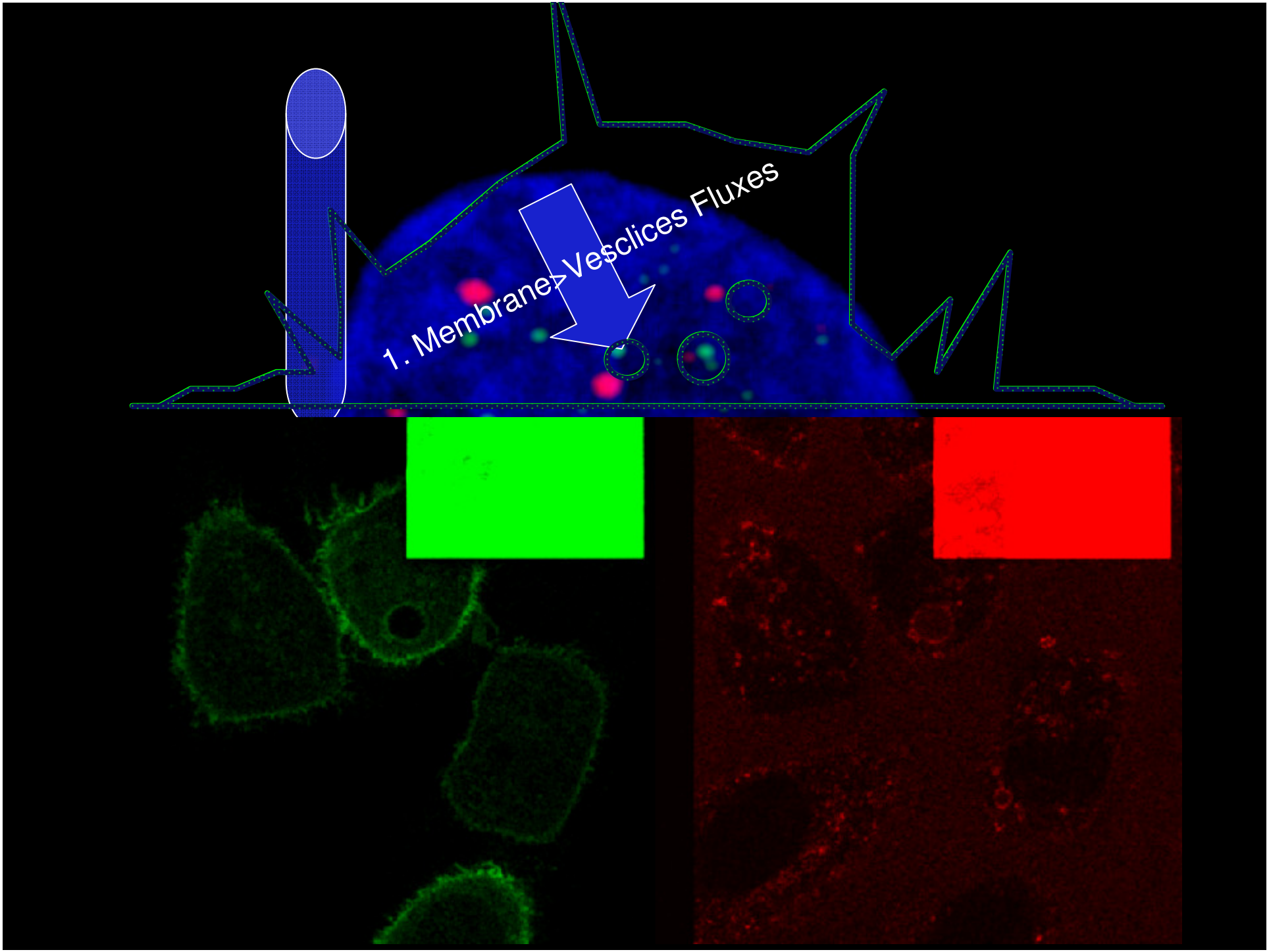
- Overexpression of specific endocytic messengers induces enhanced vescicle formation accompained by actin remodelling activity (ruffling) upon stimulation
- Vescicles accumulates both endocytic markers and actin remodelling complexes

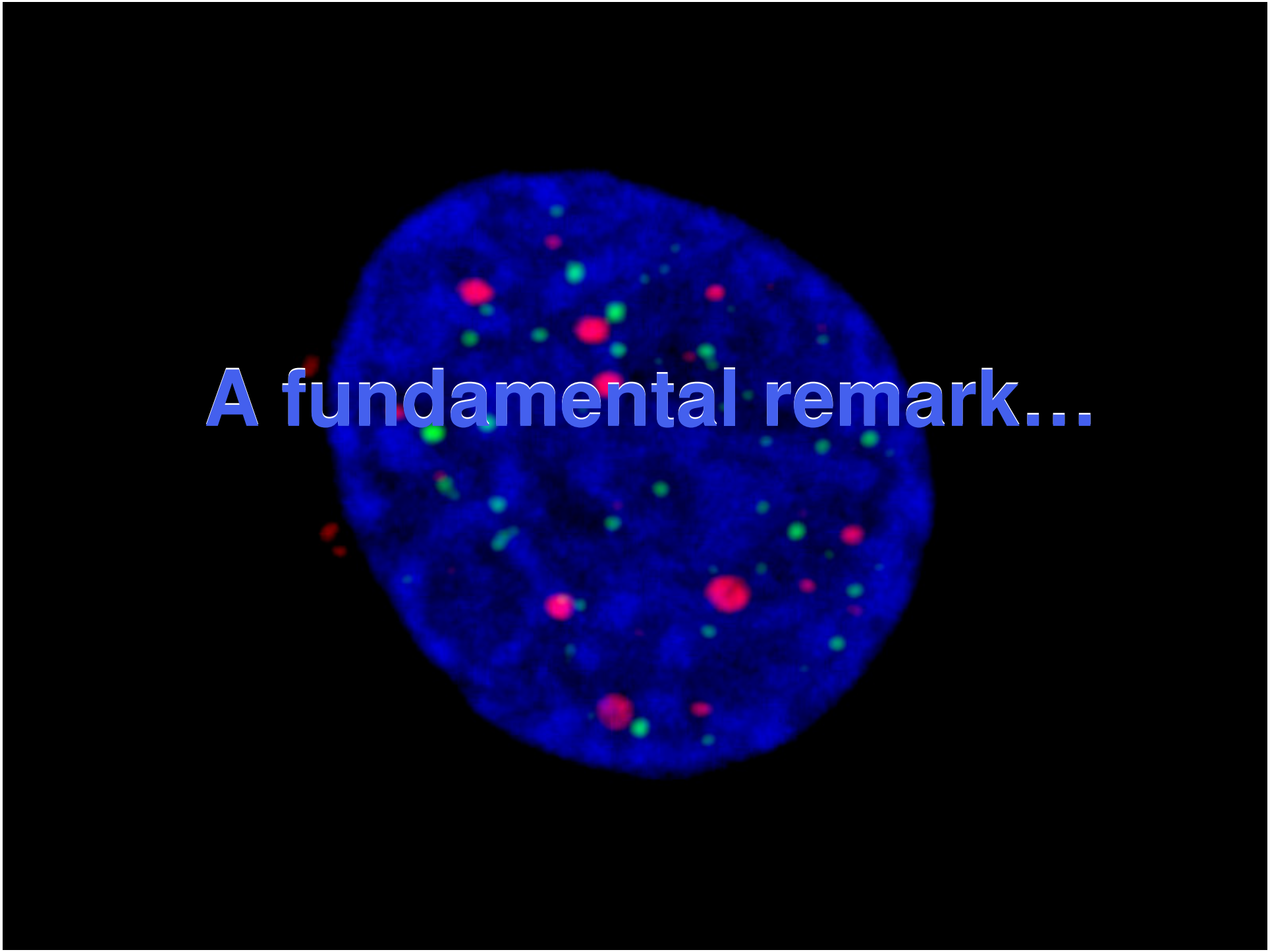


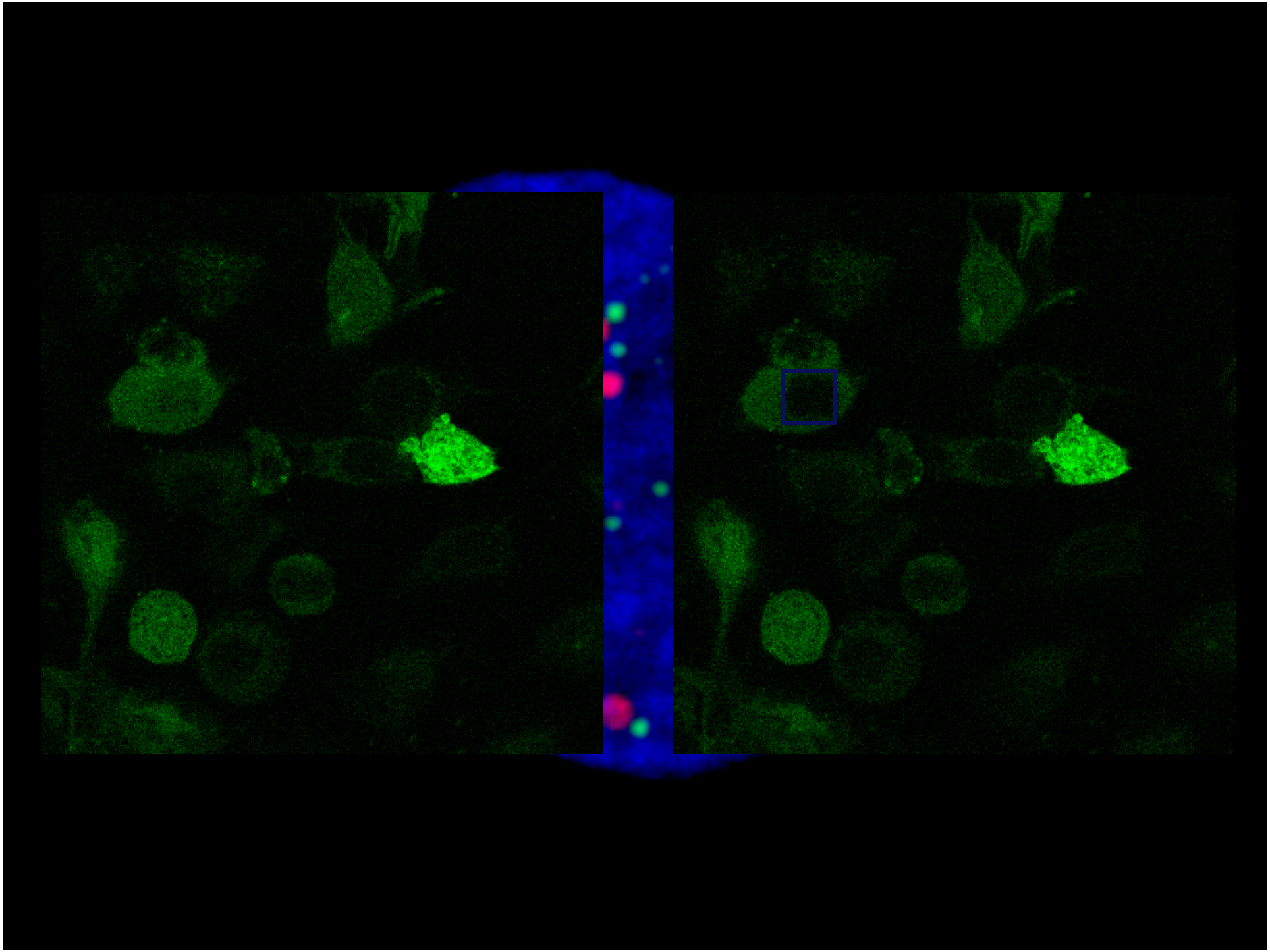
In collaboration with Dr A. Palamidessi, Dr G. Scita and Prof. P Di Fiore

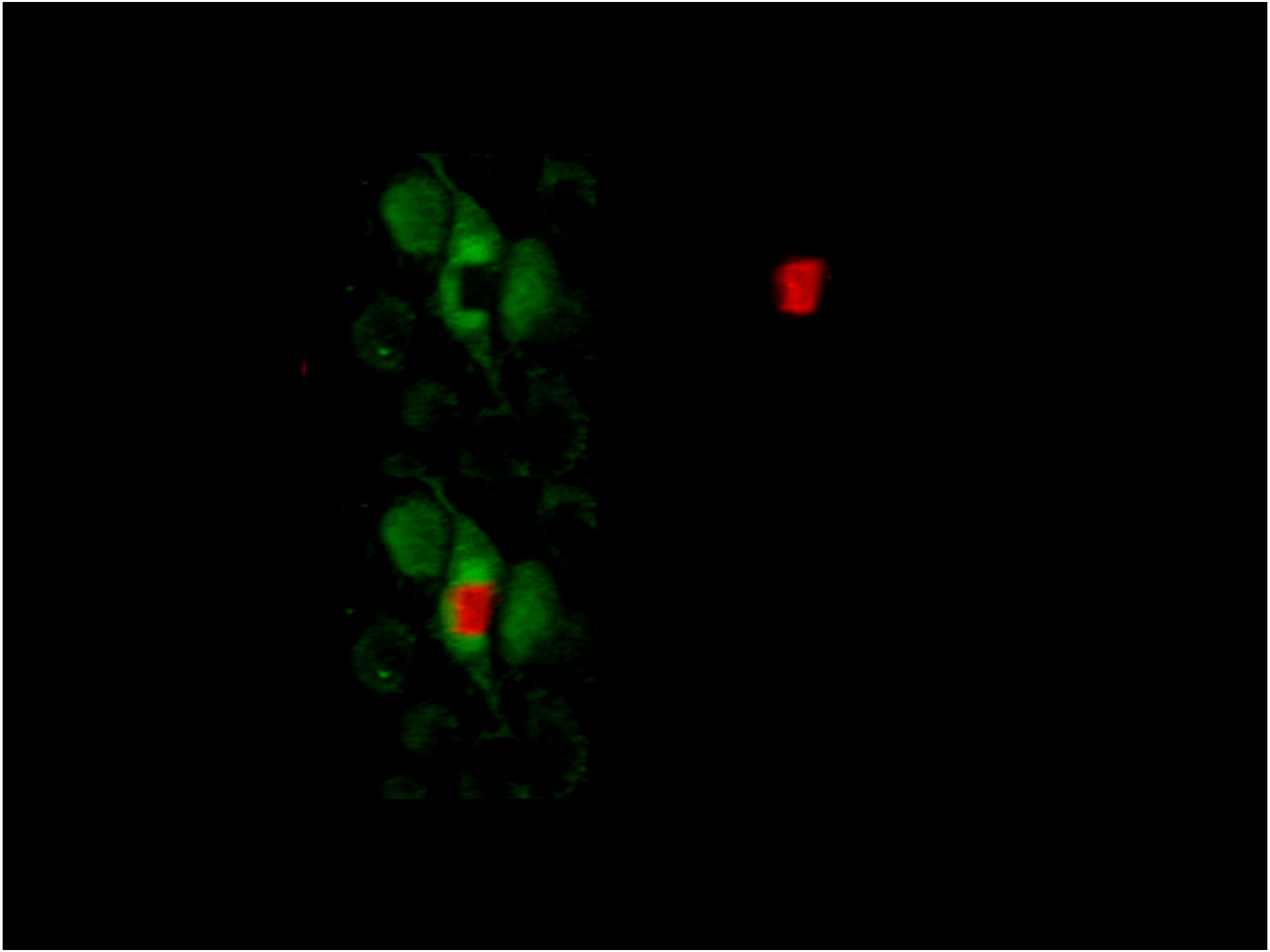
Come talk to me (Peter Gabriel 1993): Characterizing molecular fluxes between cell compartments

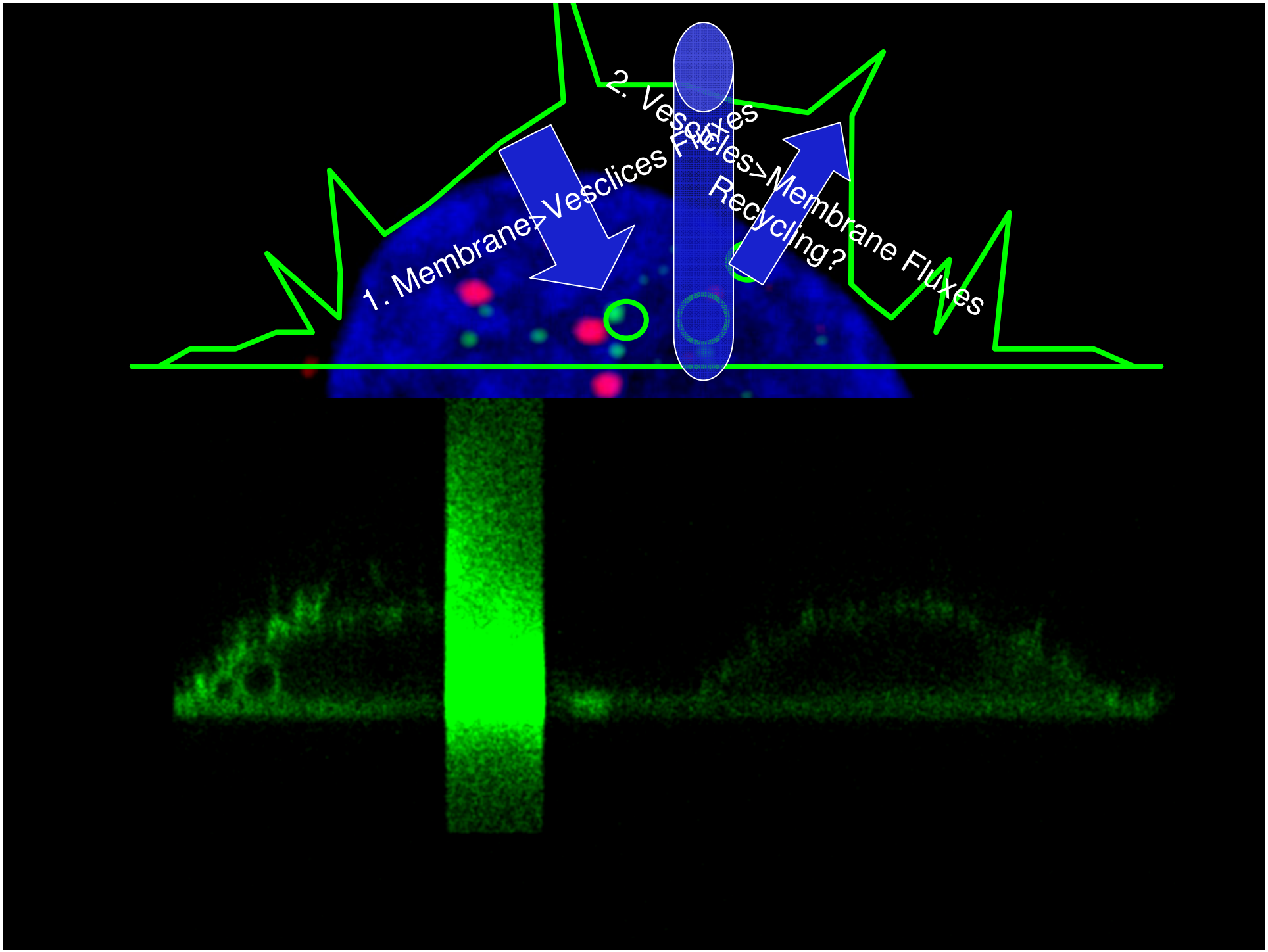



1. Membrane> Vesclices Fluxes

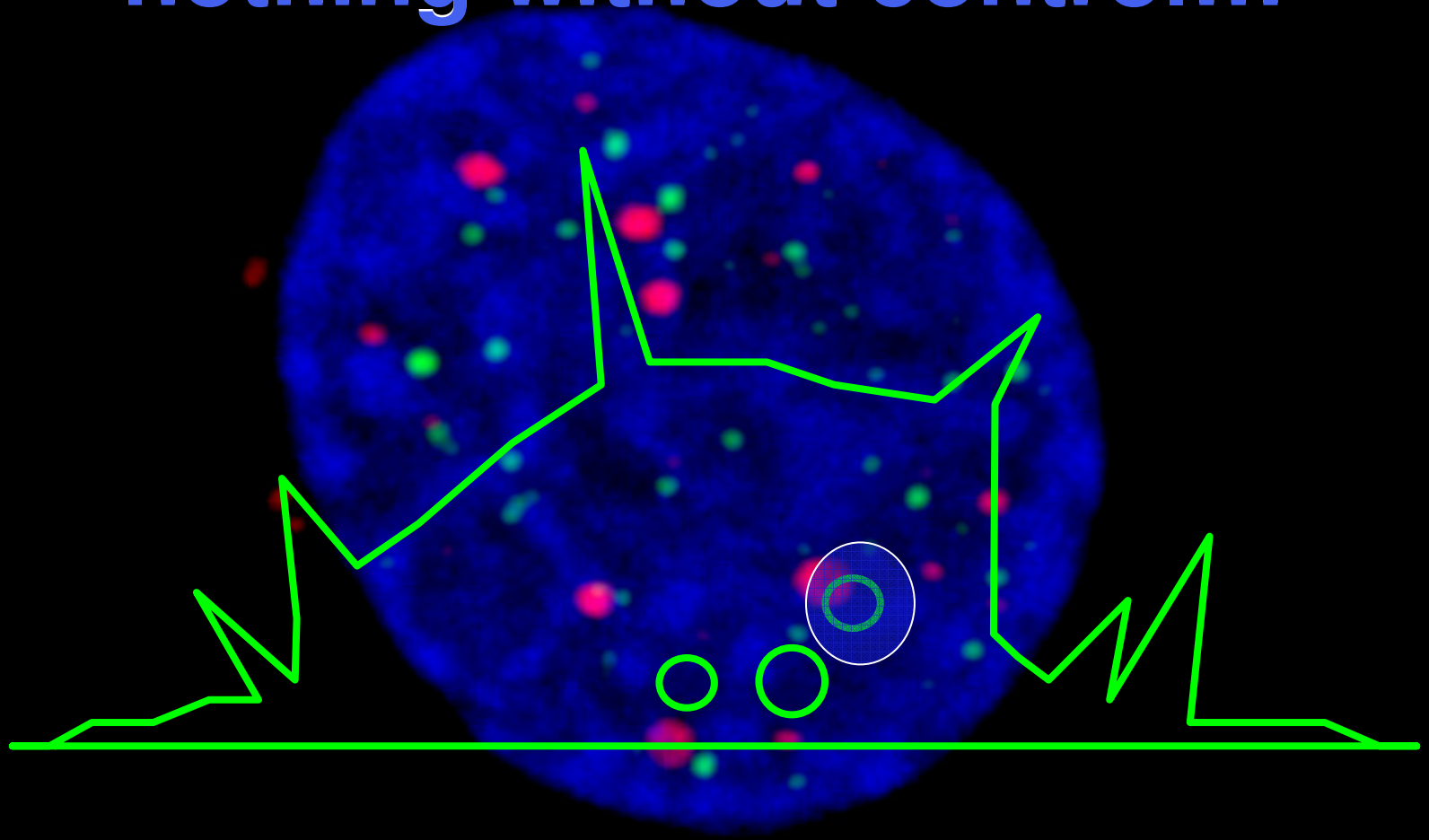

A fundamental remark…

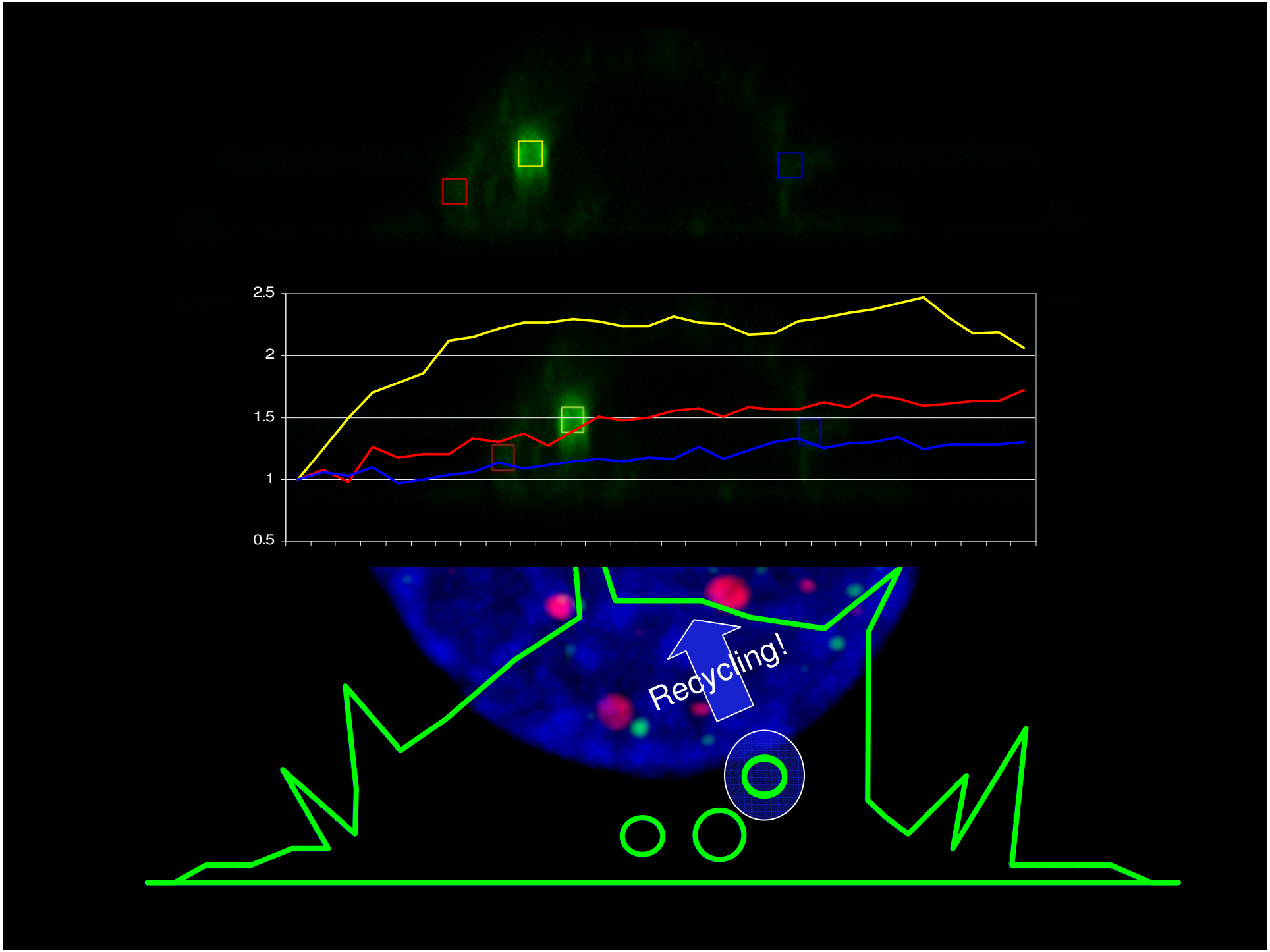





1. Membrane>Vesclices Fluxes
2. Vesclices>Membrane Fluxes
Recycling?

Two is better than one: power is nothing without control…




2.5
2
1.5
1
0.5
Recycling!

Total Internal Reflection Fluorescence Microscopy (TIRFM)

- Fluorescence excitation within a very thin layer
- High signal to noise ratio
- Single molecule detection

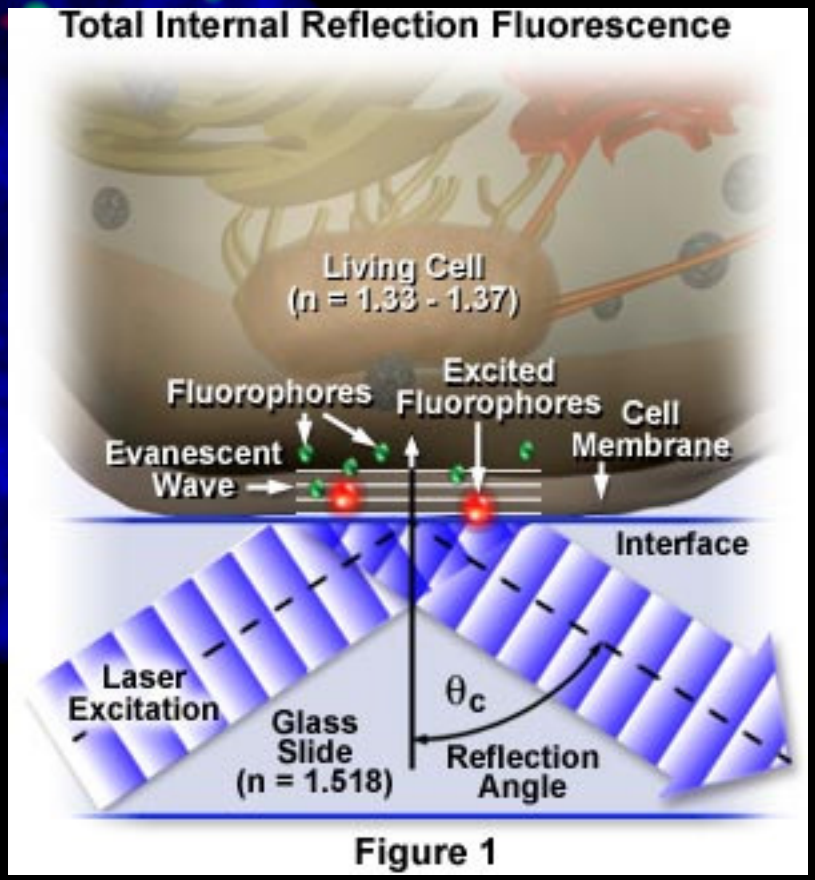
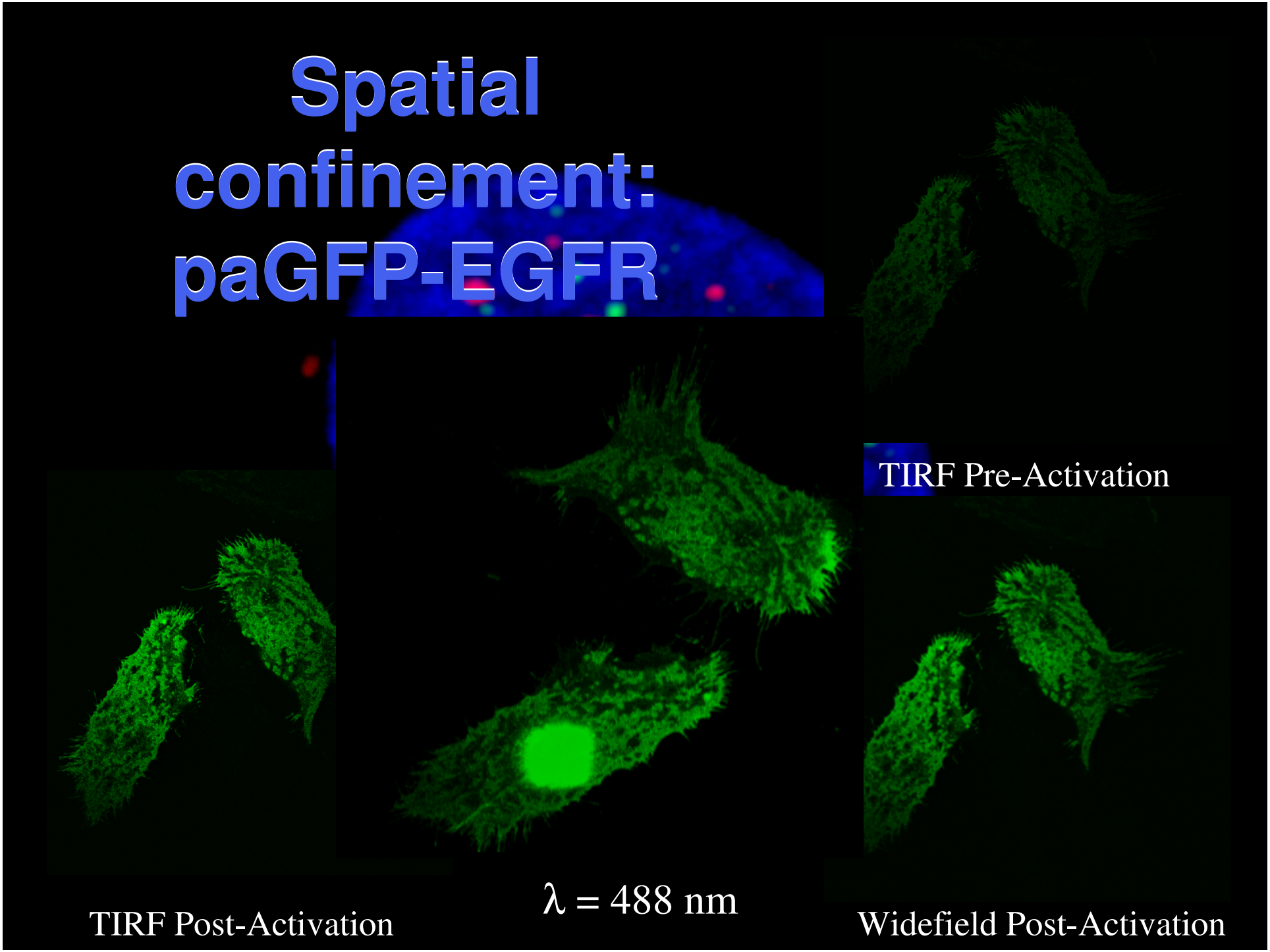
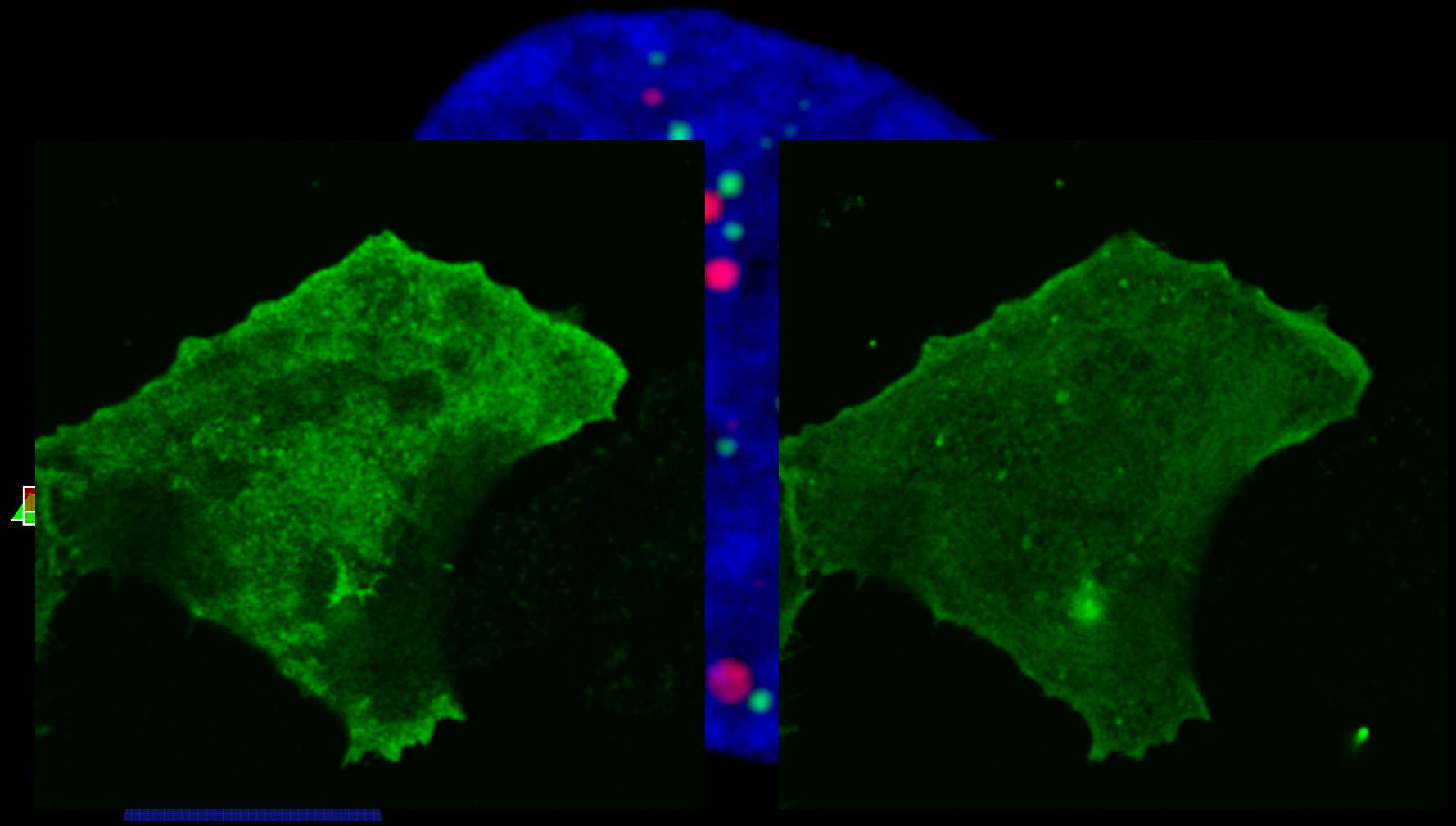


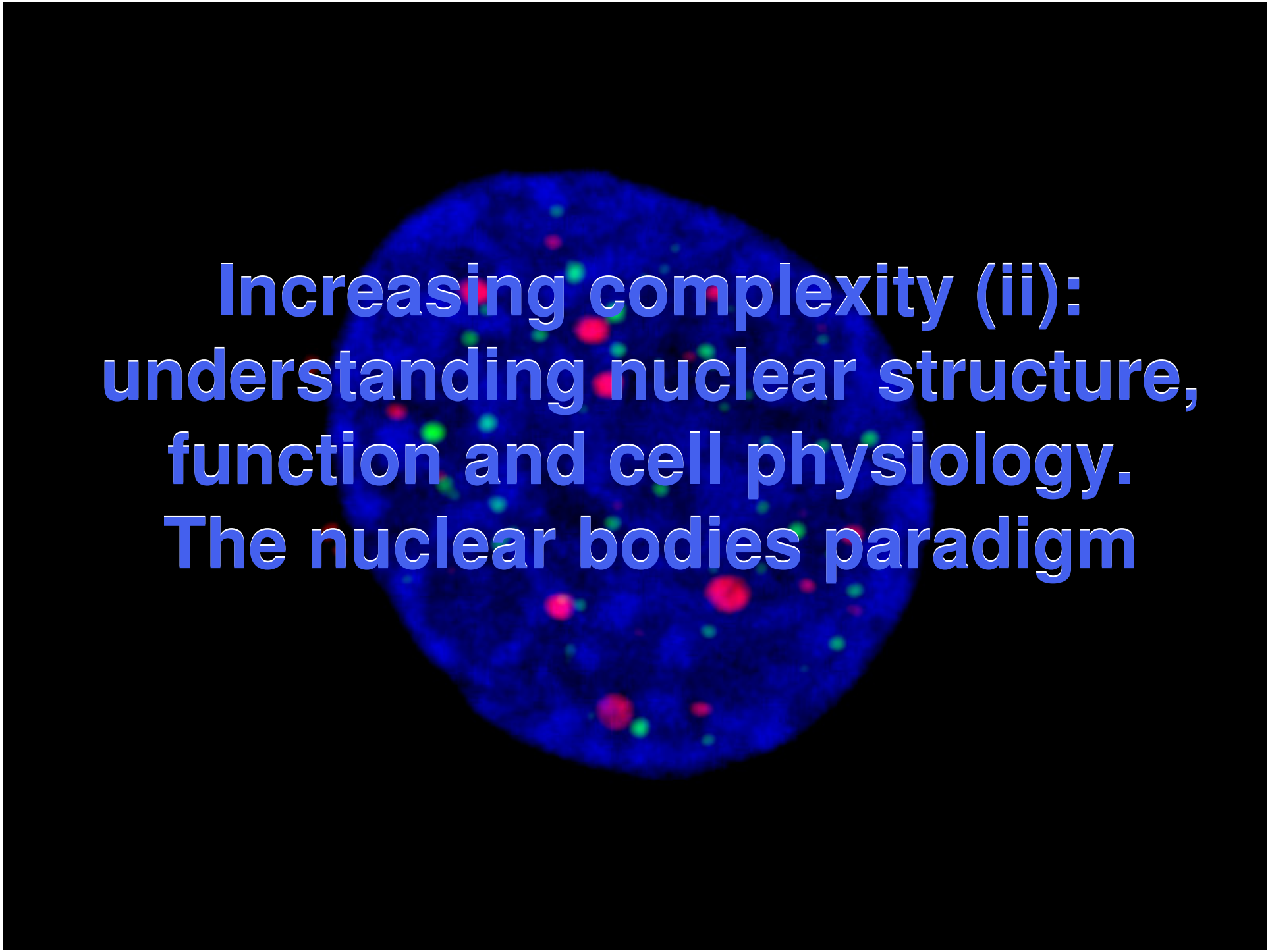
Figure 1

Spatial confinement: paGFP-EGFR



Walking around resolution…

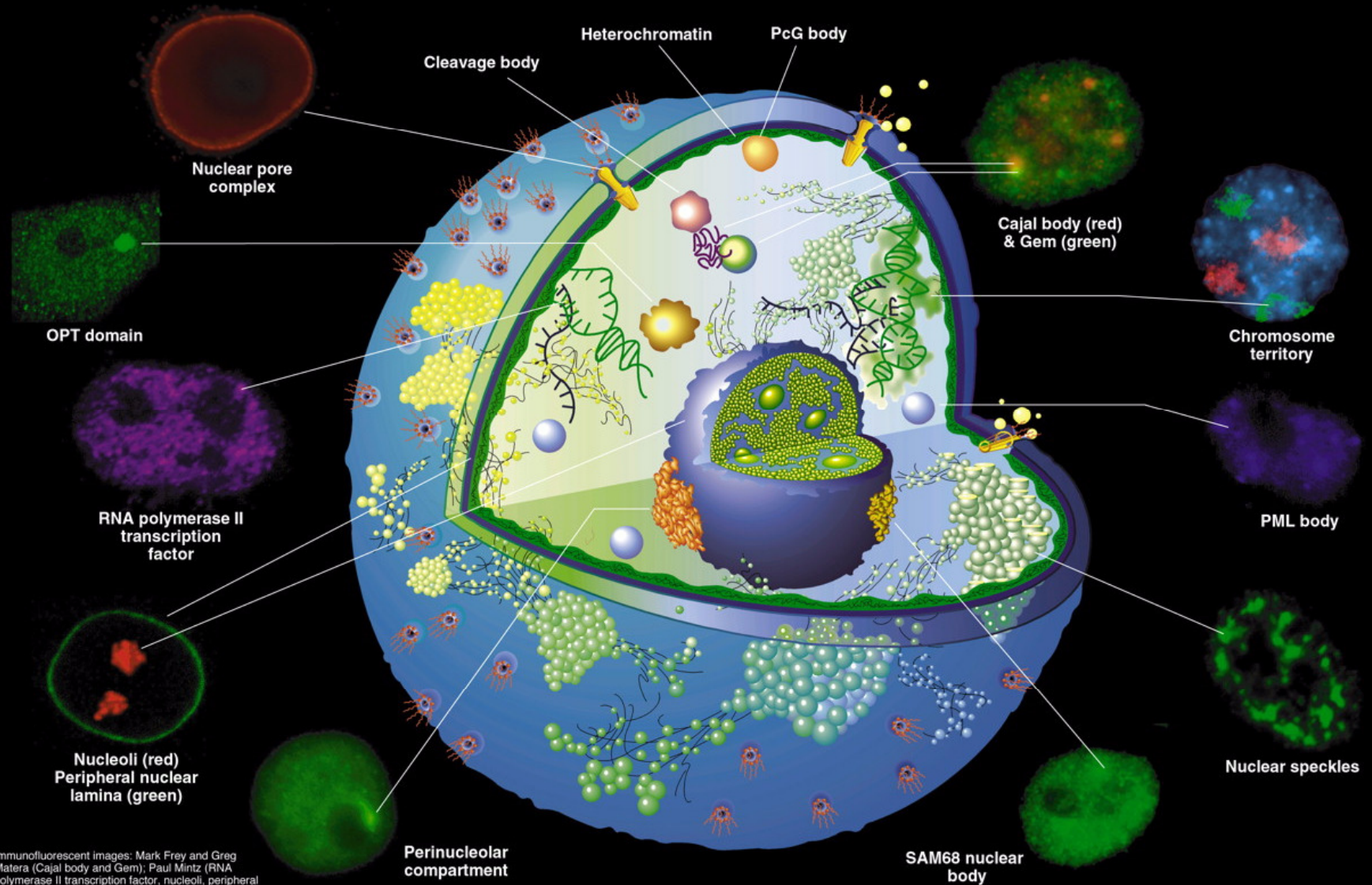



Increasing complexity (ii):
understanding nuclear structure,
function and cell physiology.
The nuclear bodies paradigm

Journal of
Cell Science

Nuclear Domains

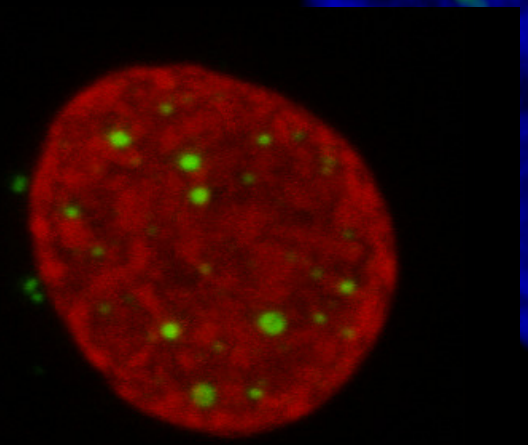
David L. Spector



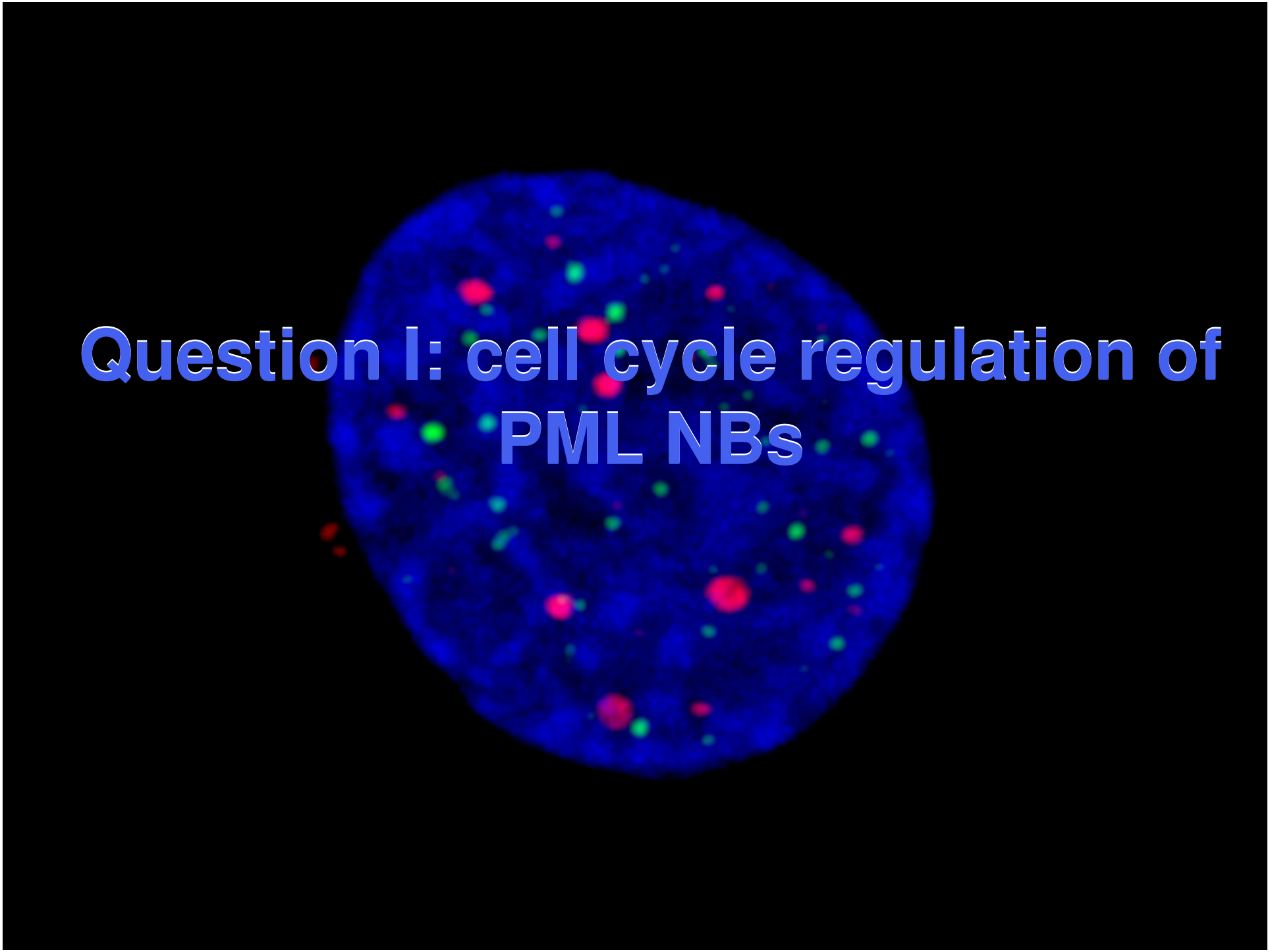
Immunofluorescent images: Mark Frey and Greg Matera (Cajal body and Gem); Paul Mintz (RNA polymerase II transcription factor, nucleoli, peripheral nuclear lamina, perinucleolar compartment, PML body and nuclear speckles); Ana Pombo (OPT domain) Stéphane Richard (SAM68 nuclear body); Thomas Ried and Evelin Schröck (chromosome territory)
Design: Jim Duffy

Pro-Myelocytic Leukemia protein (PML)

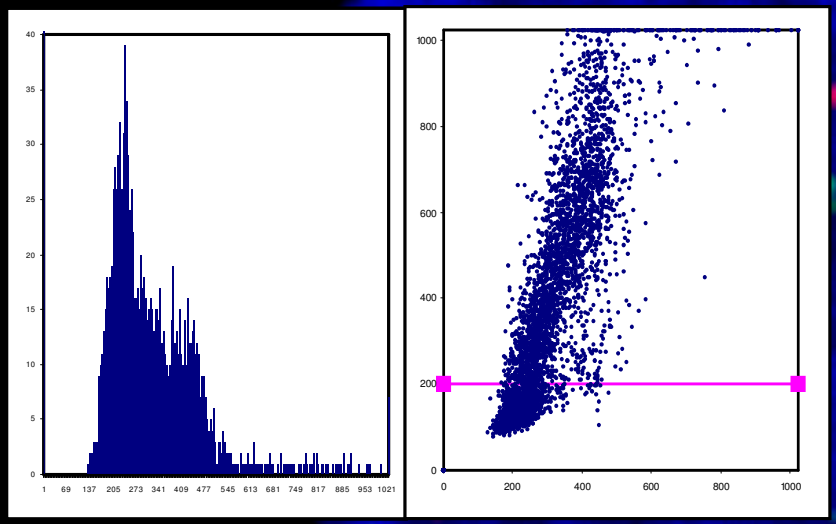
Protein structure involves strong homo-aggregation



- PML localizes in nuclear bodies: variable size (0.5 - 2 μm) and number according to cell type and state
- More than 80 proteins involved in different cell functions (DNA transcription, replication and repair, RNA splicing, ubiquitination, sumoylation…)


Question I: cell cycle regulation of
PML NBs

- Flow cytometry is generally employed for cell cycle analysis and correlated protein expression measurement



- Low protein amounts, as in the case of PML, are not detectable by flow cytometers

- PML cell cycle regulation of expression, localization and interaction requires high resolution analysis of “morphological expression patterns”

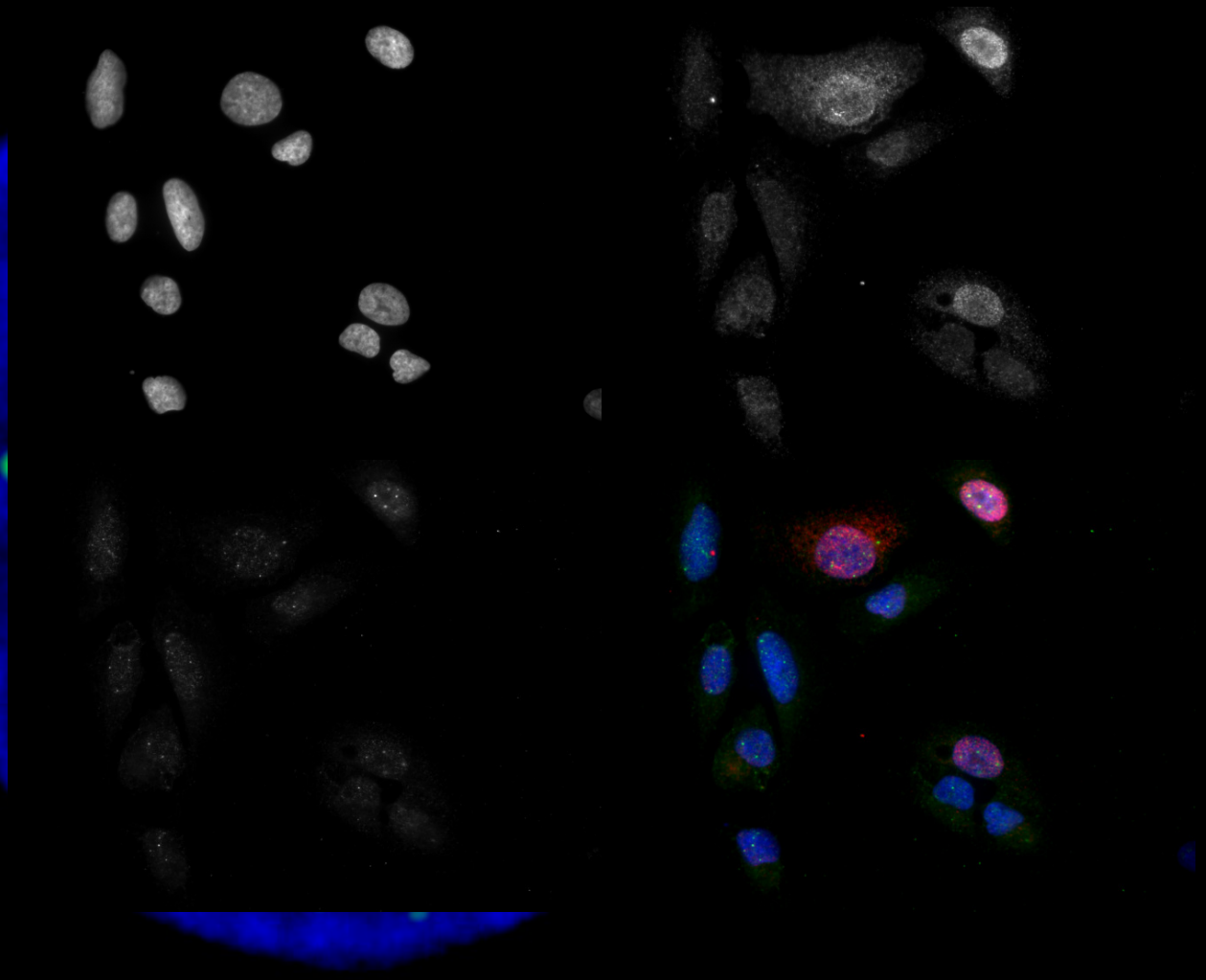
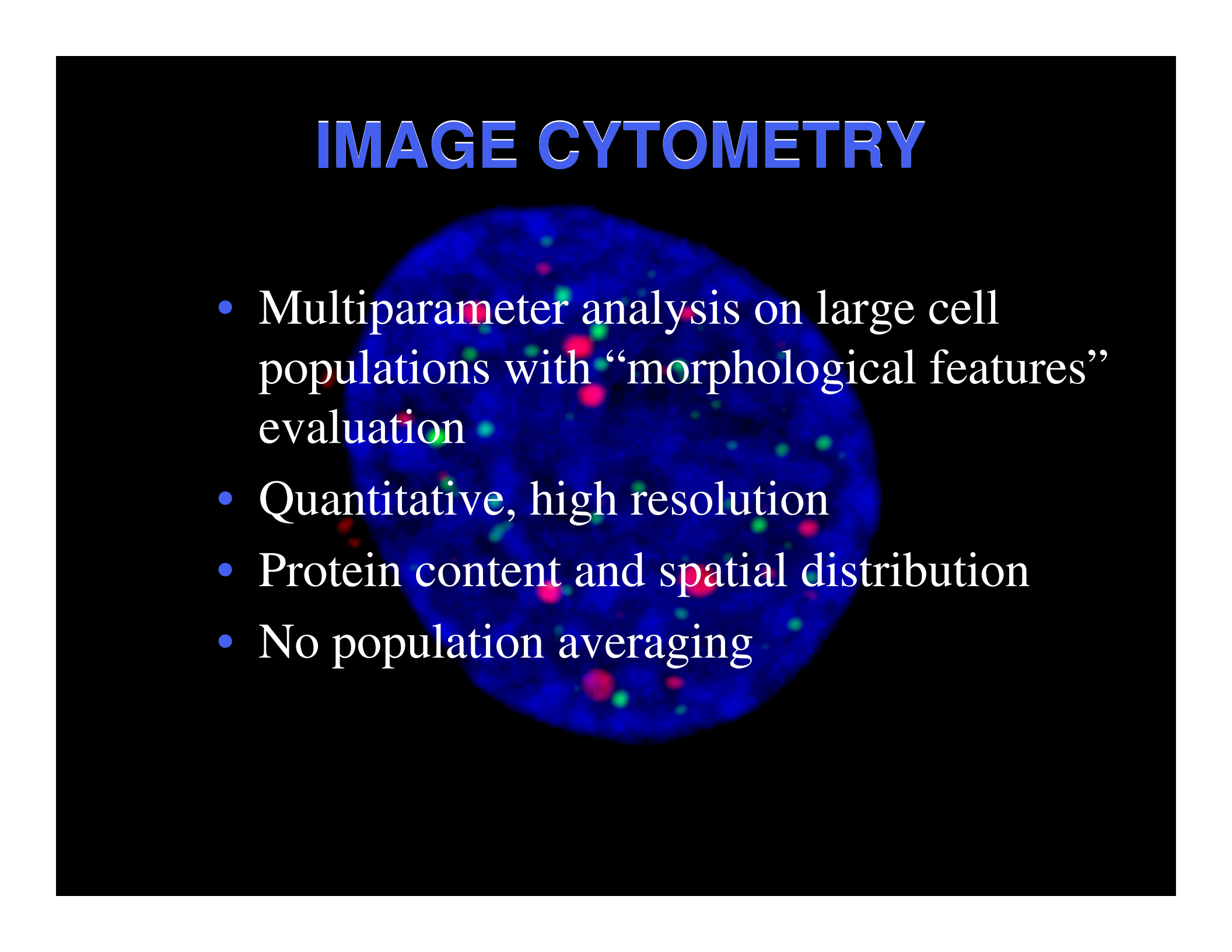
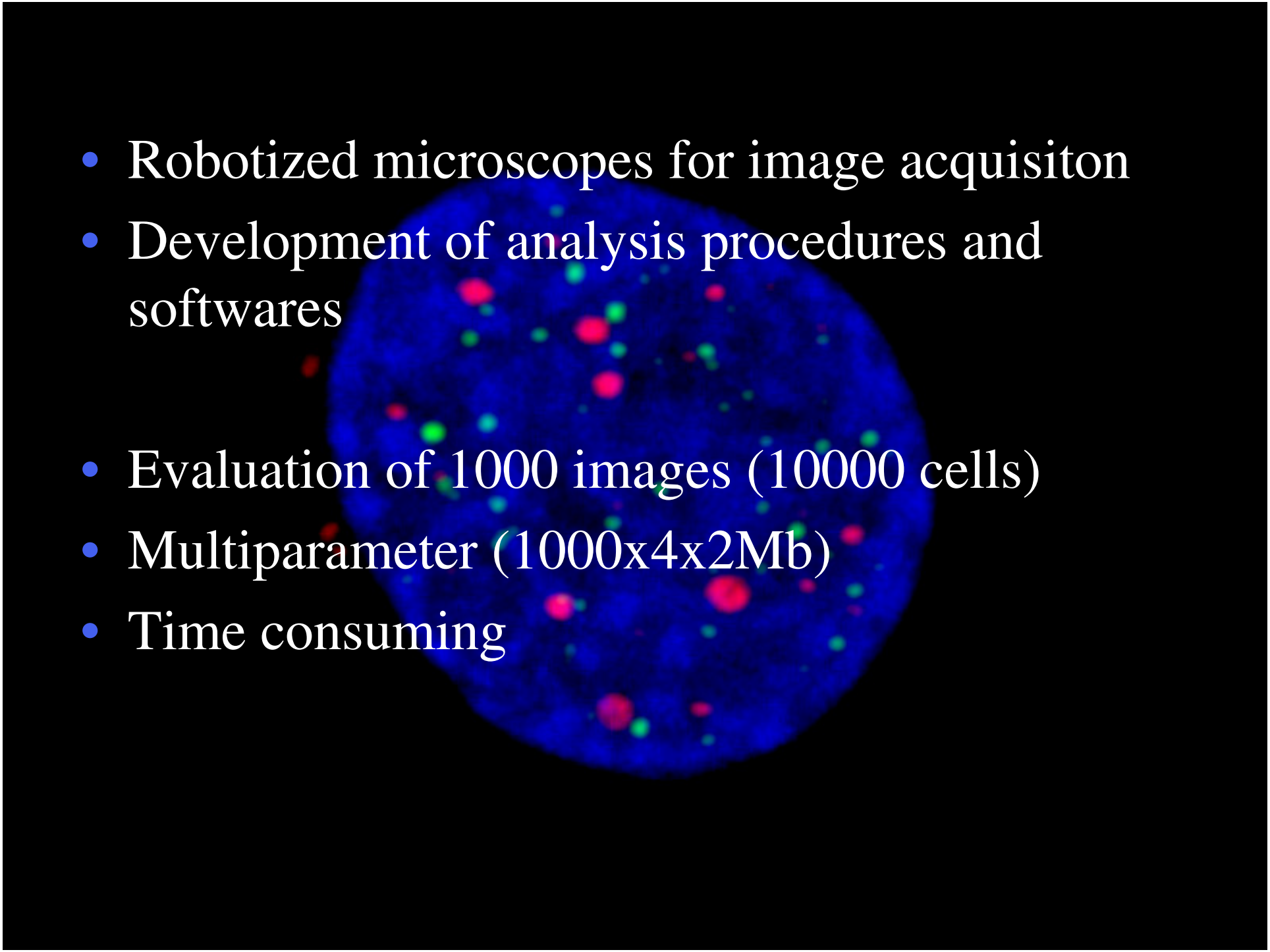
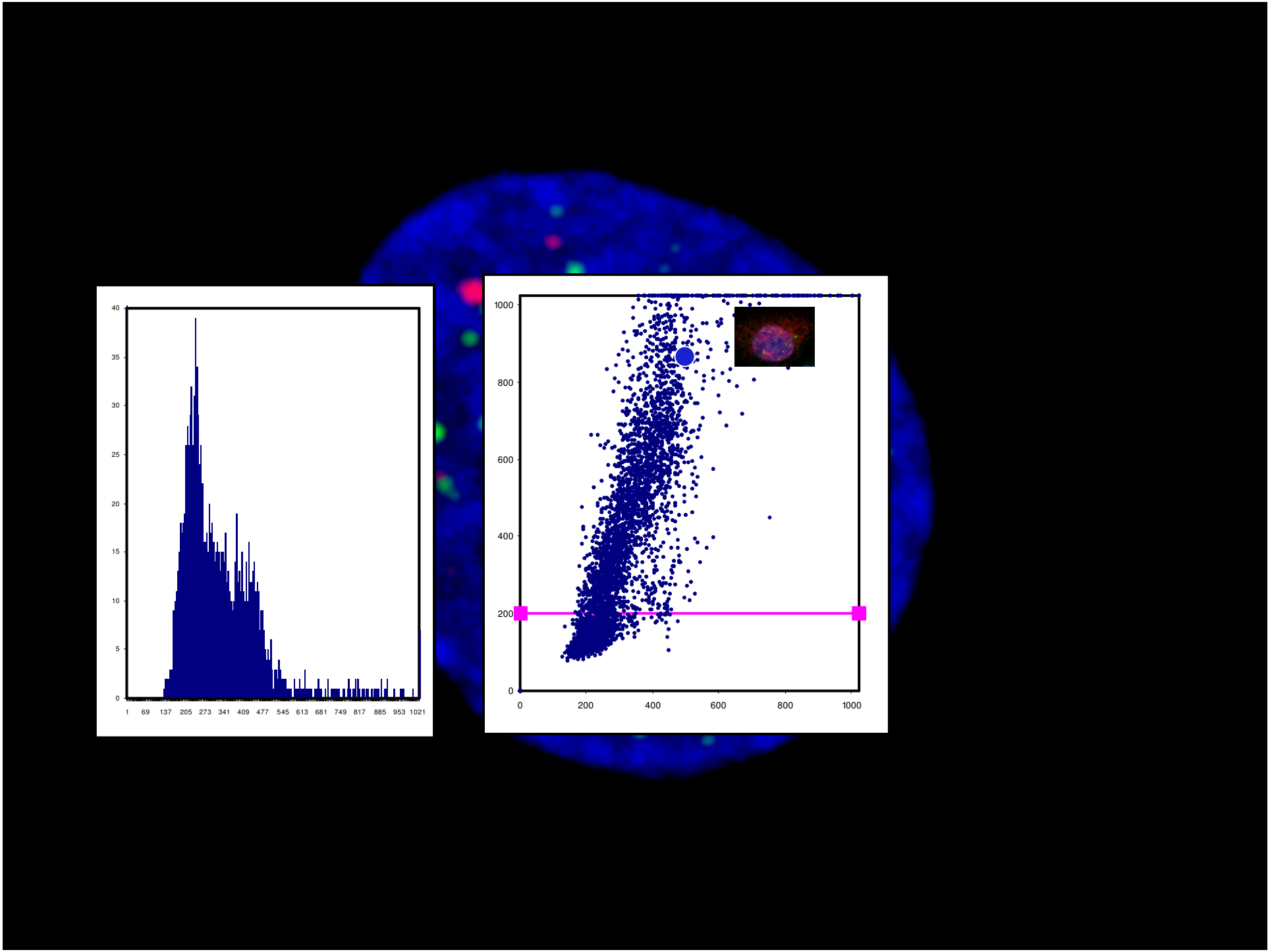


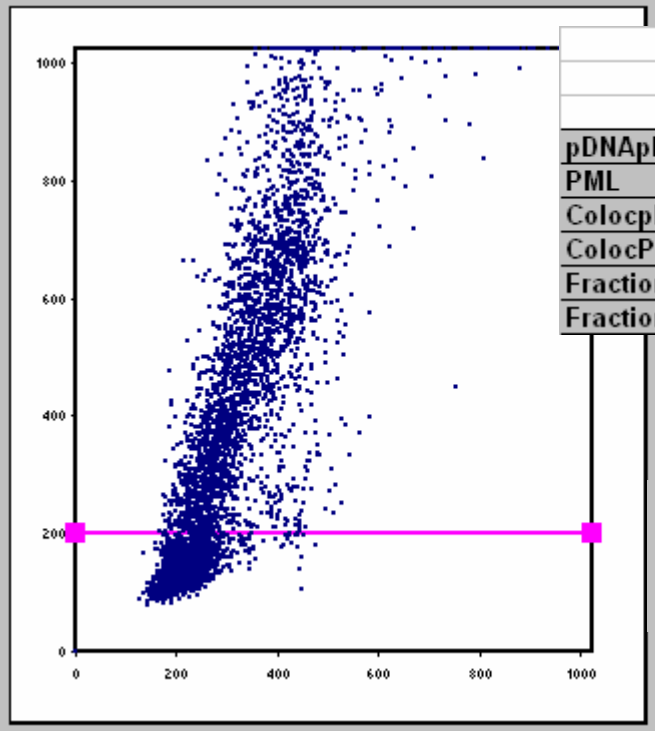
IMAGE CYTOMETRY



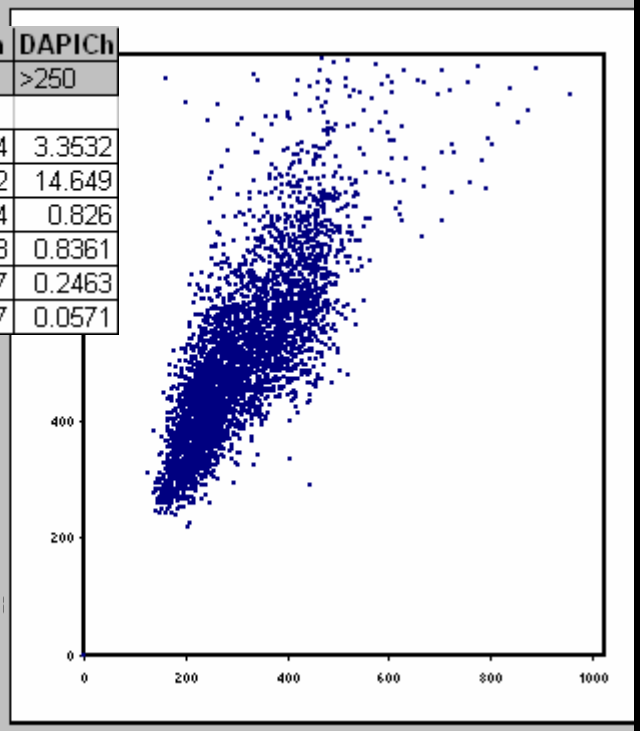
- Multiparameter analysis on large cell populations with “morphological features” evaluation
- Quantitative, high resolution
- Protein content and spatial distribution
- No population averaging

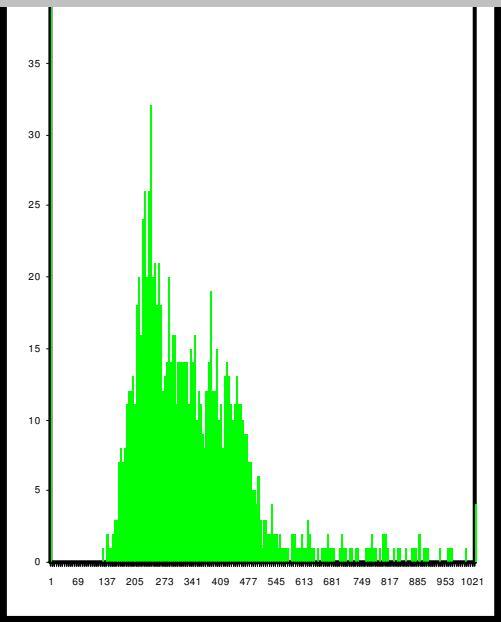

• Robotized microscopes for image acquisiton
• Development of analysis procedures and softwares
• Evaluation of 1000 images (10000 cells)
• Multiparameter (1000x4x2Mb)
• Time consuming

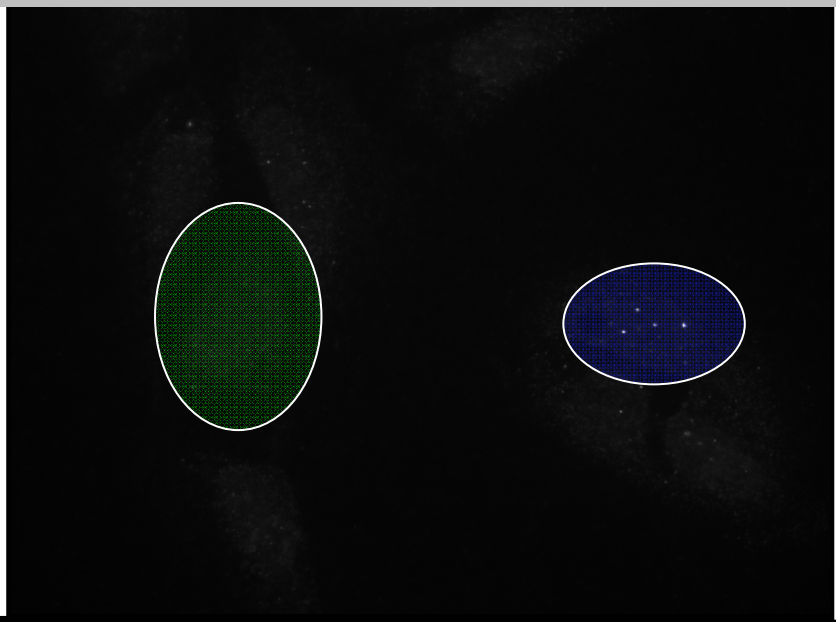

40
35
30
25
20
15
10
5
0
1 69 137 205 273 341 409 477 545 613 681 749 817 885 953 1021
1000
800
600
400
200
0
0 200 400 600 800 1000



	CyclAch <200	DAPICh <250	CyclAch >200	DAPICh >250
pDNApk	1.42753	1.4741	3.22724	3.3532
PML	9.25091	9.6797	14.5062	14.649
ColocpDNApk	0.35749	0.3823	0.80414	0.826
ColocPML	0.36114	0.3871	0.81483	0.8361
Fraction of ColocDNApk	0.25043	0.2593	0.24917	0.2463
Fraction of ColocPML	0.03904	0.04	0.05617	0.0571







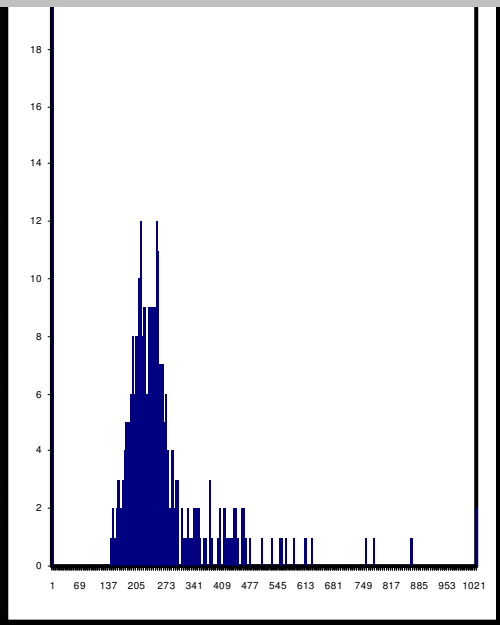
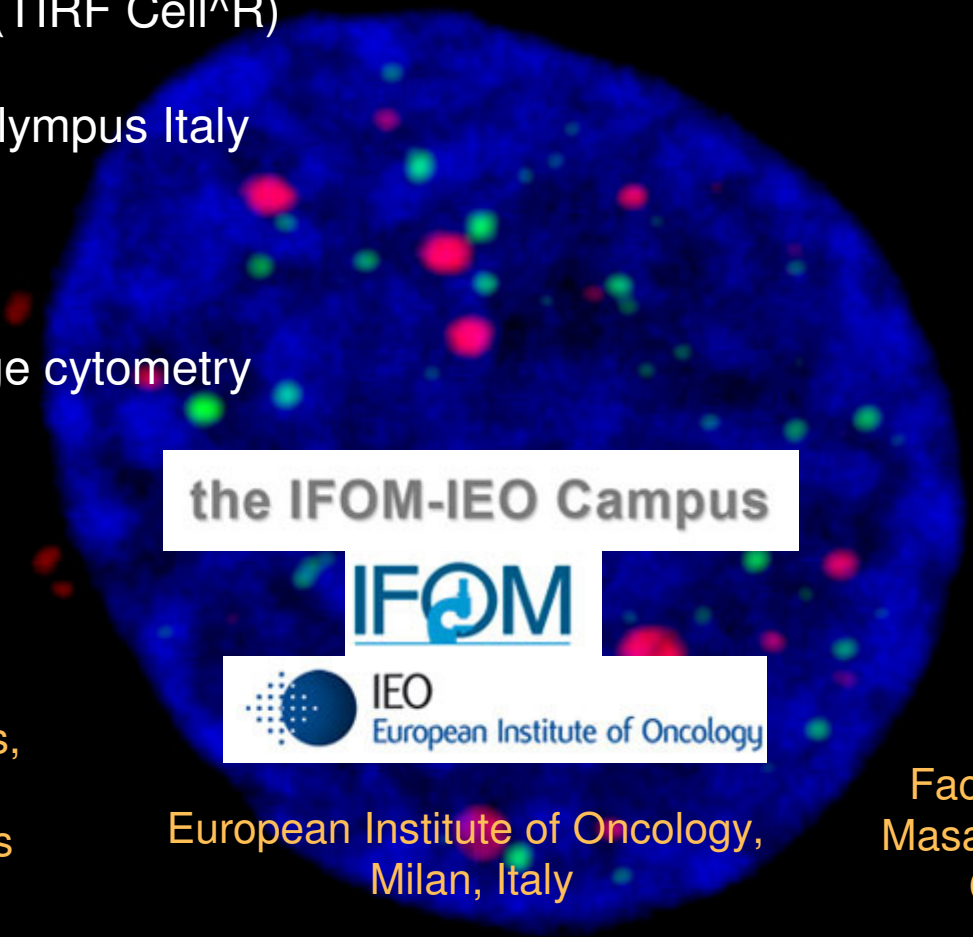


Image Cytometry (collaborative agreement for the development of the systems)
- Dr K Joanidopoulos, Dr P Zehetmayer, and Dr D Krueger (Scan^R)
- Dr P Messler (TIRF Cell^R)

Support from Olympus Italy



Confocal Image cytometry

Institute of Biophysics, Brno
Academy of Sciences of Czech Republic

Kozubek S.
Falk M.
Lukásová M.

the IFOM-IEO Campus

IFOM

IEO
European Institute of Oncology

European Institute of Oncology, Milan, Italy

Pelicci P.G.
Faretta M.
Dellino I.
Cocito A.

Faculty of Informatics, Masaryk University, Brno
Czech Republic

Kozubek M.

lambs

laboratory advanced microscopy bioimaging Spectroscopy

- Overview
- People
- Publications
- Popular readings
- Research
- Collaborations
- Awards
- Funding
- Events
- Lectures
- Links

We are a RESEARCH group of BIOPHYSICISTS

merging different backgrounds

physics, **bioengineering**, **biotechnology**,

materials science, **chemistry**, **biology**, **medicine**,

computer science

conceiving, designing, realizing, utilizing

methods and techniques

to study biological systems to advance life sciences

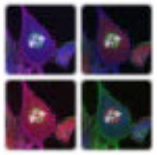
[...] Dance with wolves and count the stars, including the unseen... (L.Ferlinghetti, Challenges to young poet, 2001)

difi

IFOM
ISTITUTO FIRC
DI ONCOLOGIA MOLECOLARE

NANOMED Labs
Nanotechnology for bioMedicine

NEWS



The Imaging Center of the IEO-IFOM Campus

Personnel

Mario Faretta
Facility coordinator and Scientist.
Wide Field and Confocal Fluorescence Microscopy, Time-lapse Microscopy, Flow Cytometry, Image Analysis, and SPR molecular interaction Analysis.
(Phone:0257489880)

IEO:

Simona Ronzoni
Flow Cytometry and Cell Sorting
(Phone:0257489880)

Ivan Muradore
Flow Cytometry
(Phone:0257489880)

Sara Barozzi
Fluorescence Microscopy and Cell Microinjection
(Phone:0257489880)

Francesca Refaldi
Flow Cytometry and Cell Sorting
(Phone:0257489880)

Dario Parazzoli
Fluorescence Confocal Microscopy and Image Analysis
(Phone:0257489880)

IFOM:

Emanuela Frittoli
Fluorescence Microscopy and Cell Microinjection
(Phone:02574303324)

Massimiliano "Brutus" Garrè
Fluorescence wide field, Confocal and Time-lapse Microscopy
(Phone:02574303225)

Pietro Transidico
Fluorescence Confocal Microscopy and Image Analysis
(Phone:02574303225/258)