



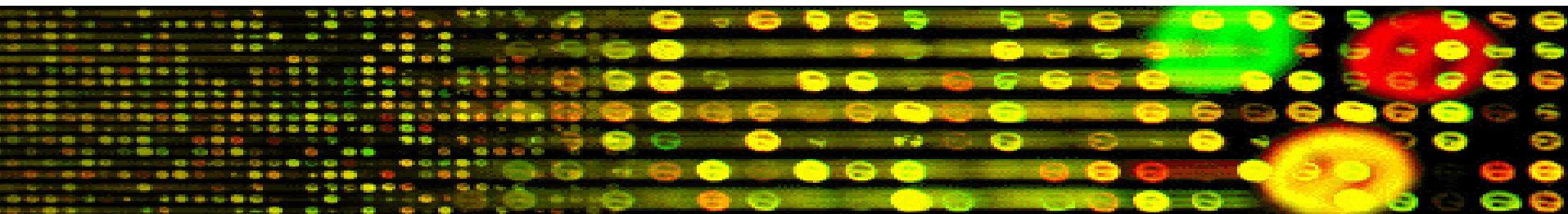
香港浸會大學
HONG KONG BAPTIST UNIVERSITY

Functional Genomics Approach to the Study of Biological Systems

Ricky N. S. Wong

Head

**Department of Biology, Faculty of Science,
Hong Kong Baptist University**



Functional Genomics

- to understand the **function** of the genes and their gene product(s) in a **holistic** manner
- two technology platforms:

1) DNA Microarray

>>> gene expression profile

2) Proteomics

>>> total protein analysis using 2-dimensional gel electrophoresis (separation) coupled with mass spectrometry (identification)

Flow of genetic information

Central Dogma:

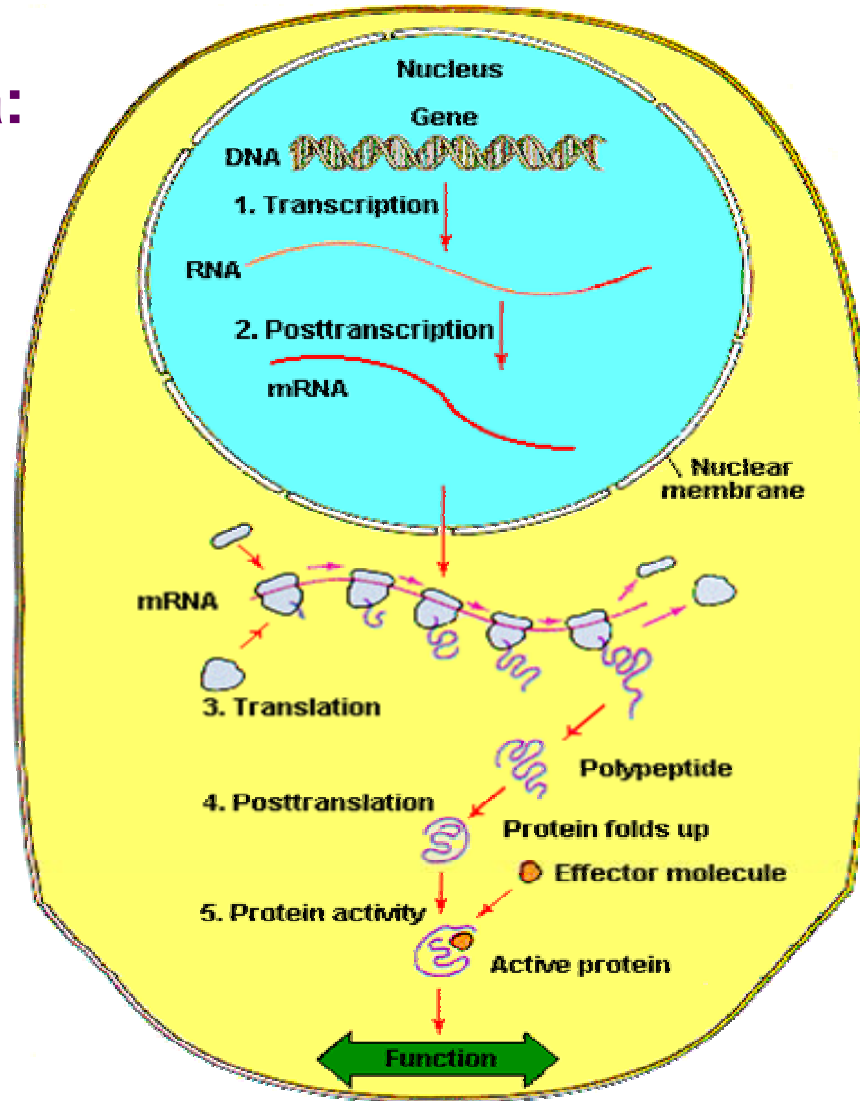
DNA

↓

RNA

↓

Protein



Sequence

↓

Structure

↓

Structural
genomics

Function

↓

Functional
genomics

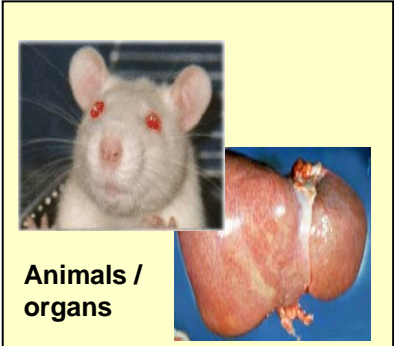
Overview on the concept of functional genomics

INPUT

BIOLOGICAL SYSTEMS

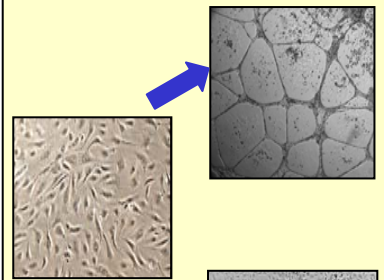
OUTPUT

Toxic Metals
e.g. Cadmium



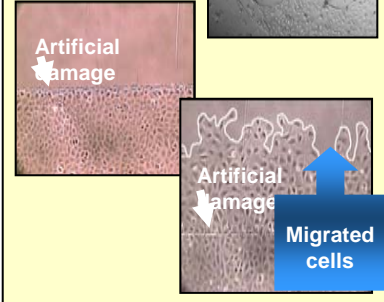
Animals / organs

Angiogenic Modulators



Cell Line
e.g. HUVECs
IEC-6

Chinese Herbal Extract



Artificial damage

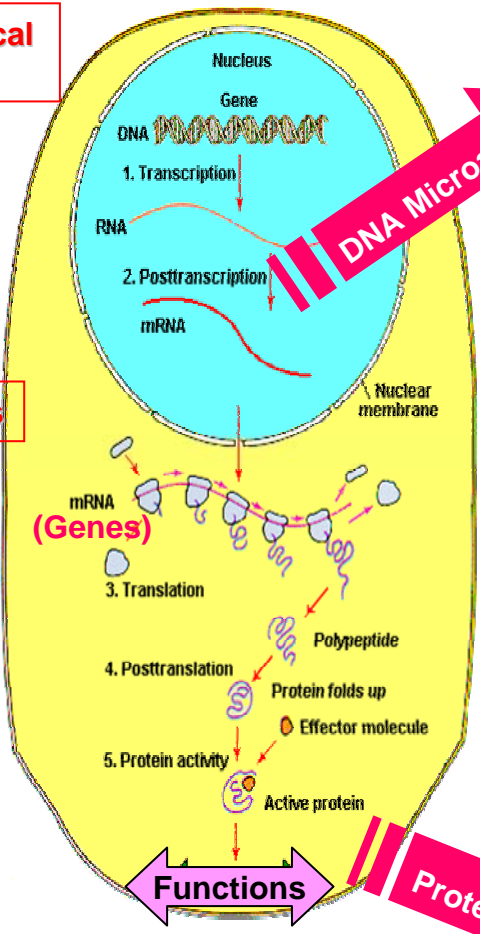
Artificial damage

Migrated cells

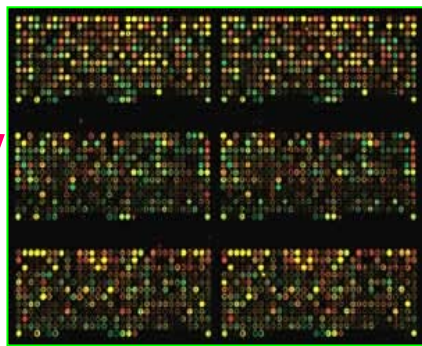
Toxicological Effect

Angiogenesis

Intestinal epithelial restitution



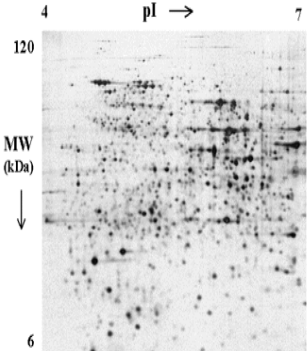
DNA Microarray



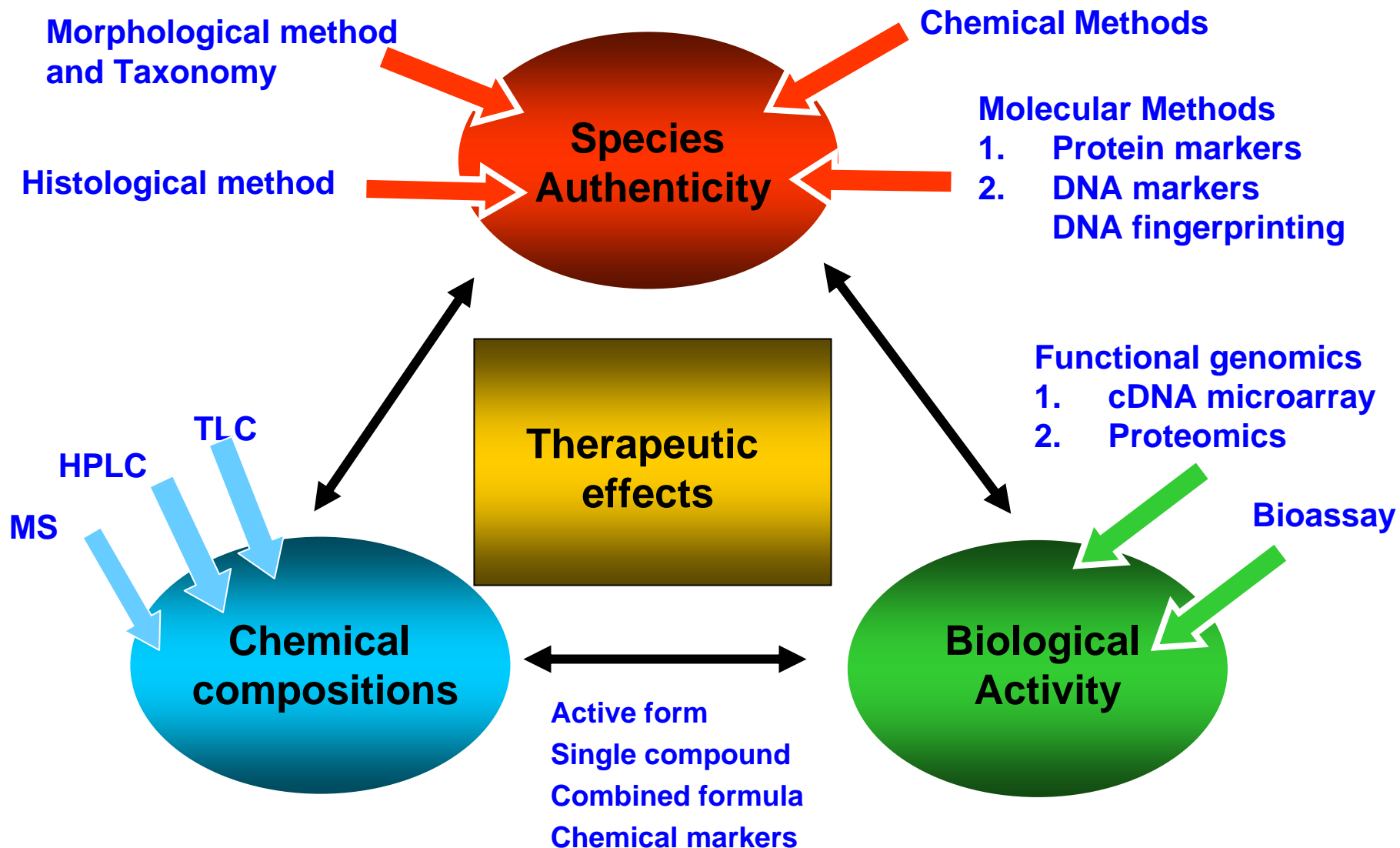
1. Therapeutic Effect Prediction
2. Drug Screening
3. Gene Discovery

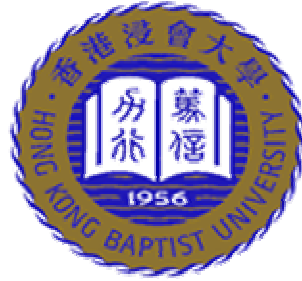
Functions

Proteomics



Quality Standardization for TCM

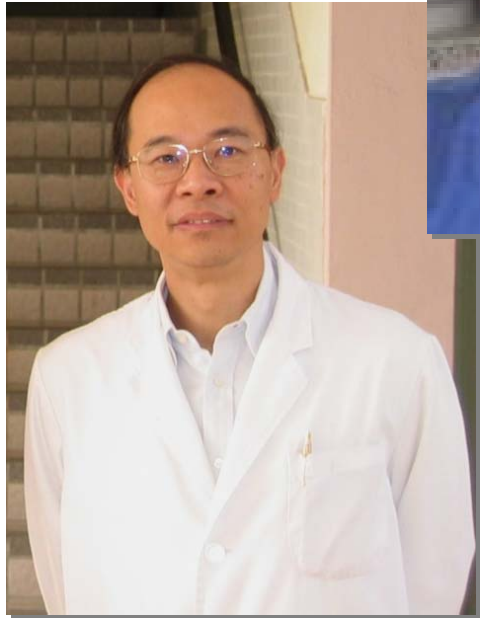
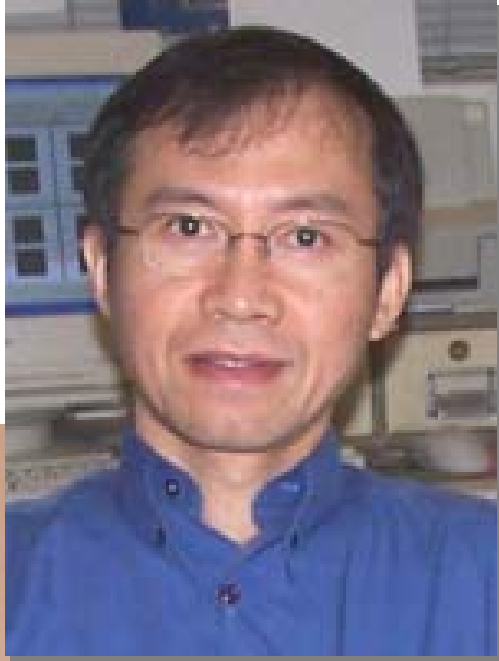




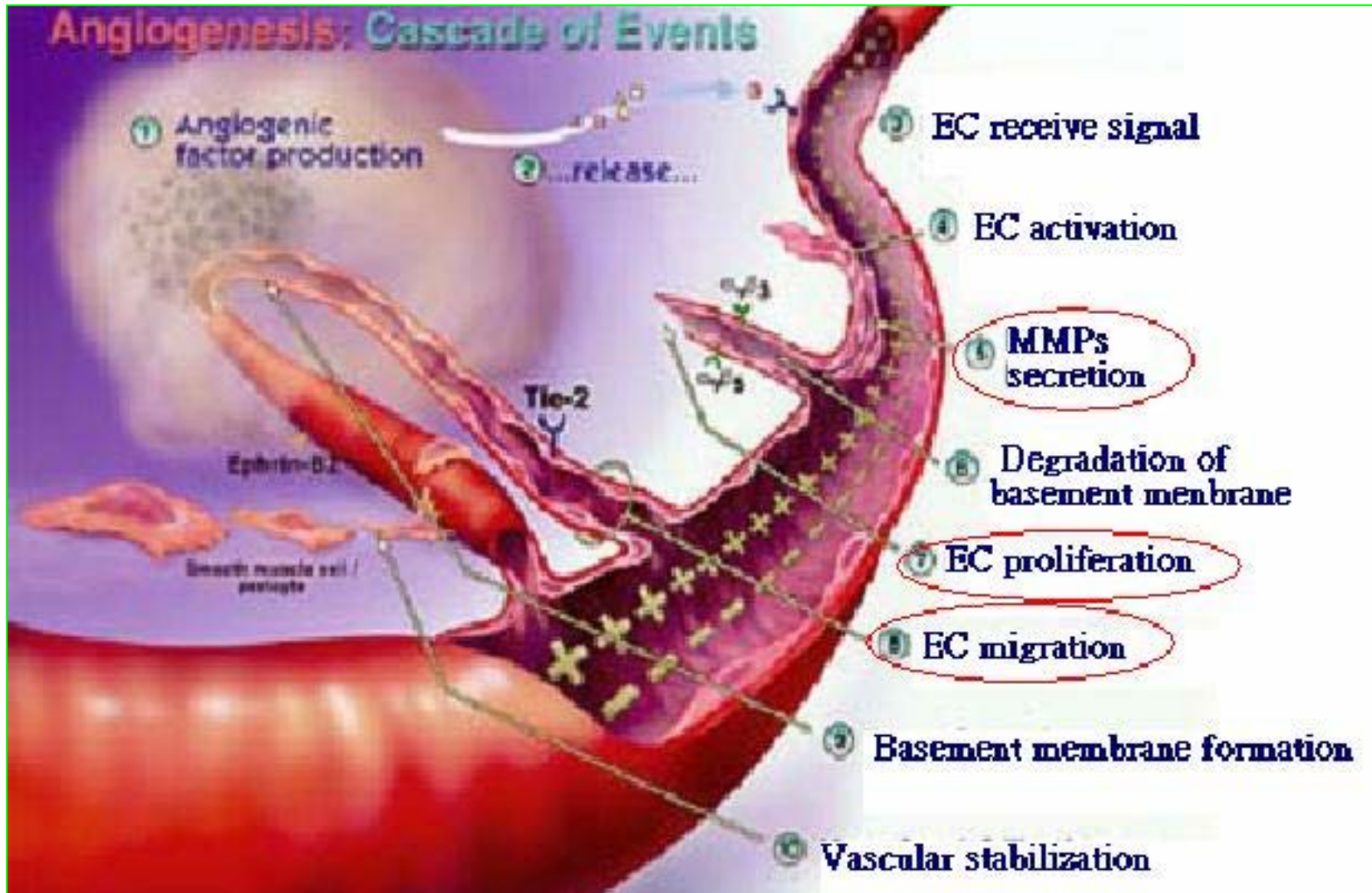
Ginseng and Angiogenesis

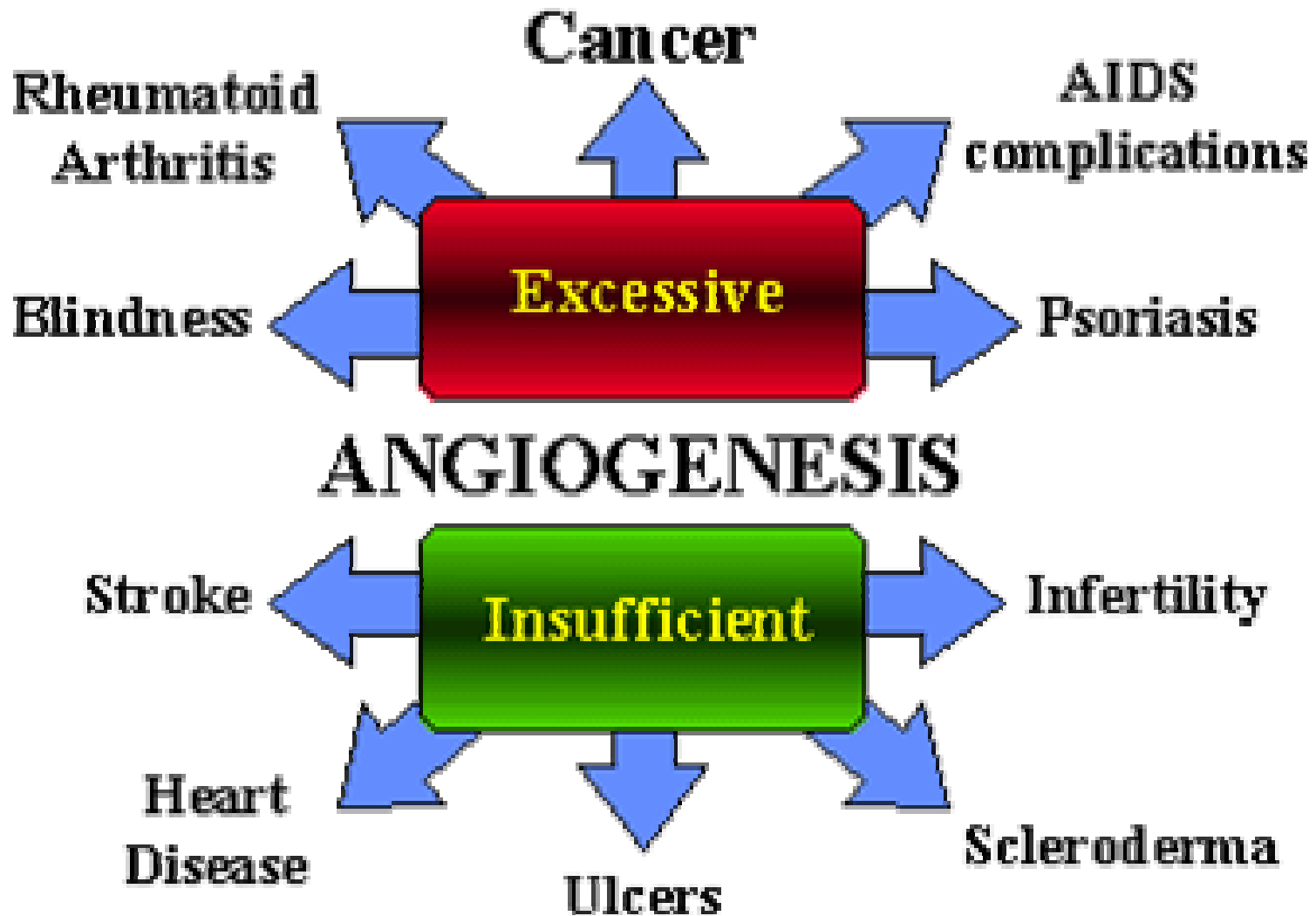
What is Chemical Biology?

- a discipline at the interface of the life sciences and the physical sciences
- an attempt to answer biological questions by directly probing living systems at the chemical levels
- a discipline spanning the fields of chemistry and biology to study and manipulate biological systems

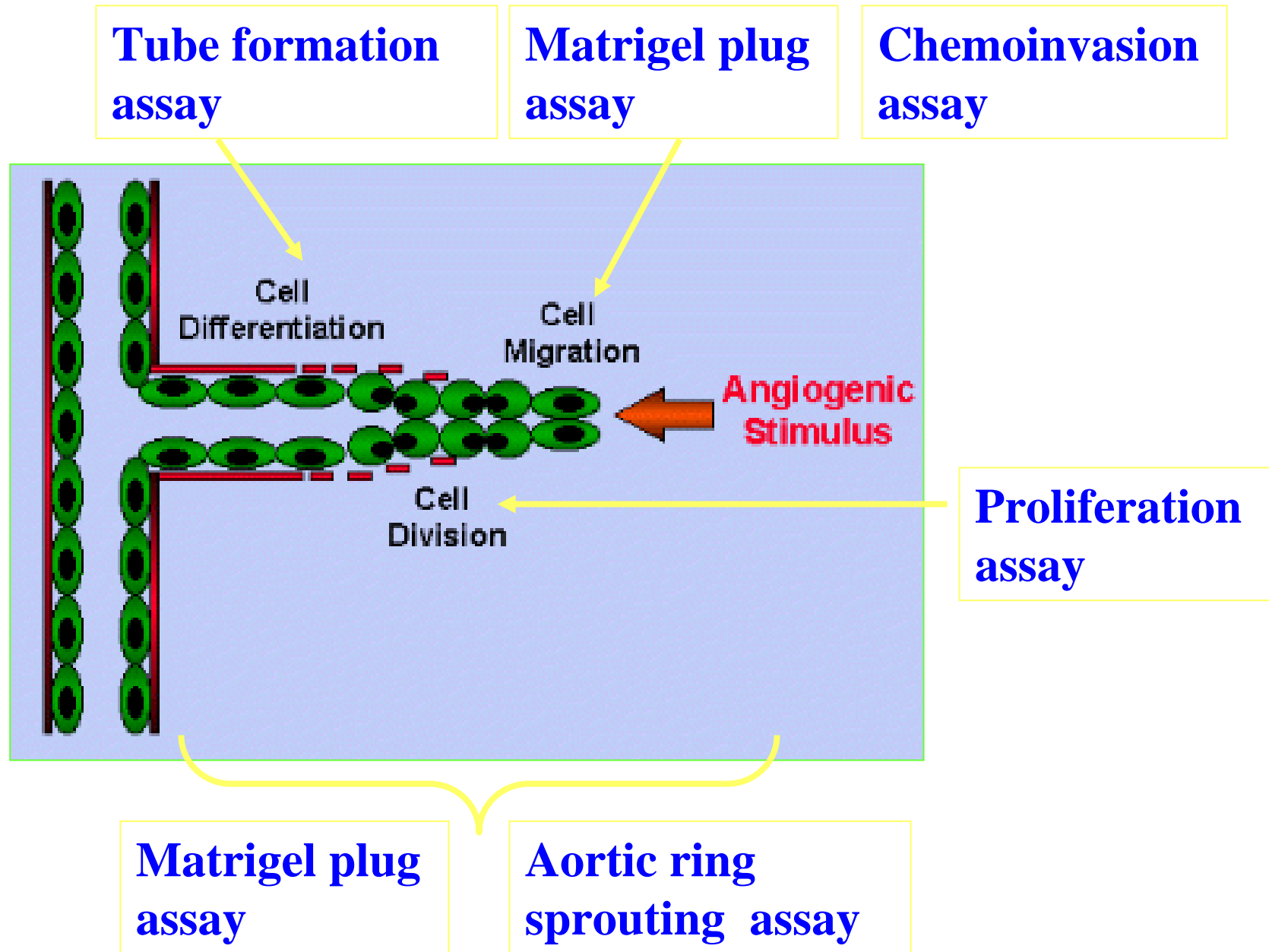


Angiogenesis



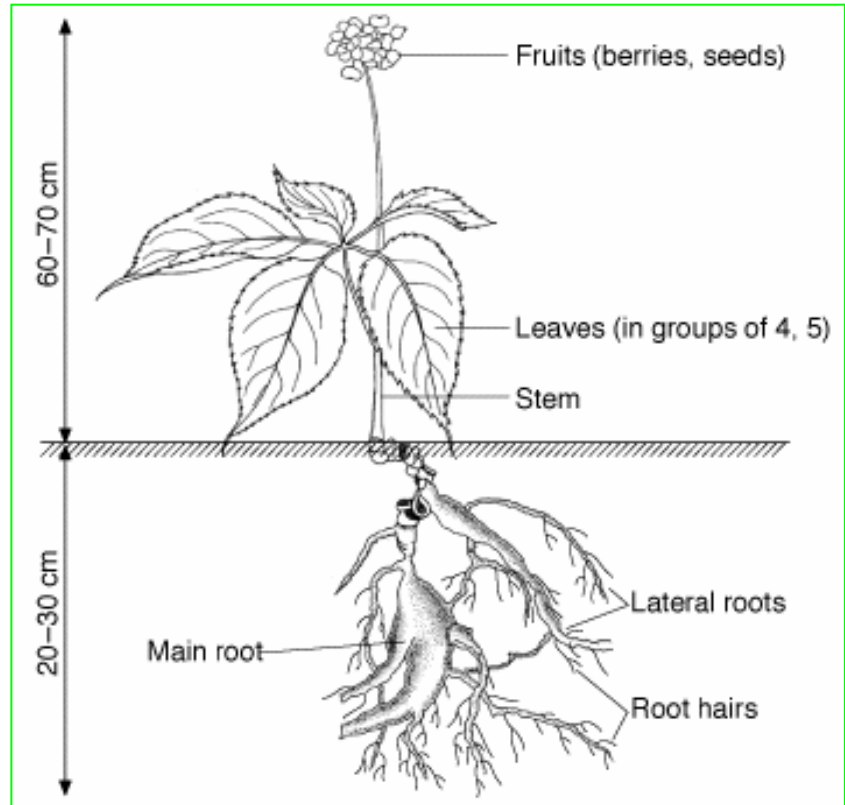


Overview on angiogenesis and bioassays



Panax ginseng

- The major constituents include:
 - *Ginsenosides*;
 - *Polysaccharides*;
 - *Peptides*;
 - *Polyacetylenic alcohols*;
and *Fatty acids*.

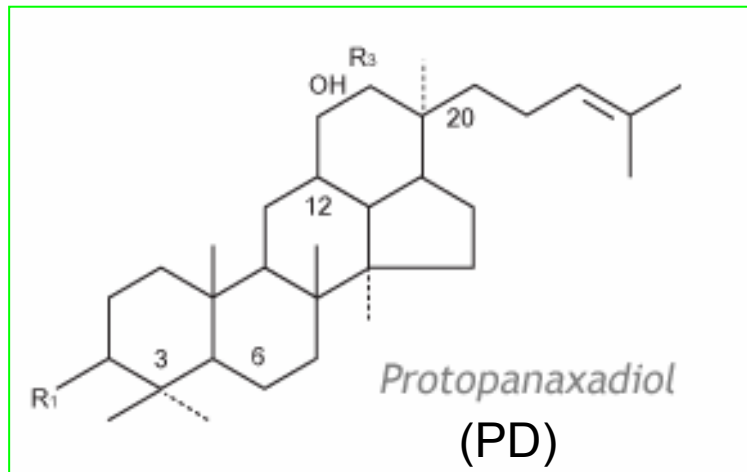


Ginsenosides

- **There are more than 30 identified ginsenosides.**
- **Most of them are *Triterpenoid Saponins*.**
- **Depending on their aglycone, they can be grouped into either**
20(S)-Protopanaxadiol (PD) OR
20(S)- Protopanaxatriol (PT).

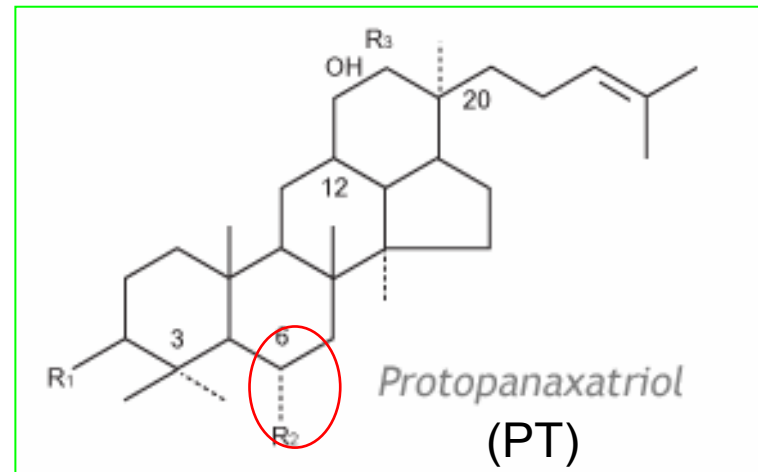
Ginsenosides

20(S)-Protopanaxadiol



(Rb1, Rb2, Rc, Rd and Rg3)

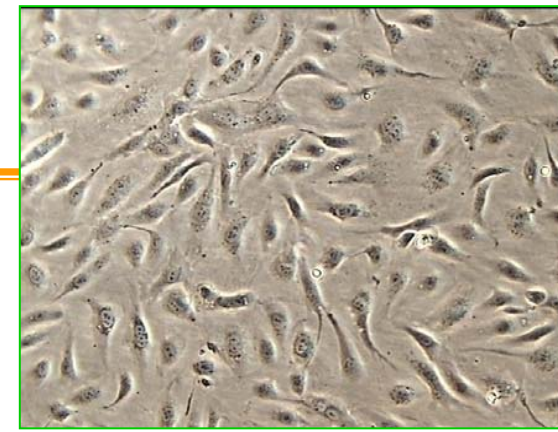
20(S)-Protopanaxatriol



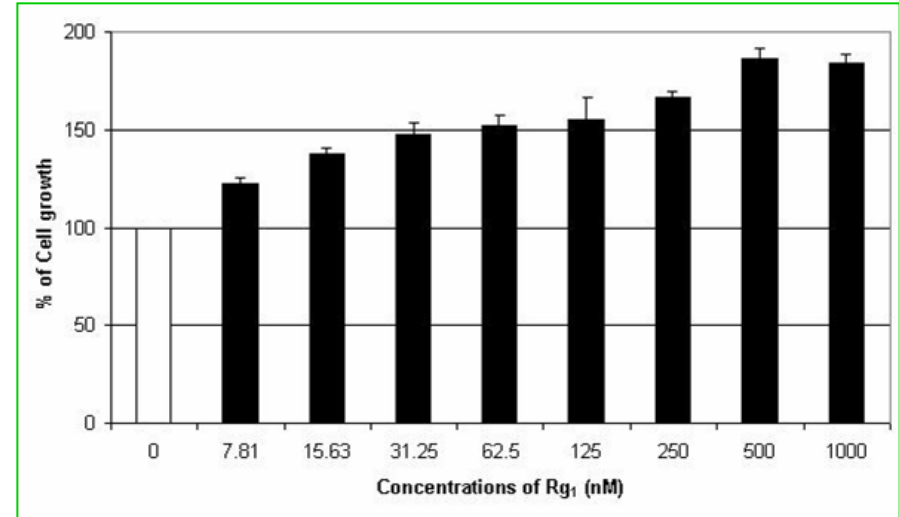
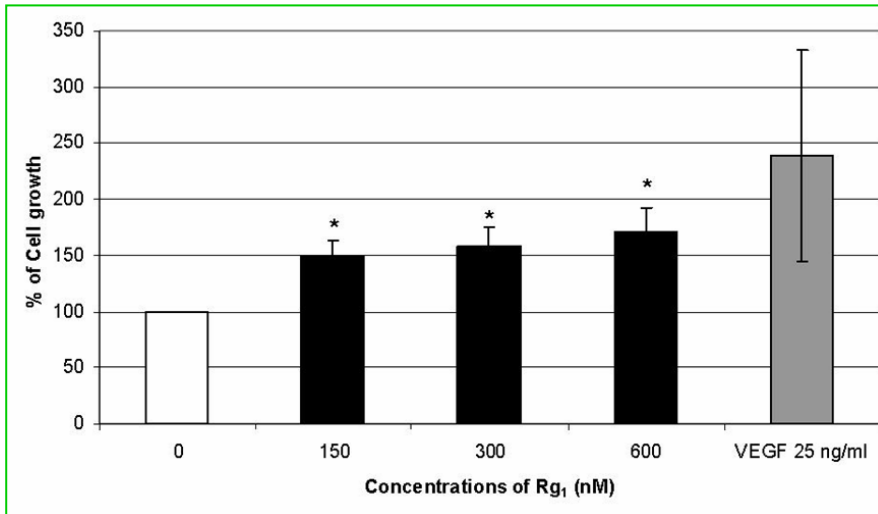
(Re, Rg1, Rg2, Rh1 and Rh3)

Proliferative effect of Rg₁

Human umbilical vein endothelial cells (HUVEC)

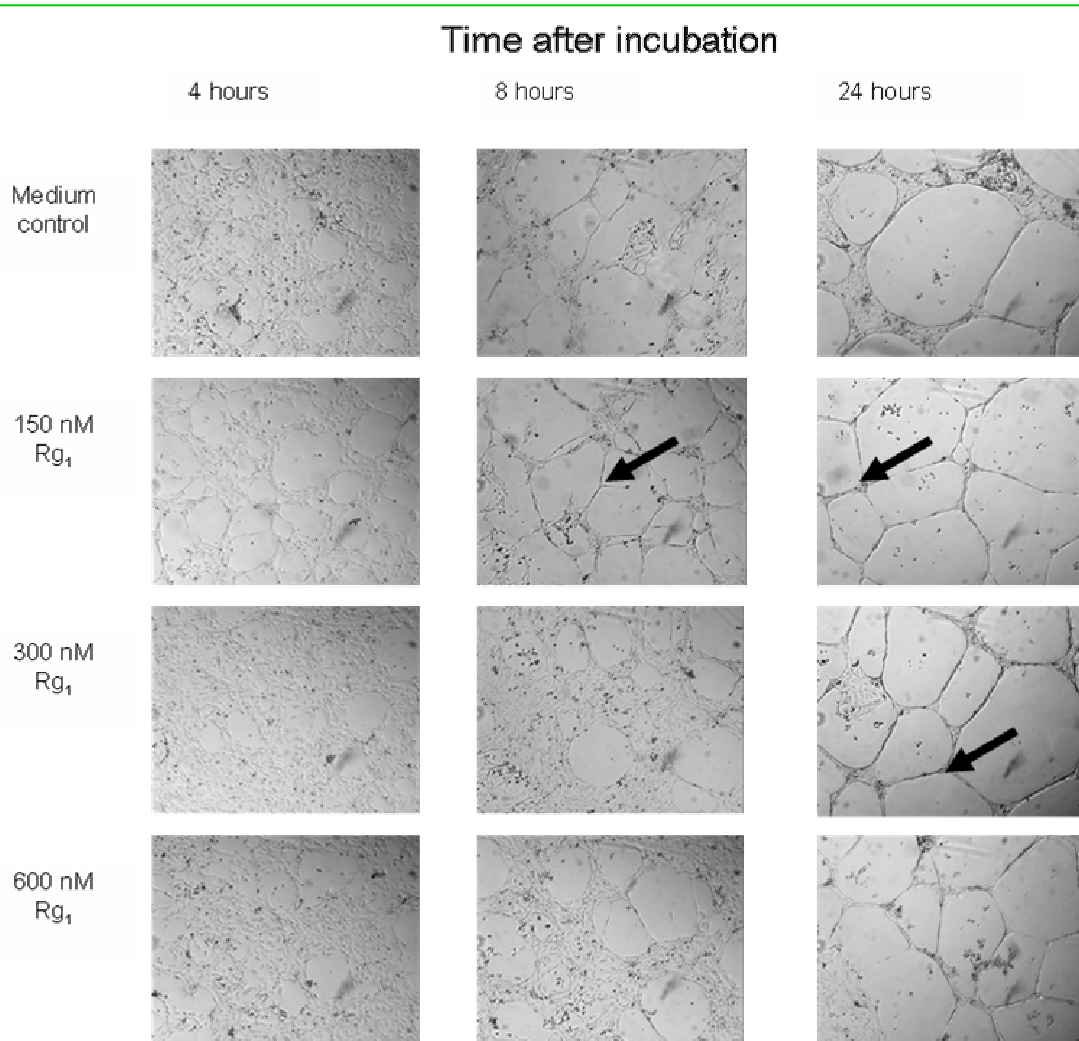


Trypan blue exclusion assay

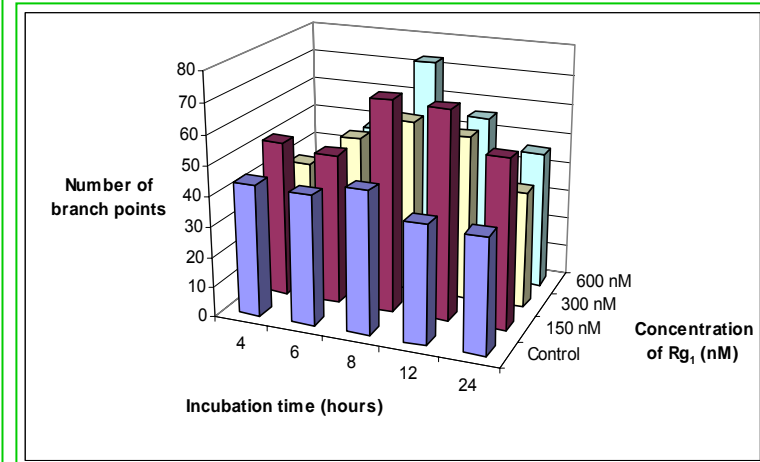


[³H]-Thymidine incorporation assay

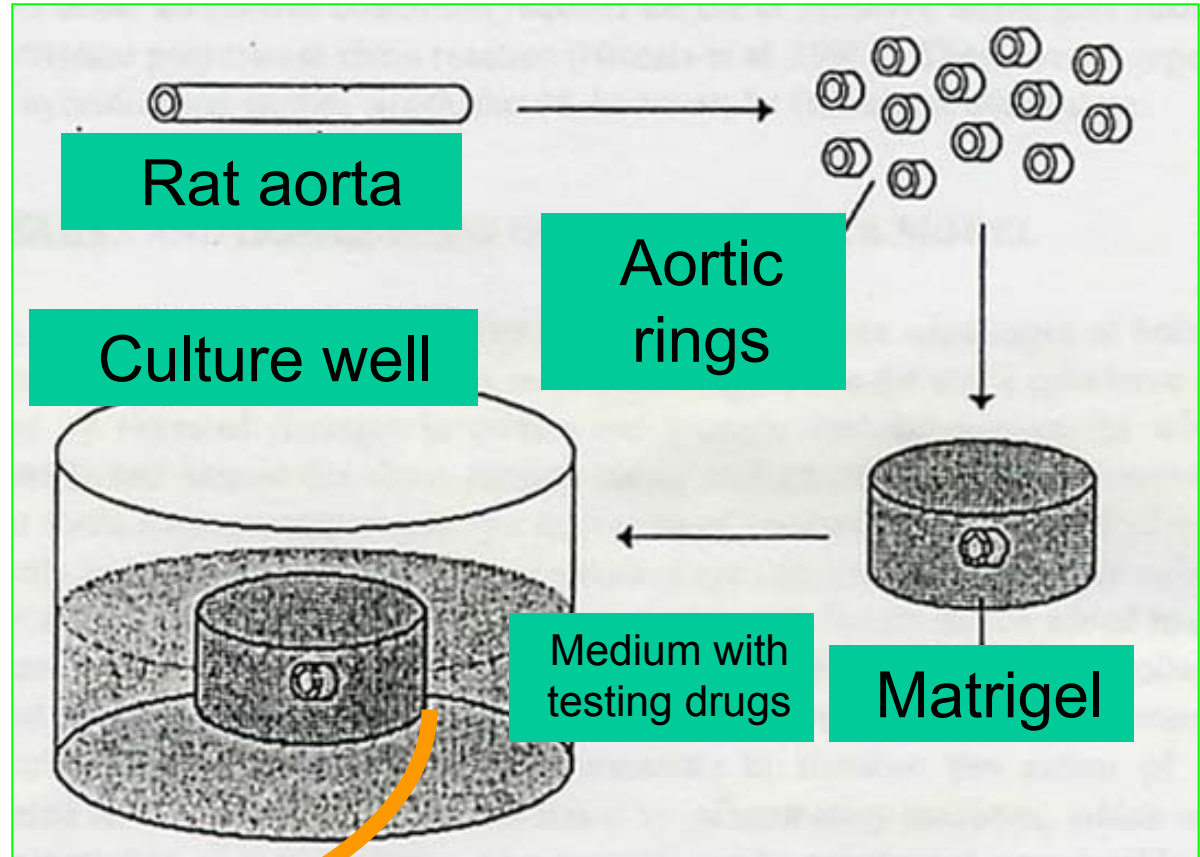
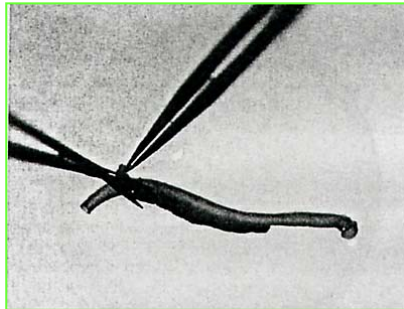
In vivo tubulogenesis



Tube formation assay

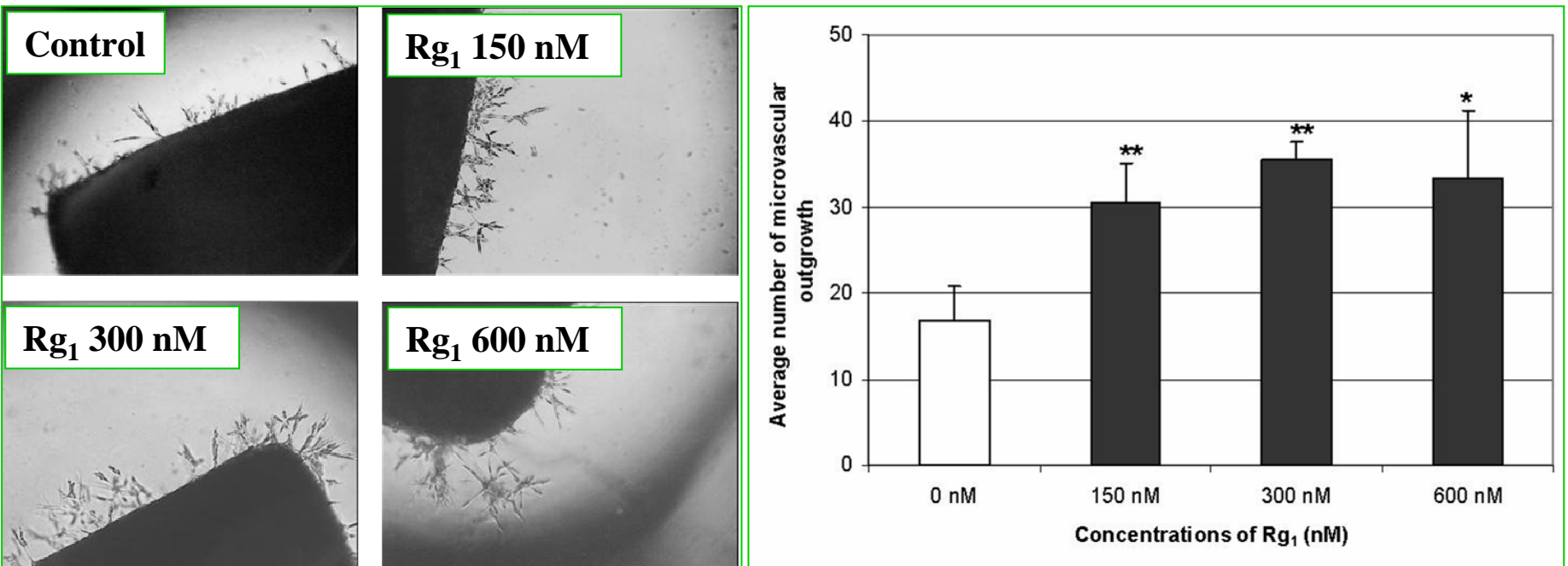


Aortic ring sprouting assay

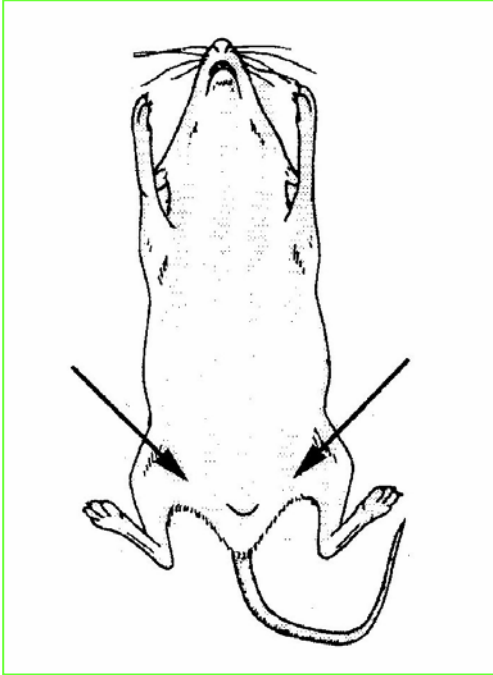


Ex vivo endothelial sprouting

Aortic ring sprouting assay

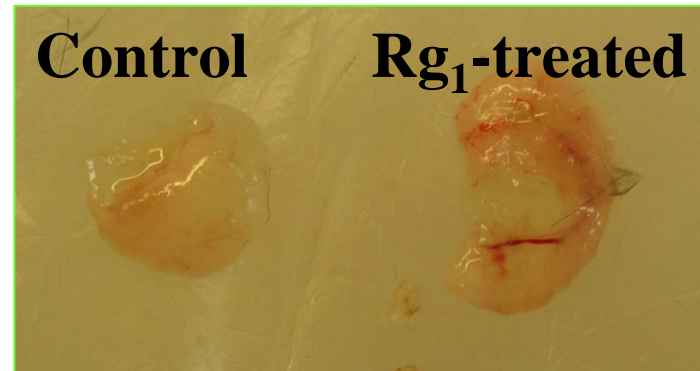
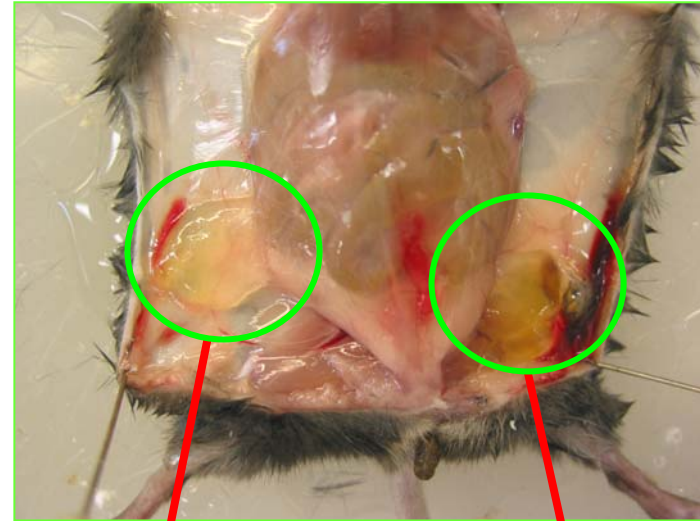


Matrigel plug assay



Subcutaneous
injection of
Matrigel with
testing drug

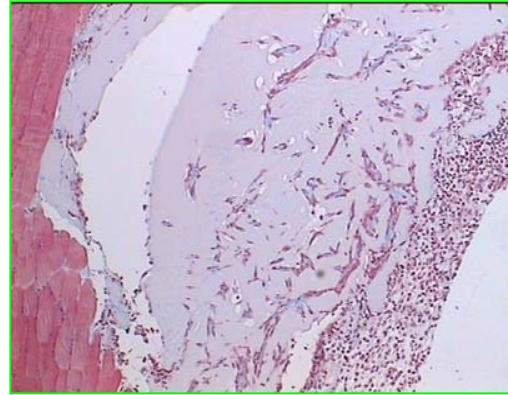
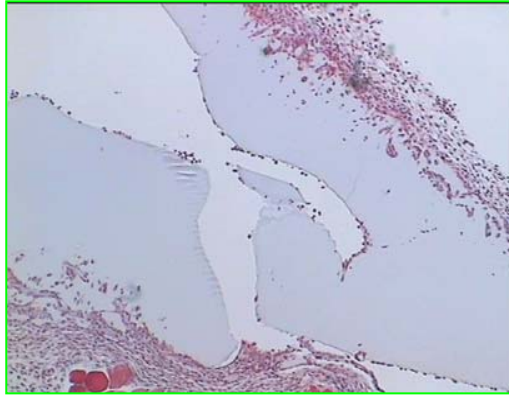
- haemoglobin measurement
- histological staining



Matrigel plug assay

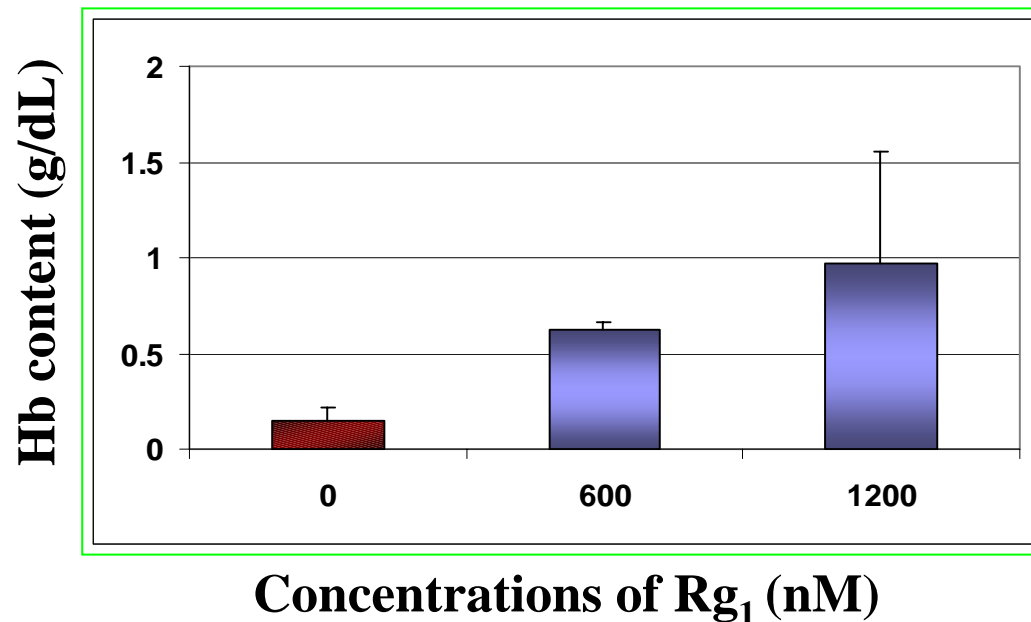
- Masson Trichrome staining

Matrigel
alone



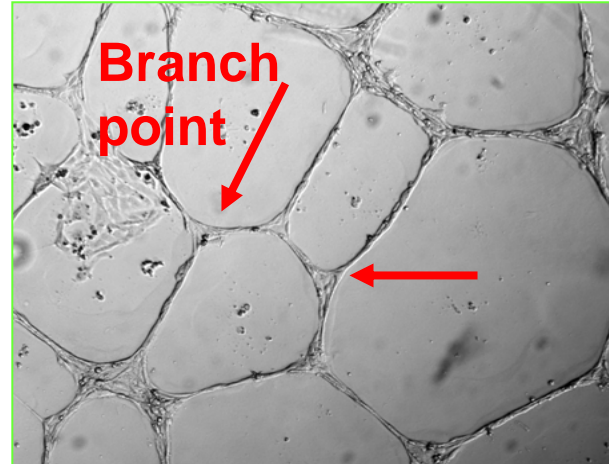
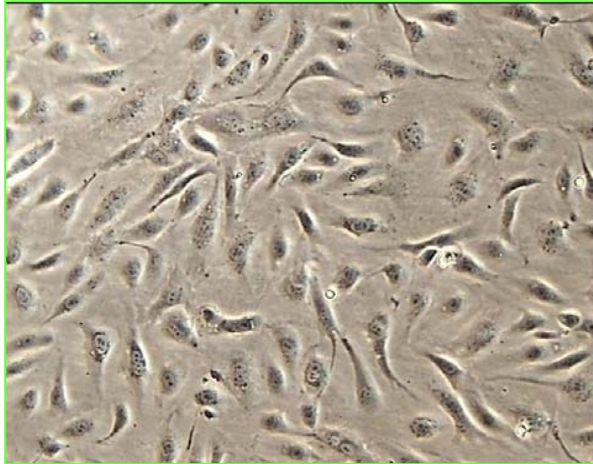
Matrigel with
600 nM Rg₁

- Quantitative analysis of hemoglobin contents



Screening of bioactivity

Tube formation assay

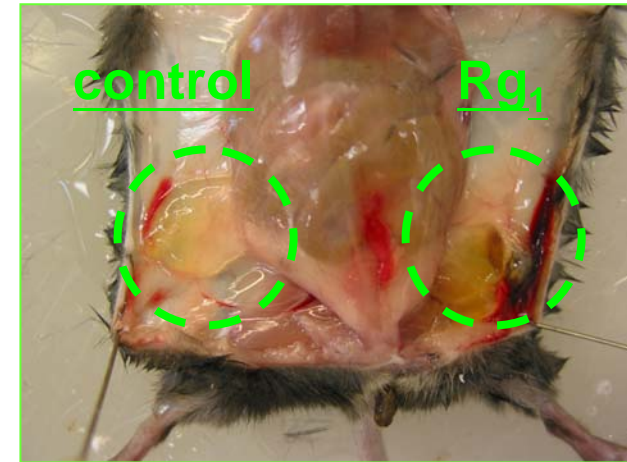


HUVEC control

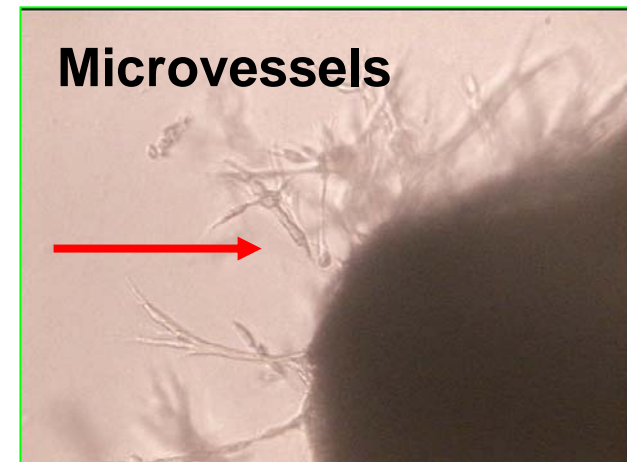
Rg₁ 150nM 24 hrs

Rg₁ can stimulate the HUVEC proliferation in a dose dependent manner

- induce tube formation *in vitro*
- increase the endothelial cells invasion and migration *in vivo*
- increase the microvessels formation *ex vivo*

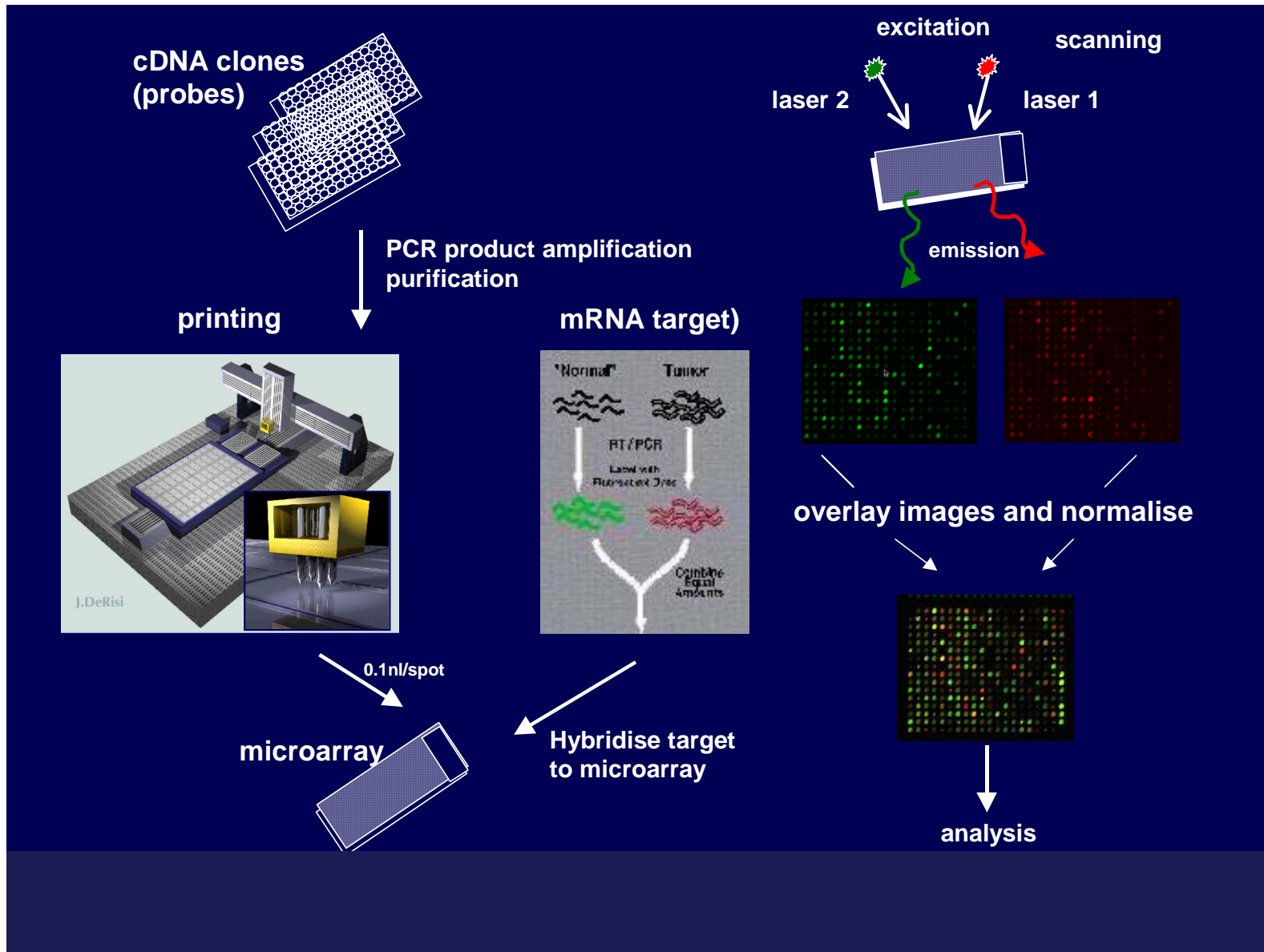


Matrigel plug assay



Aortic ring sprouting assay

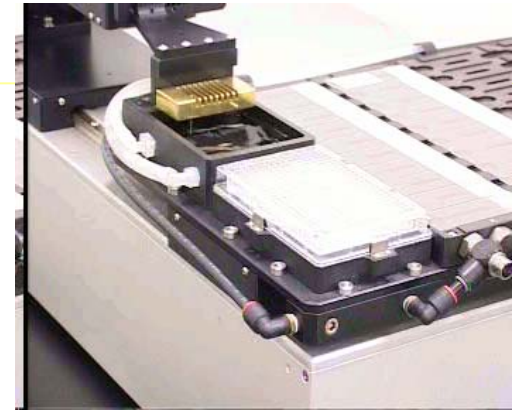
Overview of Microarray Technology



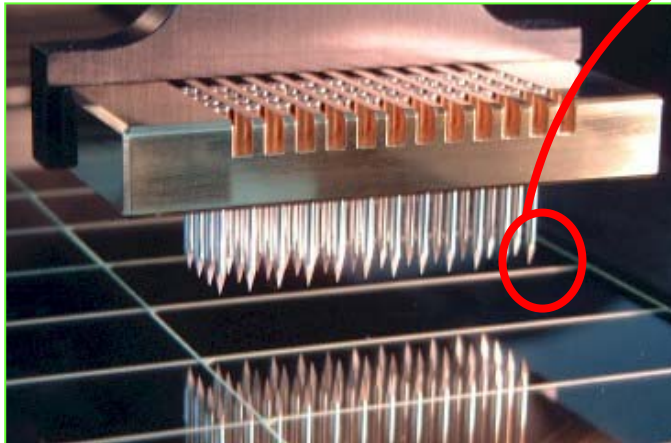
Fabrication of microarray



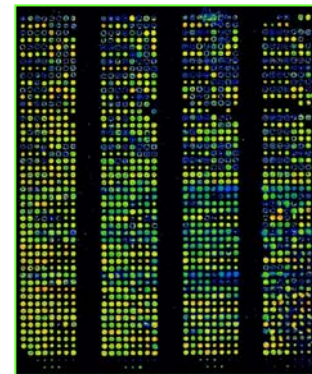
Arrayer



Pin tip



Pin head



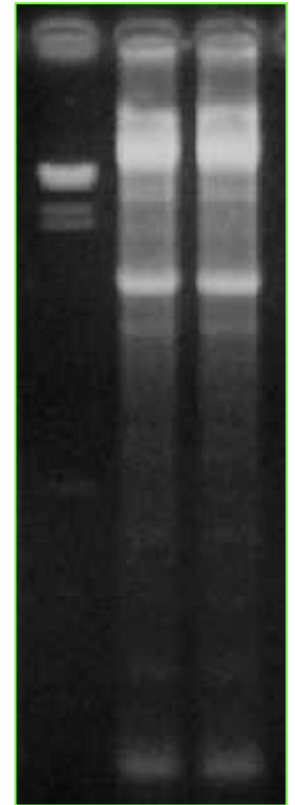
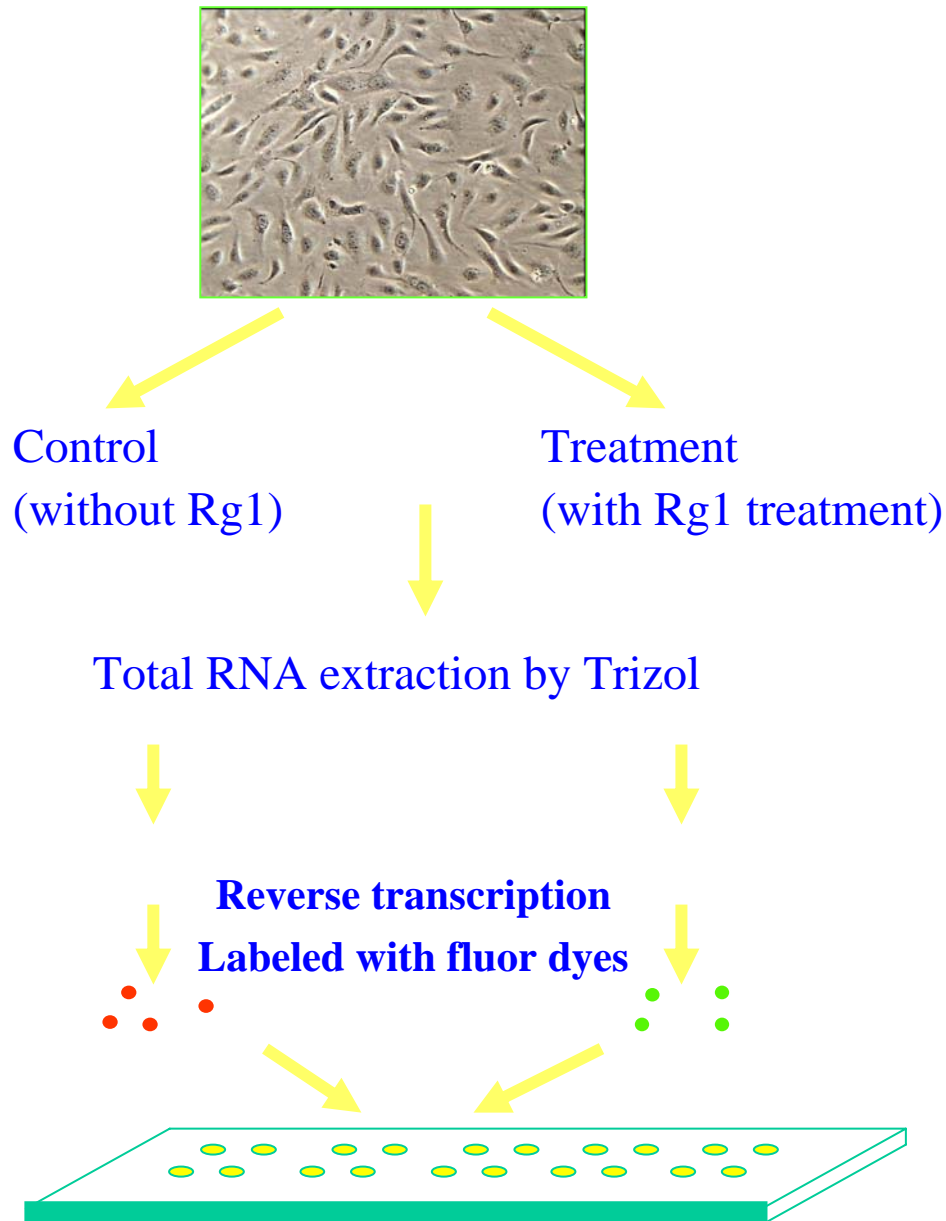
Array pattern

2 cm



Spot size ~ 200 μ m

Overviews of Microarray experiment



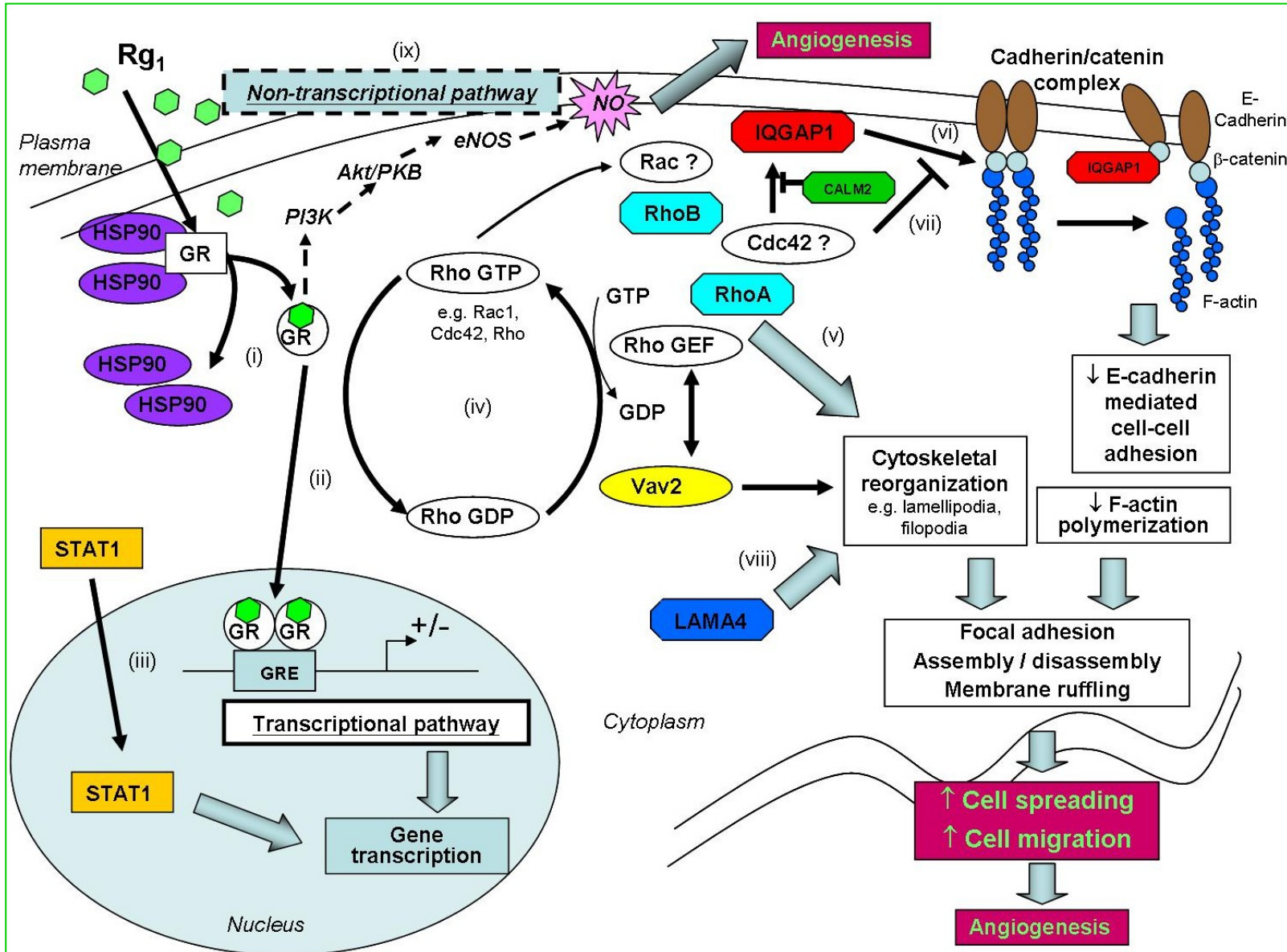
Gene expression profiling

Short Gene Name	Gene Name	Name	Average Cy3/Cy5 ratio	Categories
Aquaporin 9	Aquaporin 9	AB008775	3.7	blood inflammation
BRCA2	Breast cancer susceptibility (BRCA2) gene	U43746	2.7	cancer
VAV 2	VAV oncogene homolog	S76992	2.5	cancer
STAT1	Signal transducer and activator of transcription 1	M97935	2.4	transcription factors
ATM	Ataxia telangiectasia gene	U33841	2.3	cell cycle
C-yes-1	V-yes-1 Yamaguchi sarcoma viral oncogene homolog 1; C-yes-1	M15990	2.1	cancer
BRG1	SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily a, member 4	D26156	2	transcription factors
PAG-A	Proliferation-associated gene A (natural killer-enhancing factor A)	X67951	2	blood inflammation
LAMA4	Laminin alpha 4	NM_002290	2	neuro
Rho	Ras homolog gene family, member A; Rho	L09159	1.9	cancer
IQGAP1	Ras GTPase-activating-like protein; IQGAP1	L33075	1.8	cell cycle
Hsp89-alpha-delta-N	Hsp89-alpha-delta-N	AF028832	1.7	heat shock
HSP86	Heat shock protein HSP86	X07270	1.7	heat shock
Protein Kinase C-L	Protein kinase C-L	M55284	1.7	neuro
PRK2	Lipid-activated protein kinase PRK2	U33052	1.7	cell cycle
EB1	EB1	U24166	1.7	cancer
PAC1	Ual-specific phosphoprotein phosphatase	U23853	1.6	aging
90-kDa HSP	90-kDa heat-shock protein 1, beta	M16660	1.6	heat shock
RhoB	RhoB	X06820	1.6	aging
BRCA 1	Breast and ovarian cancer susceptibility	U14680	1.6	cancer
Thymidine kinase 2	Thymidine kinase 2 (TK2)	U77088	1.5	cell cycle
Calmodulin	Calmodulin (CALM2)	U94728	1.5	neuro

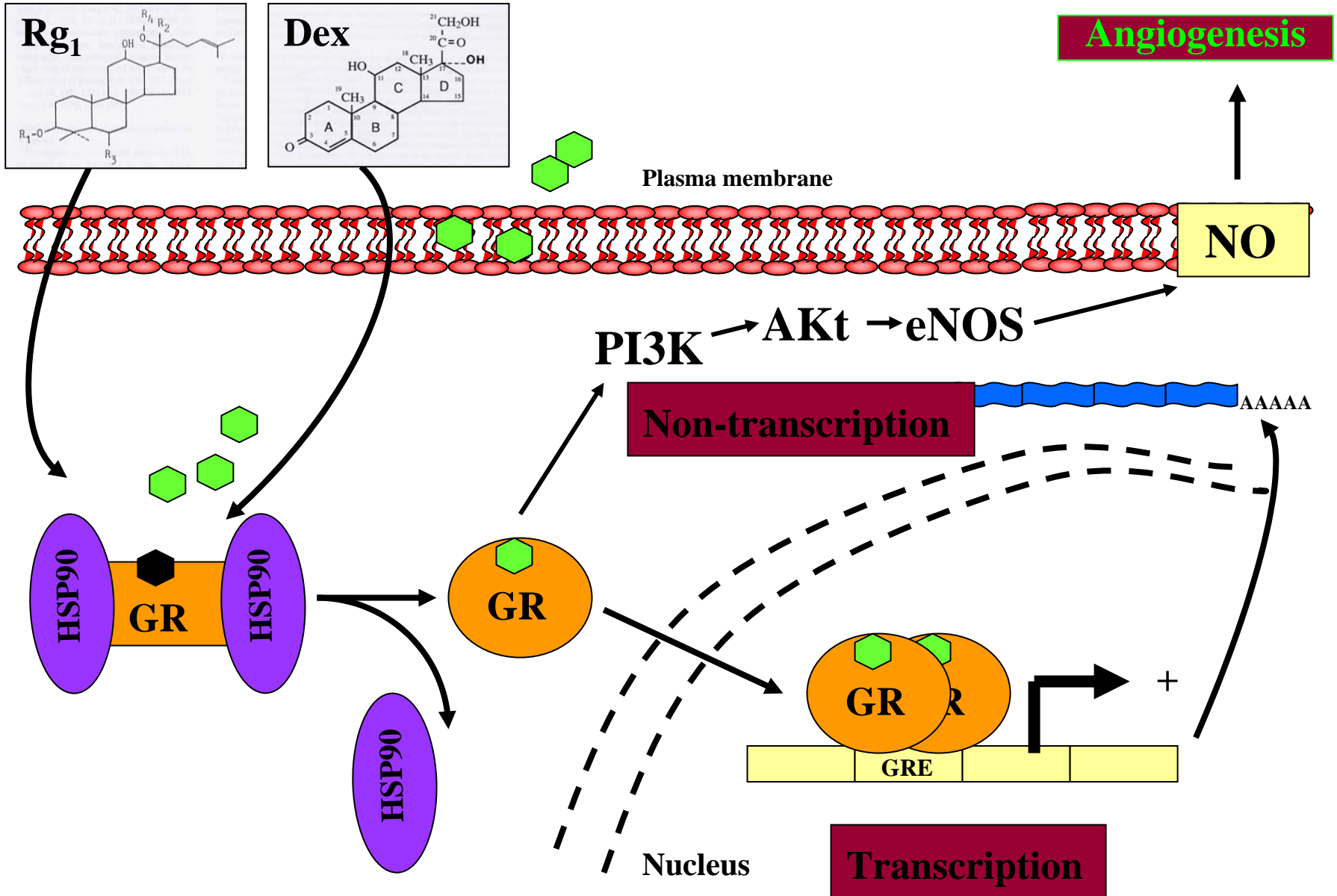
- related to cellular migration, proliferation, adhesion and cytoskeleton

Cell architectures and dynamics

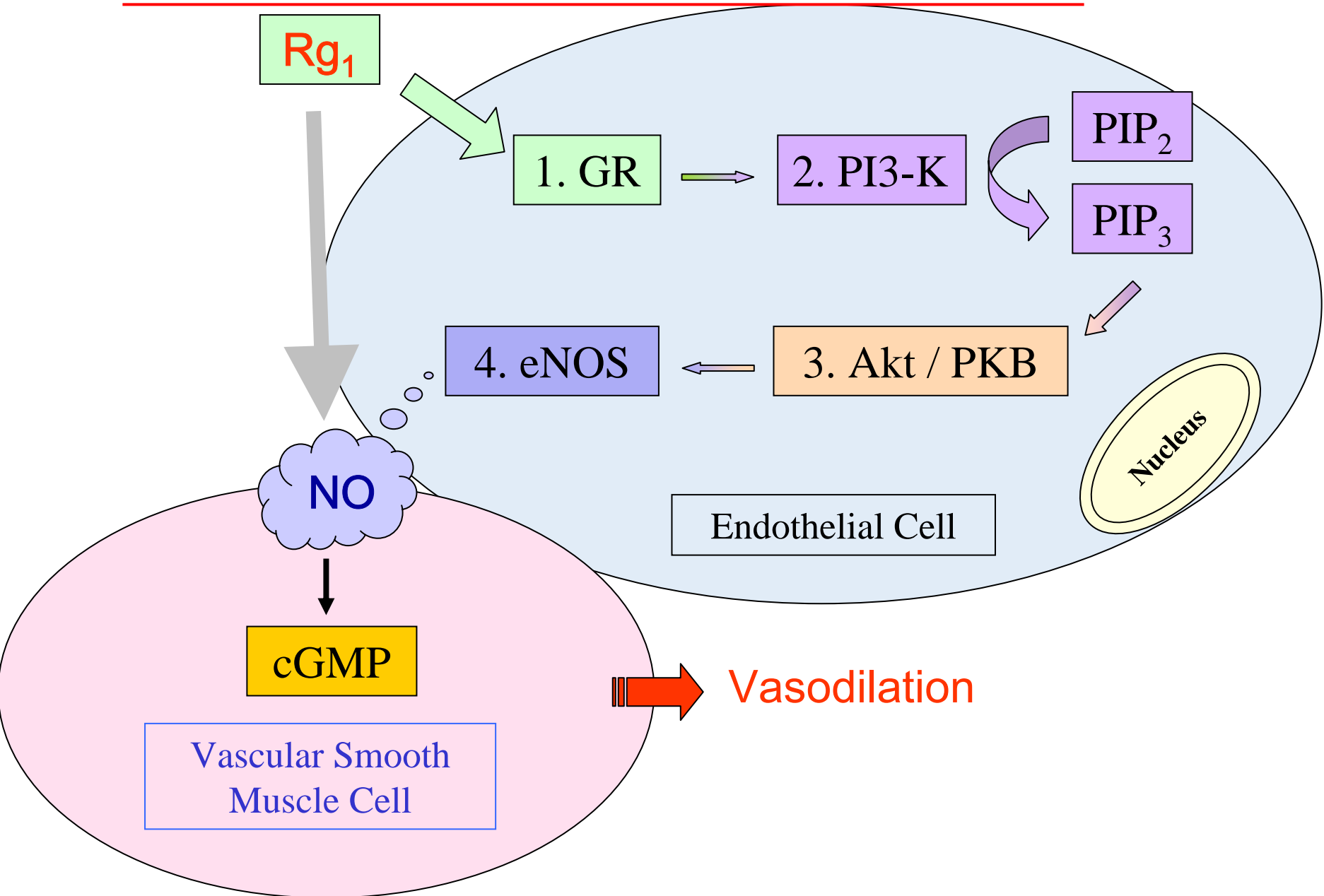
Gene expression profiling



Signaling pathway - transcriptional vs non-transcriptional

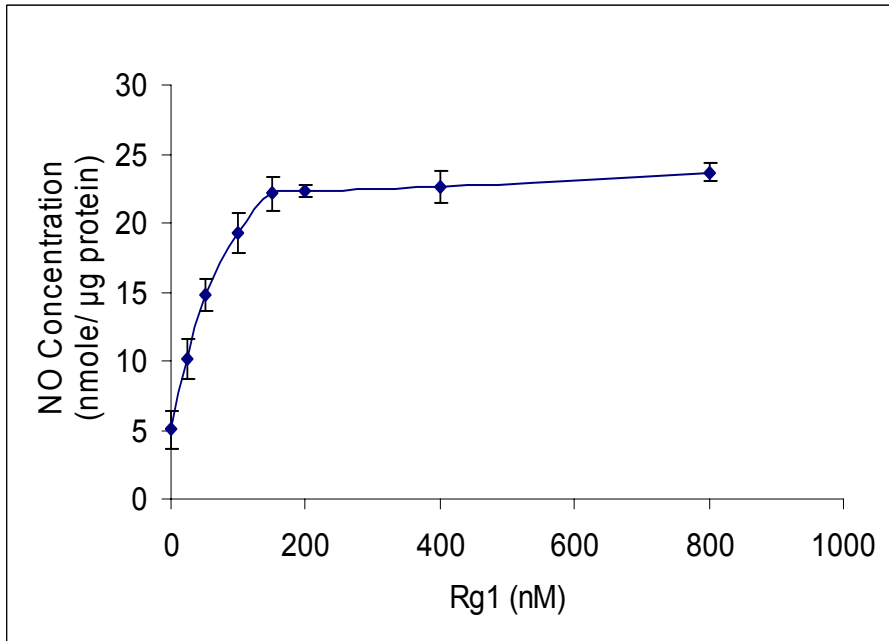


Rg₁-induced NO production

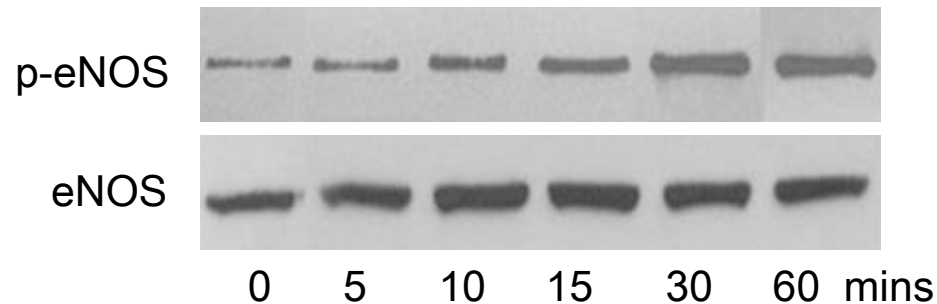
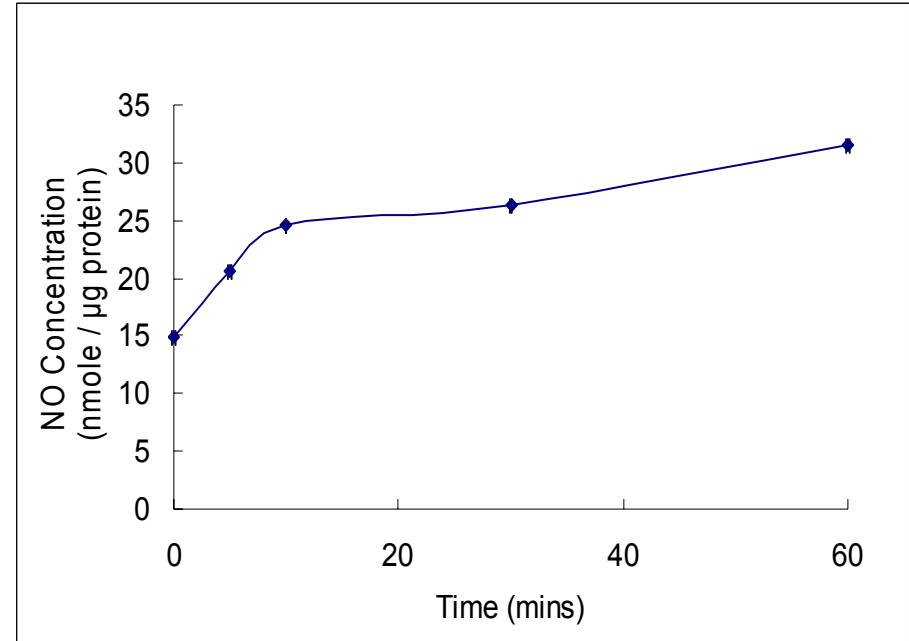


Rg1 induces NO production

A. Dose-dependent

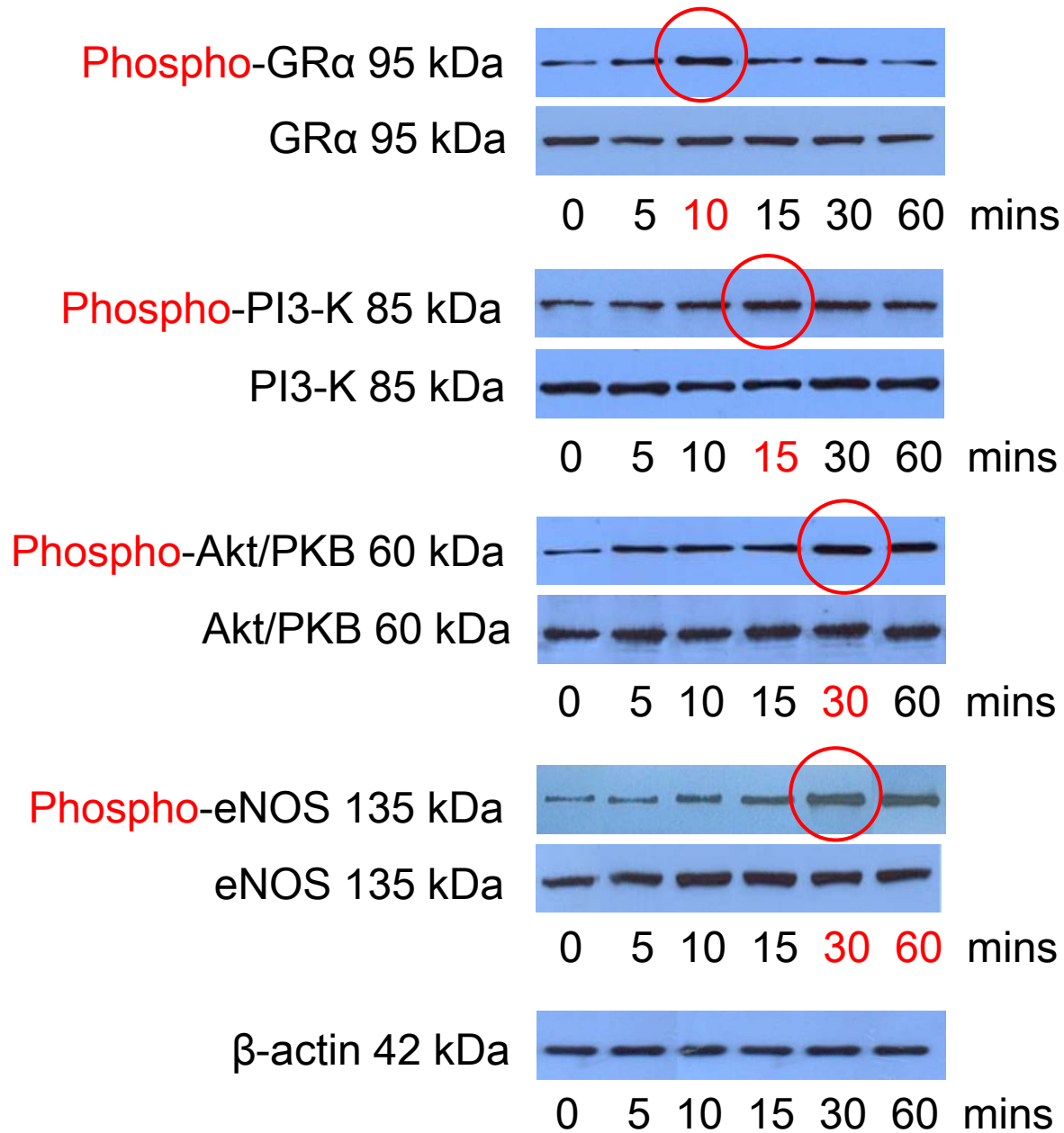


B. Time-dependent

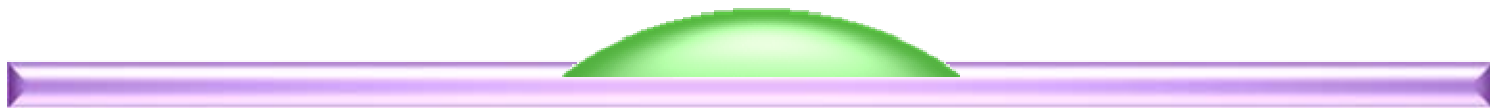
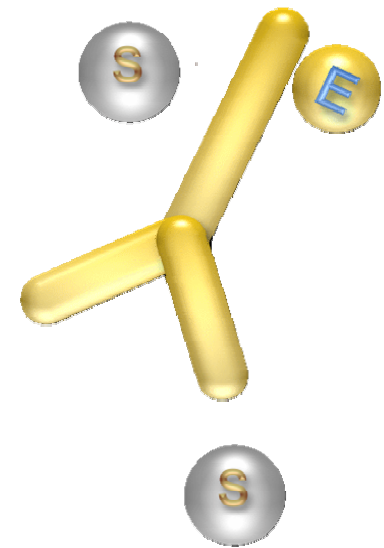
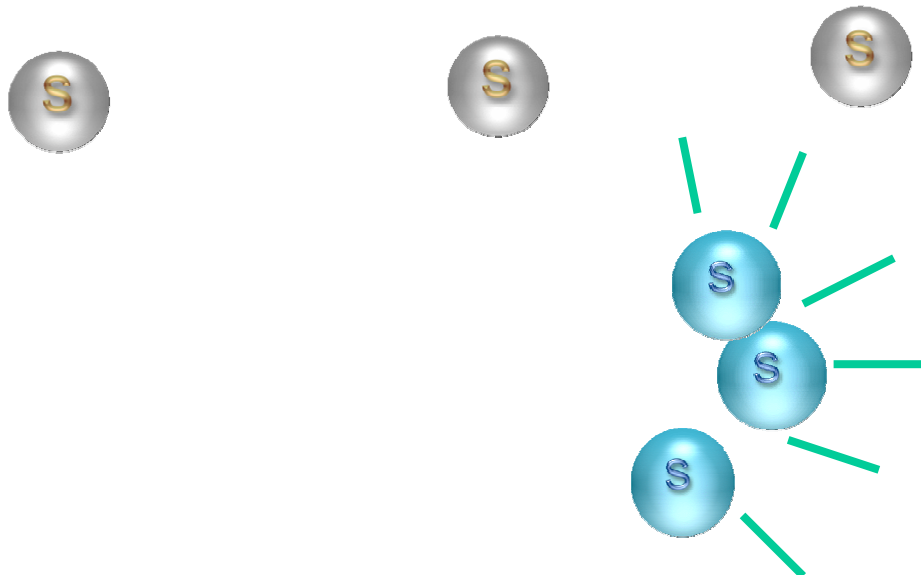
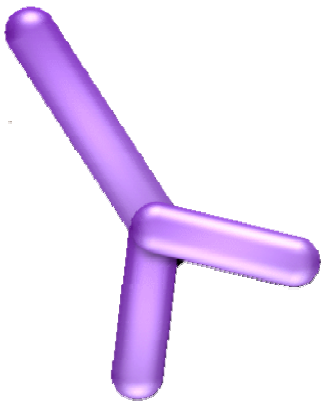


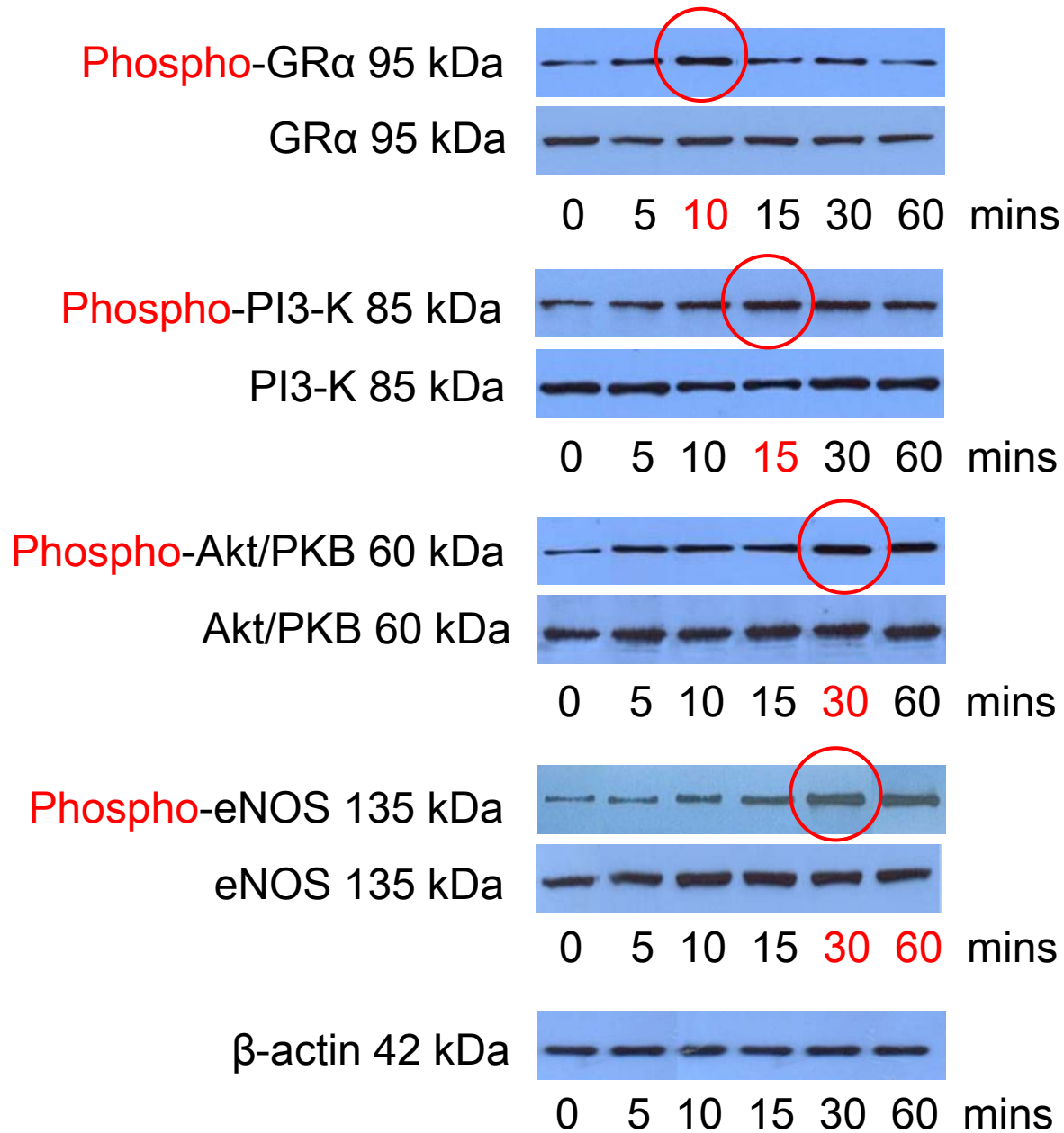
Results and Discussions 1

- **The NO concentration reached plateau at 150 nM and maintained up to 1200 nM.**
- **HUVEC was challenged with 150 nM Rg₁ for up to 60 mins, a significant increase in the total NO level was detected at 5 to 10 minutes after Rg₁ treatment.**
- **Therefore, 150 nM of Rg₁ treated for 5 and 10 mins were chosen for further studies.**



Immunodetection





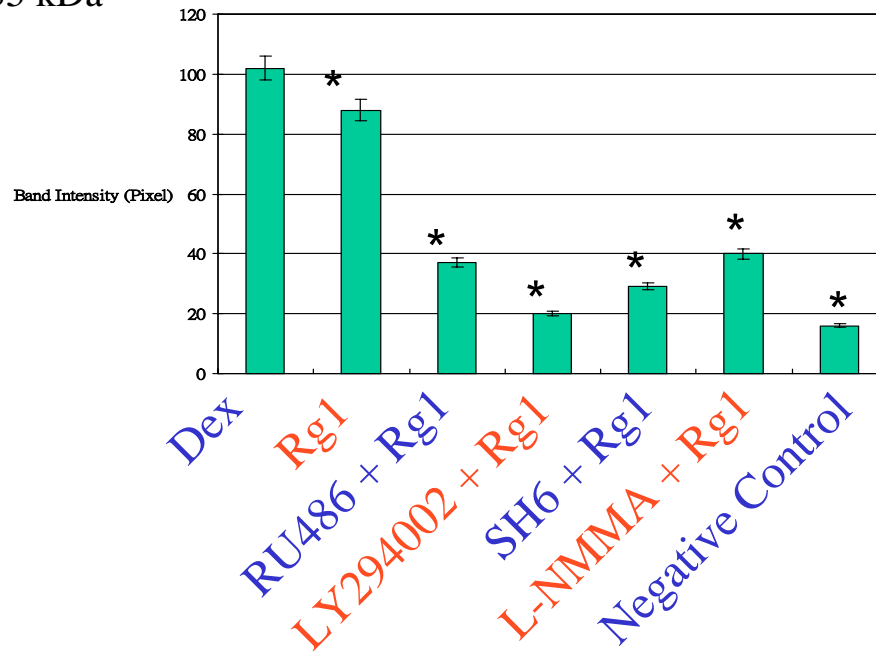
Results and Discussions 2

- **Activation of NO production was accompanied with the activation phosphorylation of eNOS at Ser1177.**
- **An approximately 2.5-fold increase in phosphorylation of eNOS was seen in the first 5 to 10 minutes after exposure of HUVEC to 150 nM Rg₁.**
- **the phosphorylation of GR; PI3-K and Akt/PKB were increased after treatment of 150 nM Rg₁ for 5 and 10 mins.**
- **Therefore, we conclude that Rg₁ can rapidly activate eNOS to produce NO in HUVEC.**

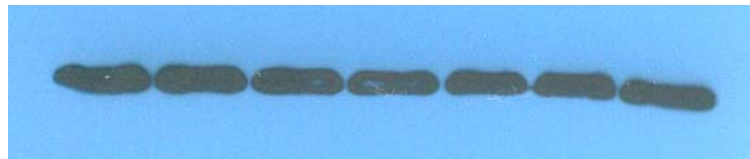
Phospho-eNOS (Ser1177) in the Presence of Inhibitors/ Antagonists

Phospho-eNOS

135 kDa



β -Actin 42 kDa

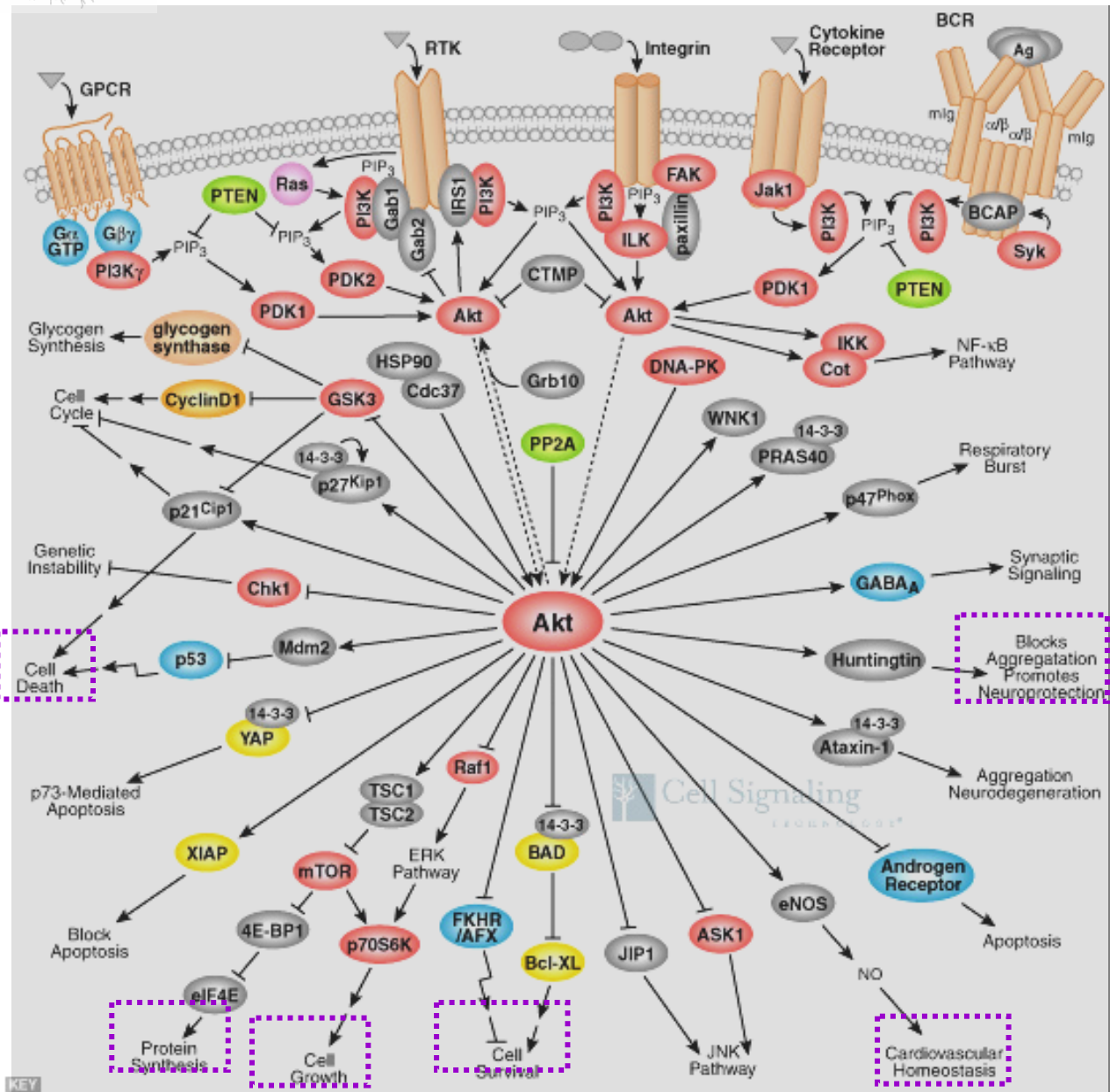


Results and Discussions 3

- **In the presence of RU486, LY294002 and SH6, Rg₁-induced eNOS phosphorylation at Ser1177 was abolished.**
- **Therefore, the involvement of GR and PI3-K/Akt pathway in eNOS activation is confirmed.**
- **Further, the functional role of GR and PI3-K/Akt pathway in Rg₁-induced NO production is examined by NO detection assays.**

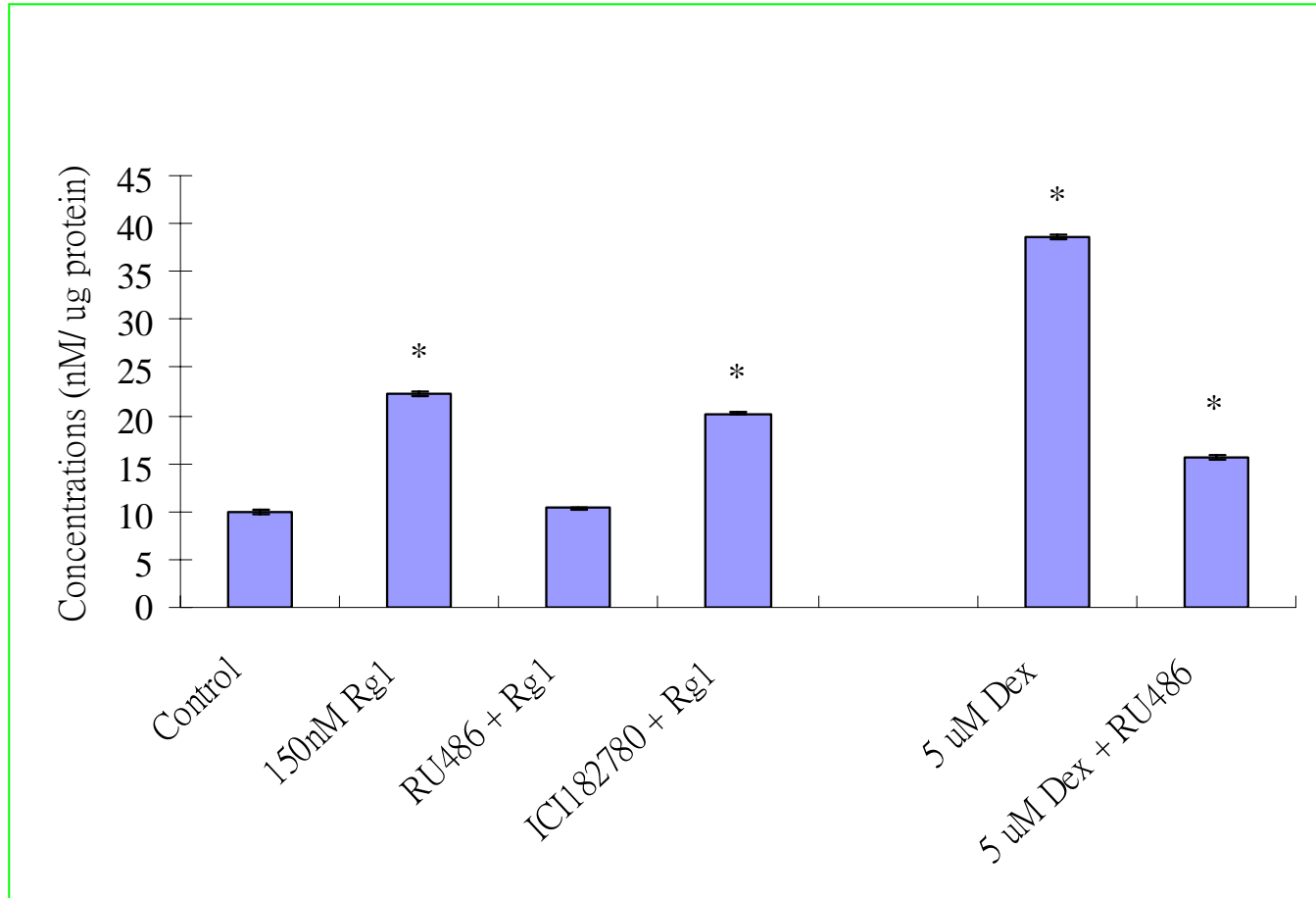


Akt/PKB Pathway – survival kinase



Binding of Rg1 to GR triggers the *PI3-K/Akt* pathway → diverse biological effects

Reduction of Rg₁-induced NO production by RU486 & ICI782180

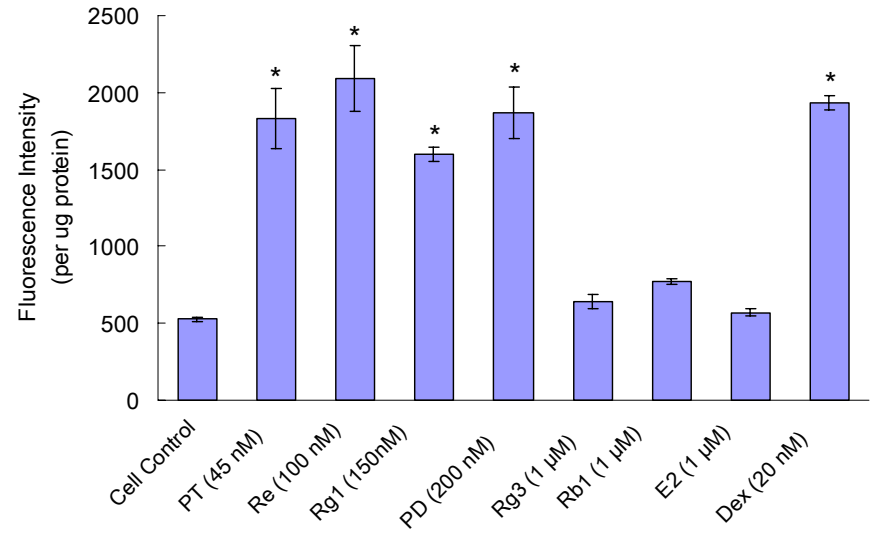
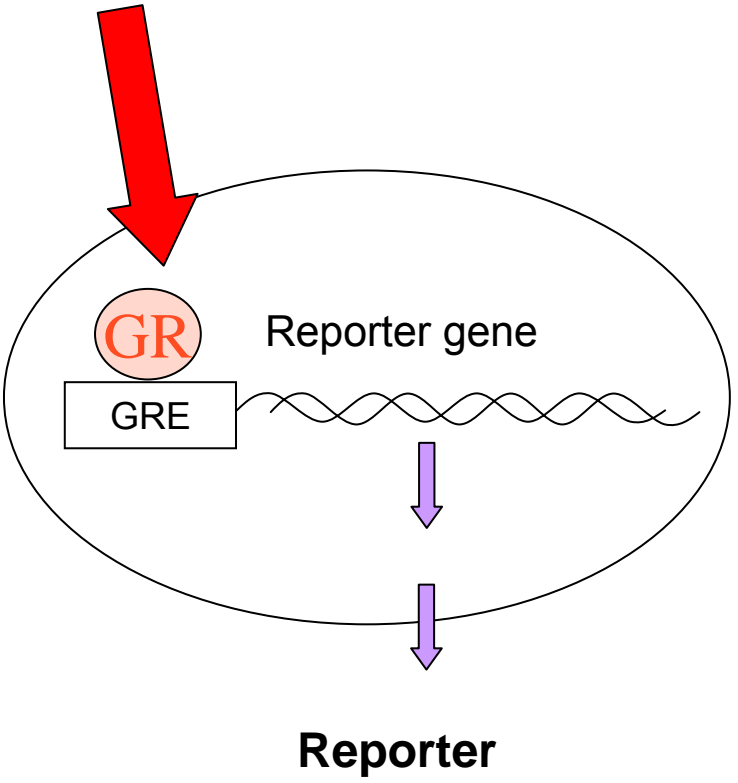
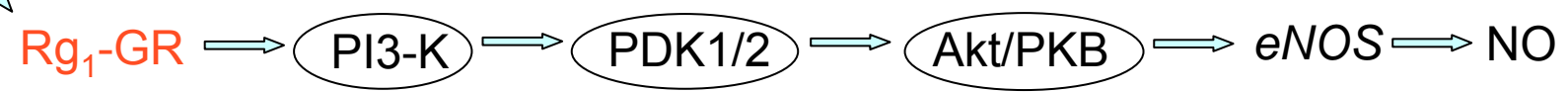


Results and Discussions 4

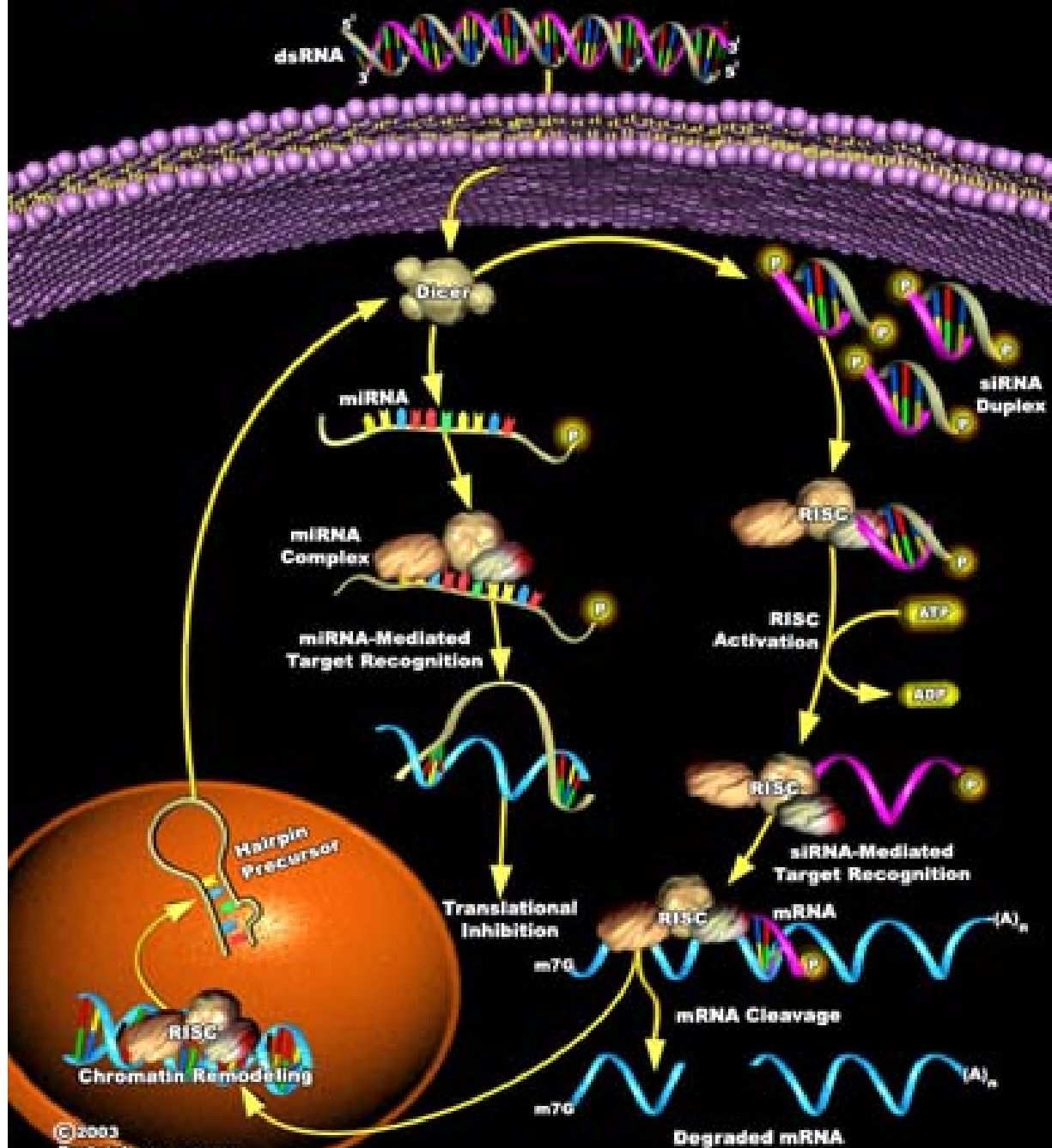
- **The production of NO was blocked by RU486, a GR antagonist.**
- **However, ICI182,780, an estrogen receptor (ER) antagonist, has no effect on Rg₁-induced NO production.**
- **These data indicates that GR is predominantly involved in Rg₁-induced eNOS phosphorylation activation and NO production.**
- **But ER plays no role in this issue.**



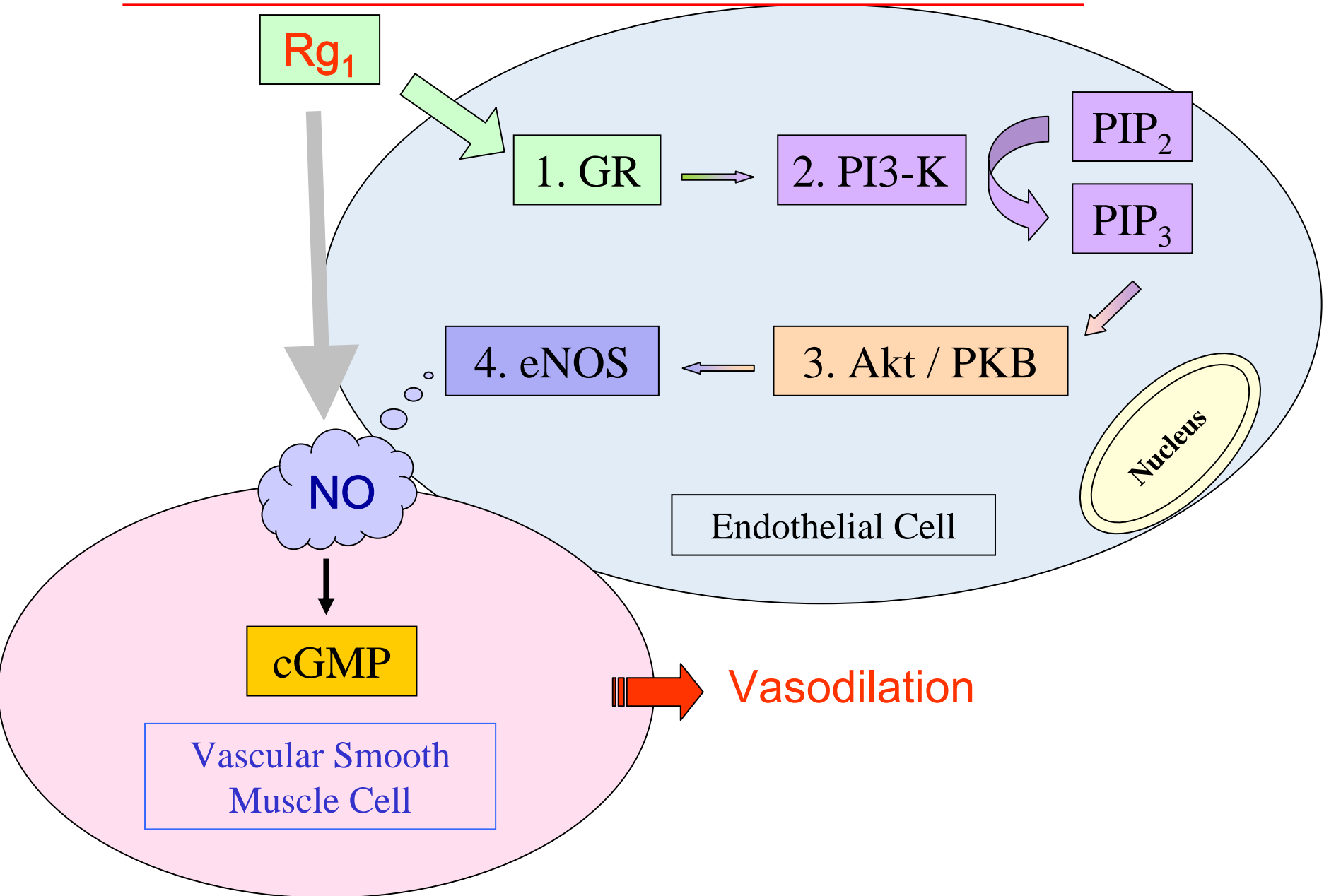
Induction of pGRE-SEAP in HUVEC by various Treatments



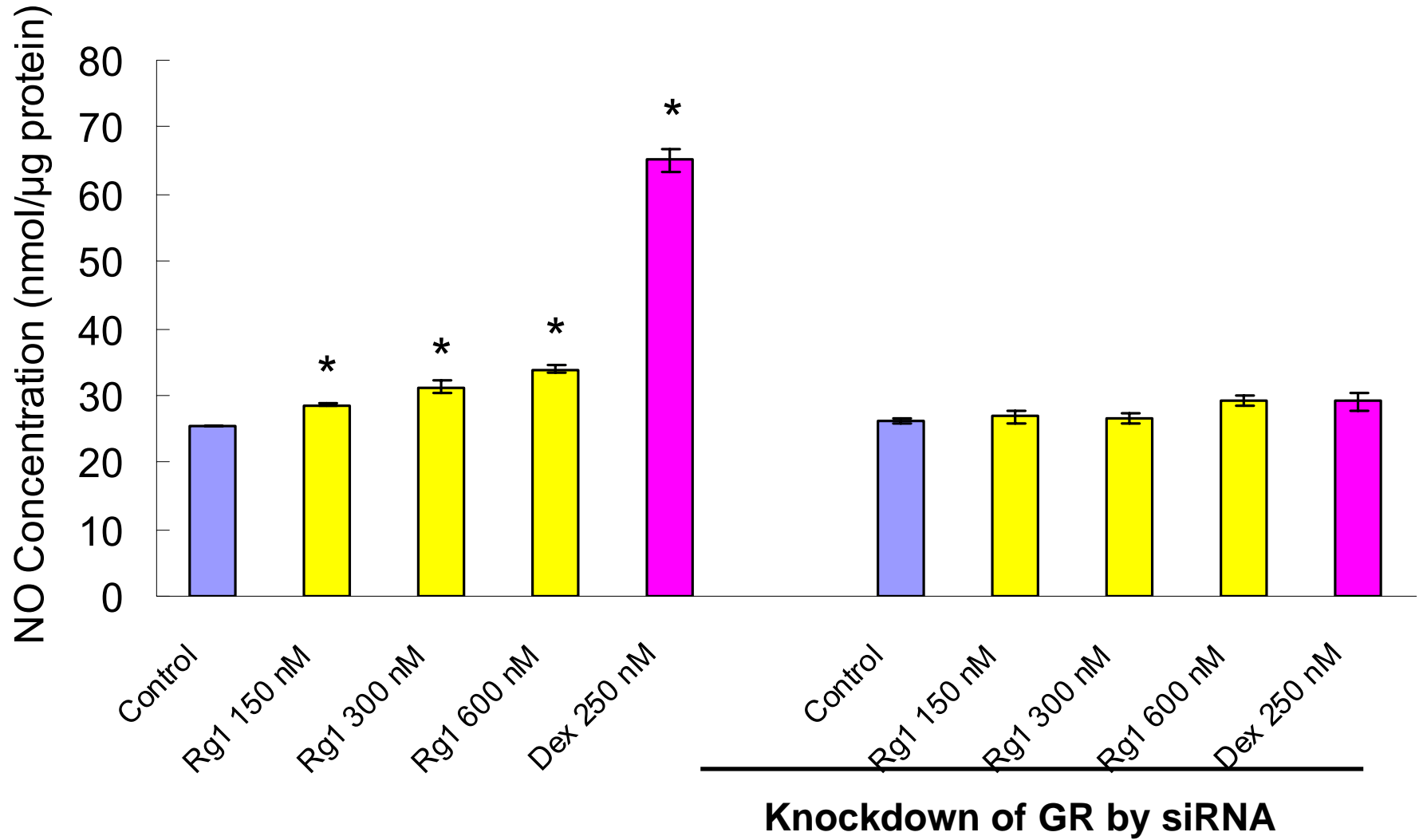
SMALL INTERFERING RNA PATHWAY



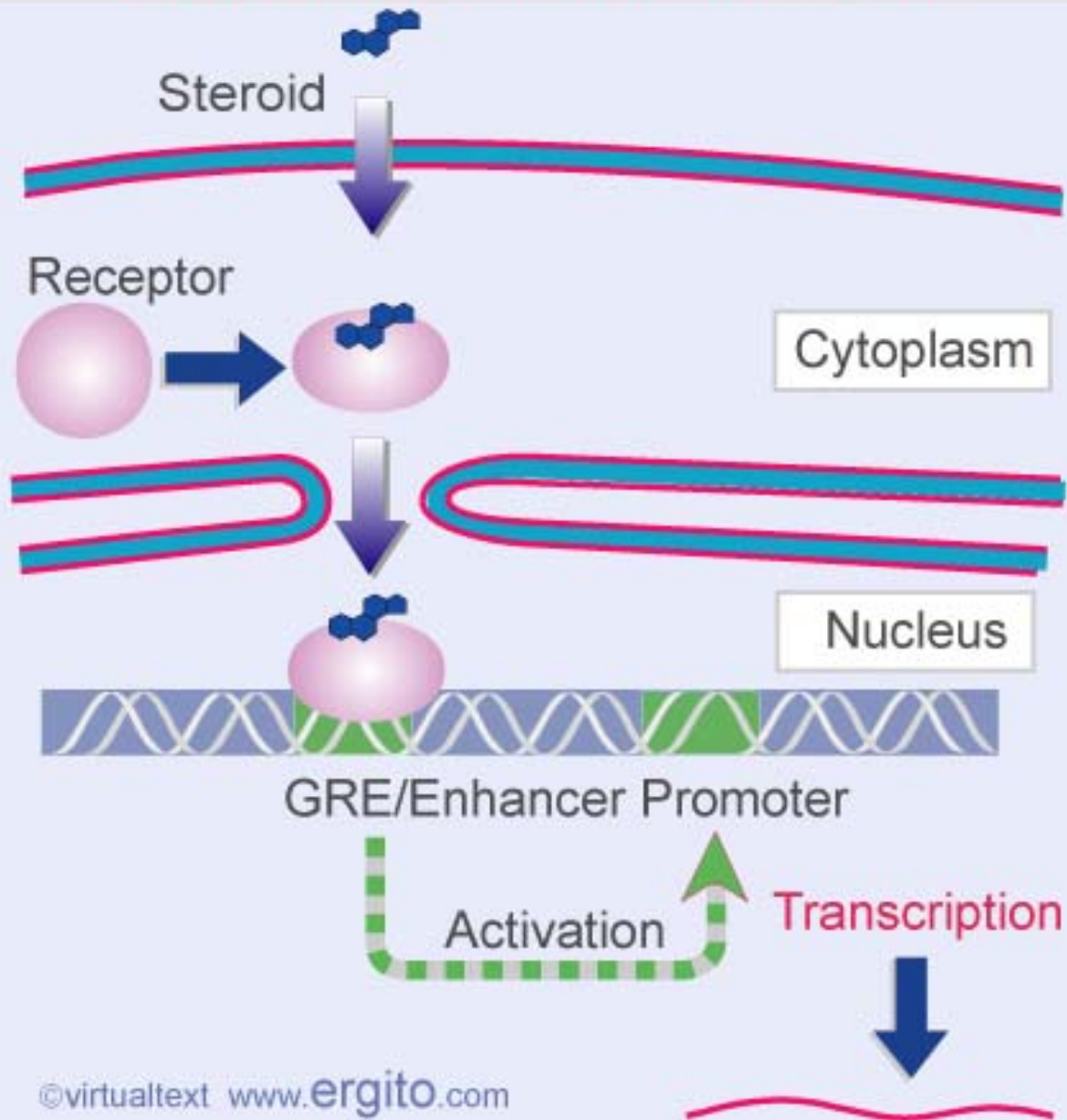
Rg₁-induced NO production



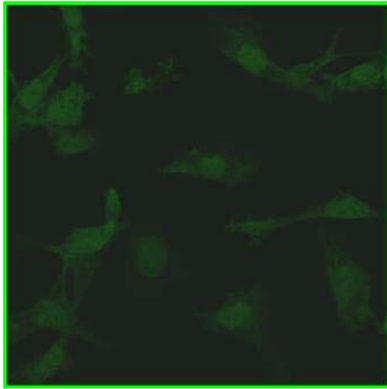
Application of small interference RNA (siRNA) to knockdown GR



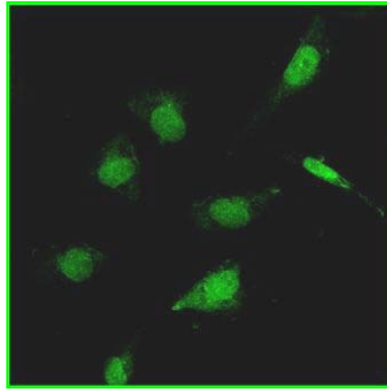
Ligands activate steroid receptors



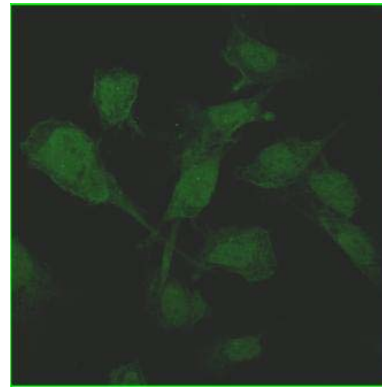
Localization of GR



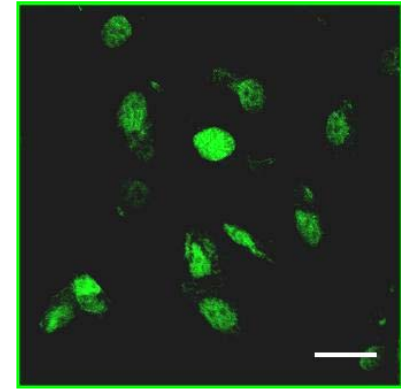
**Negative
Control**



150 nM Rg₁



RU486 + Rg₁



Dexamethazone

N/C: 1.34 (SD = ± 0.31)

3.03 (SD = ± 0.52)

1.27 (SD = ± 0.29)

10.29 (SD = ± 0.40)

Scale bar = 20 μ m

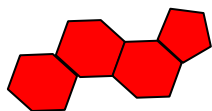
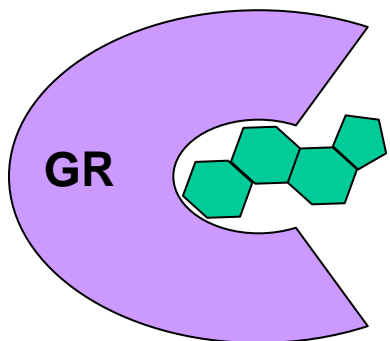
Valid to all micrographs

- HUVEC stained with Phospho - GR
- Confocal Microscopy 63X

GR- α Competition ligand-binding Assay

HIGH Polarization

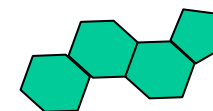
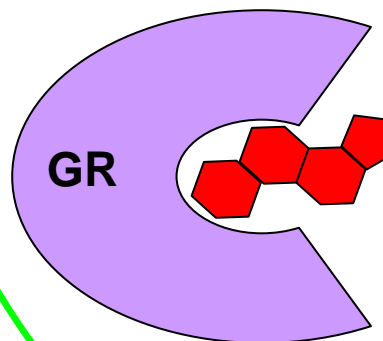
GR-Fluormone
Complex



Test Compounds:
Dex or Rg1



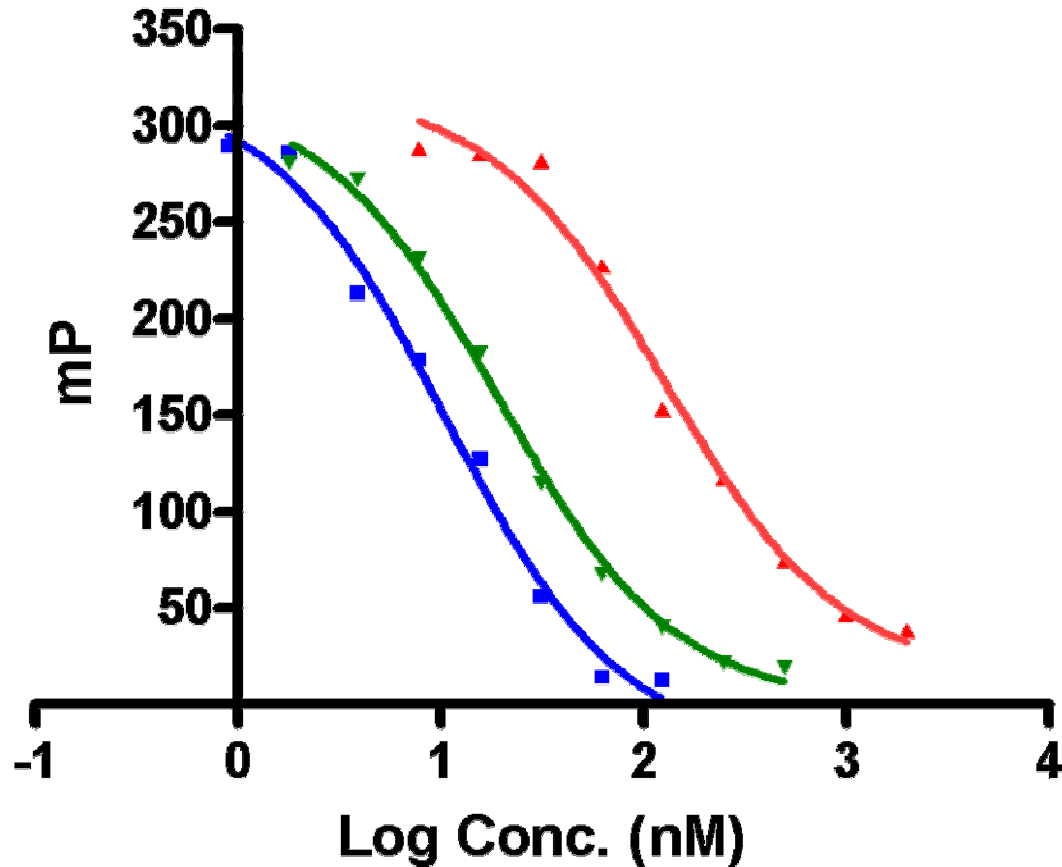
GR-Test Compound
Complex



Fluormone
Displaced

LOW Polarization

GR-alpha Competition Assay (n=3)

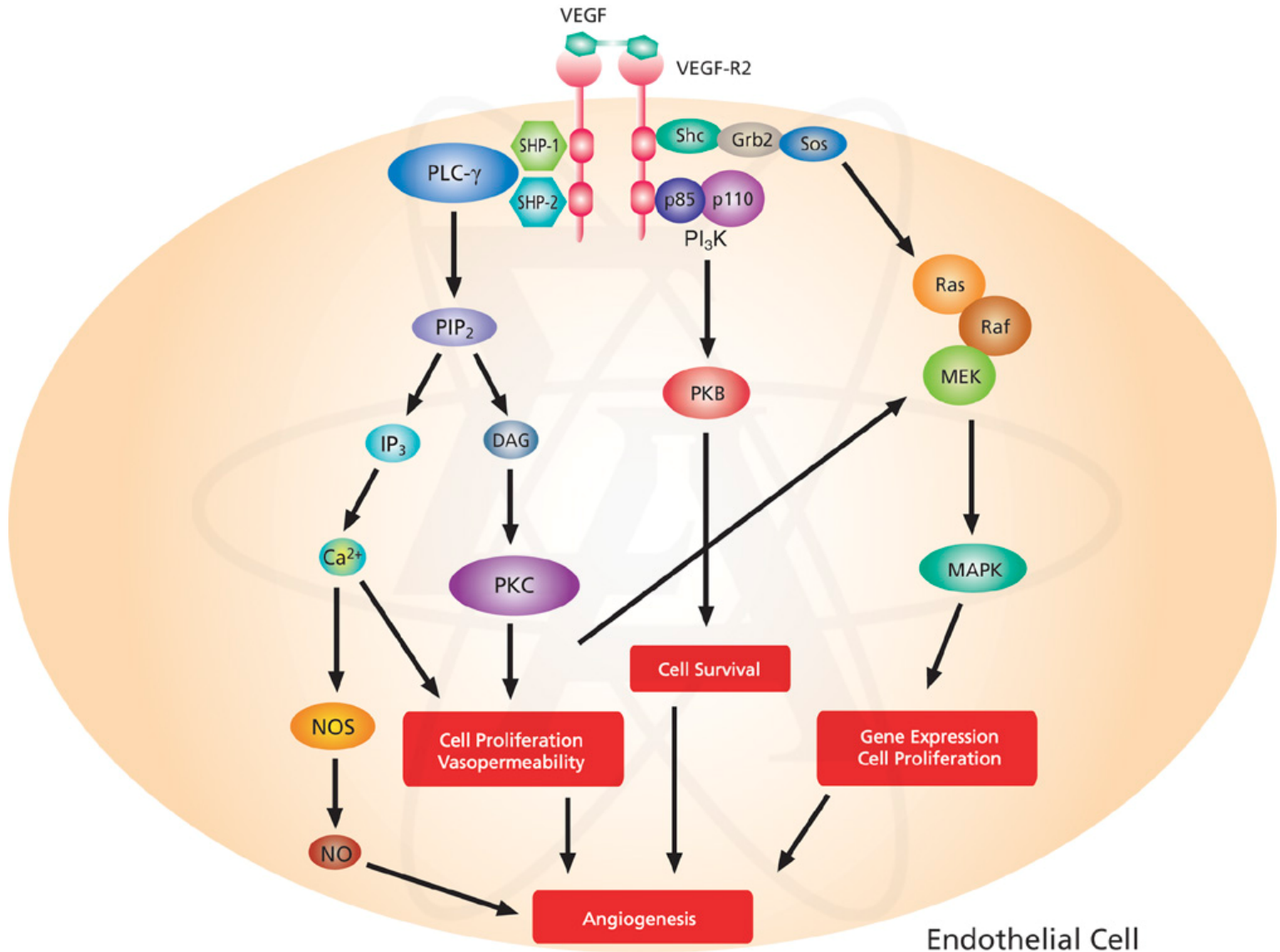


■ Dex
▲ Rg1
▼ RU

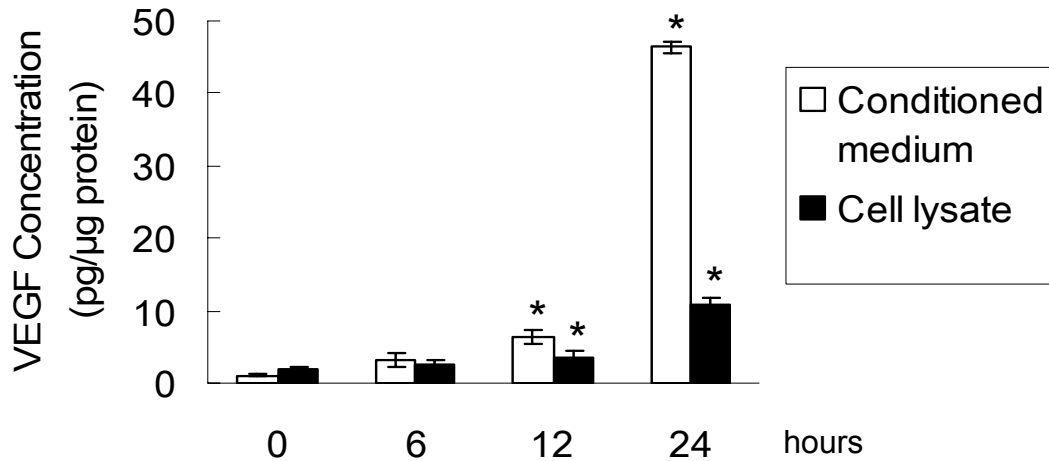
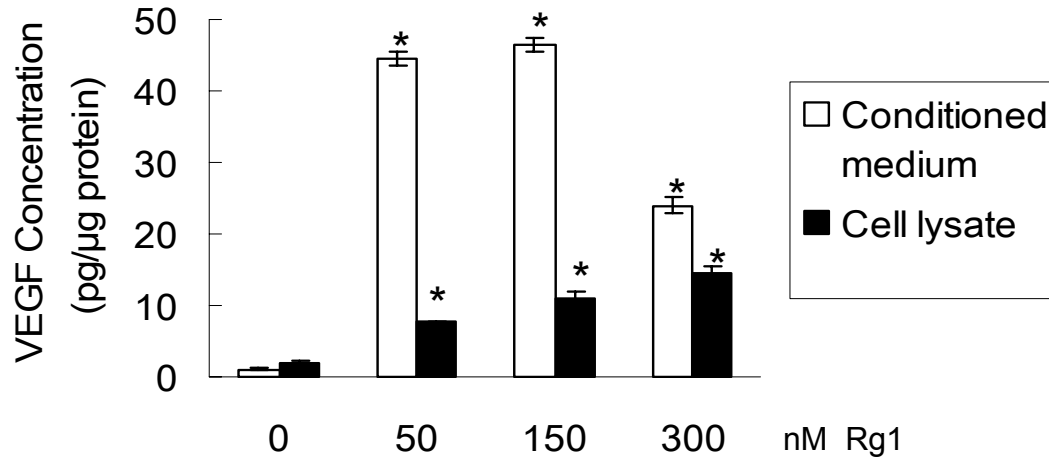
	Dex	RU	Rg1
K_d (nM)	3.35	7.52	39.40
IC_{50} (nM)	10.55	23.68	128.50
R^2	0.99	0.99	0.98

Signaling Pathways Activated by VEGF

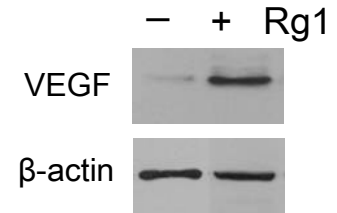
SIGMA-ALDRICH



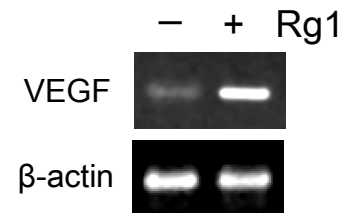
Rg1 induces VEGF production



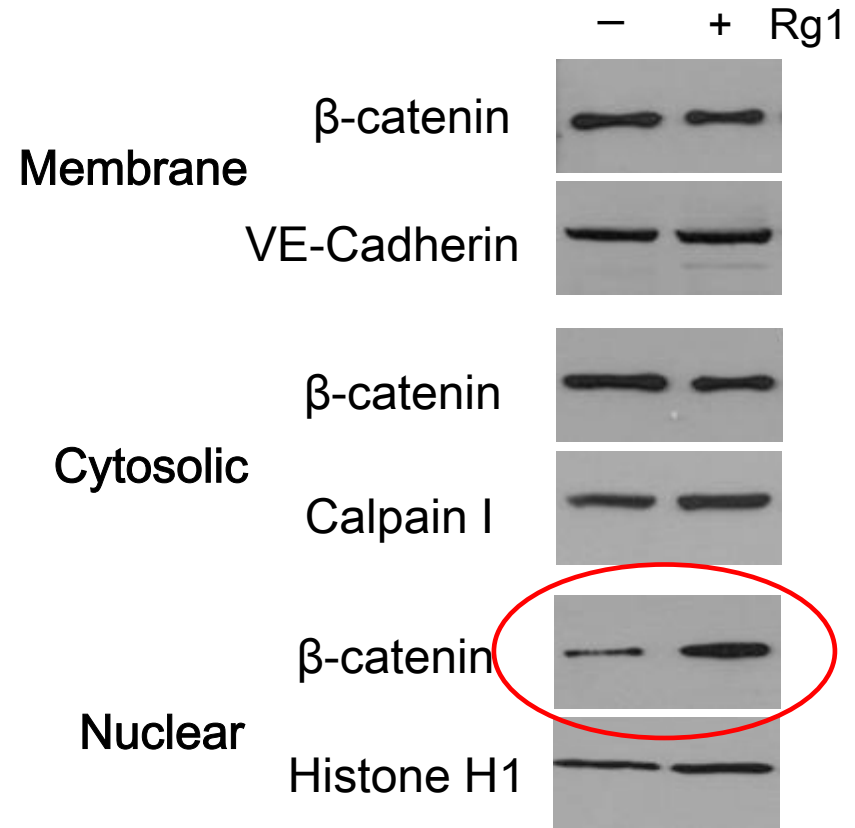
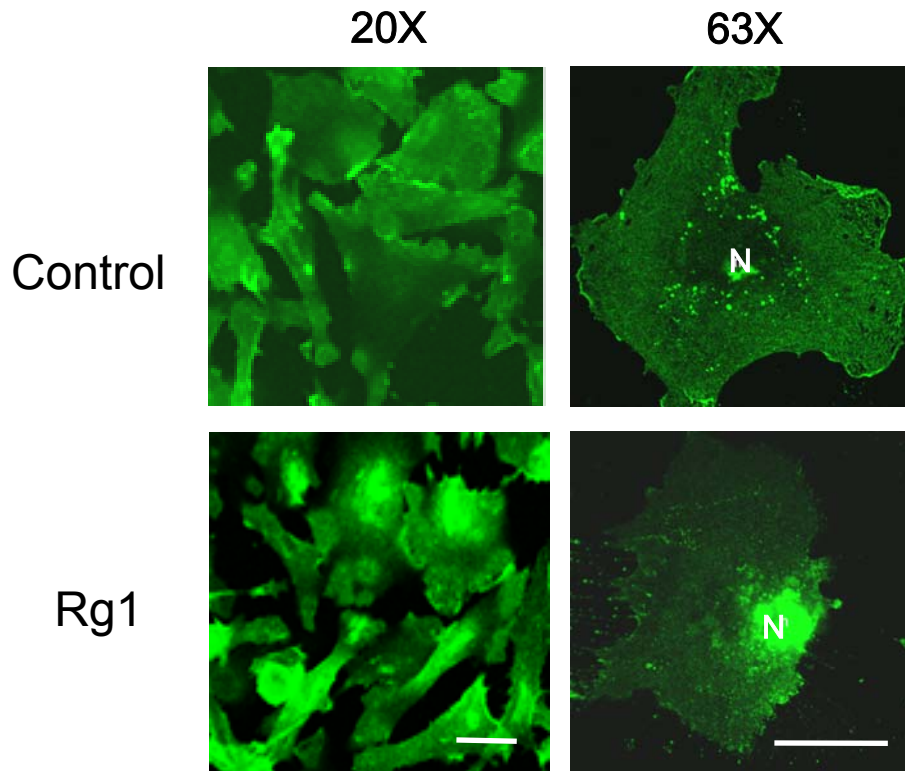
Western blot



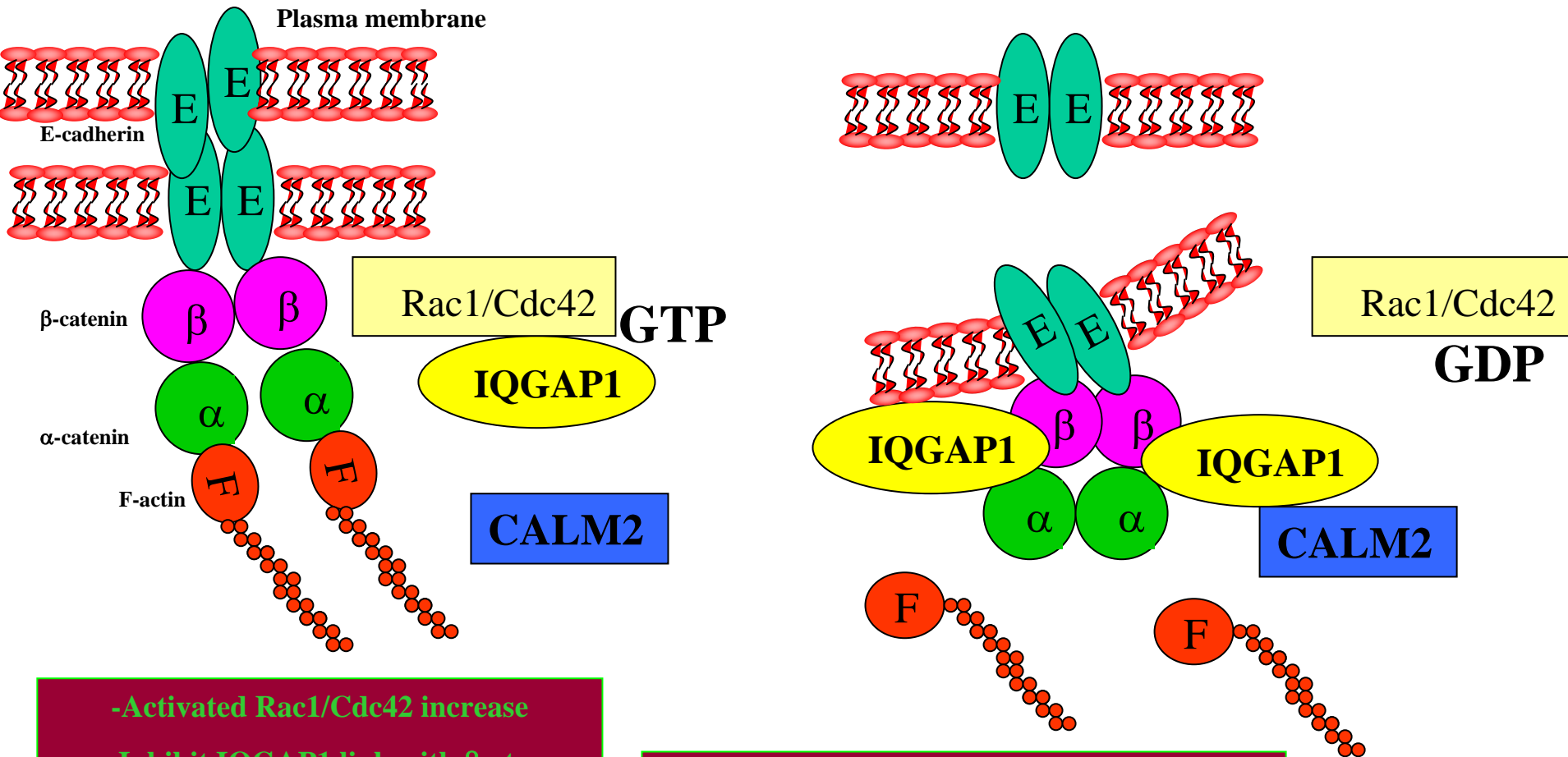
RT-PCR



β -catenin translocates into the nucleus upon Rg1 treatment

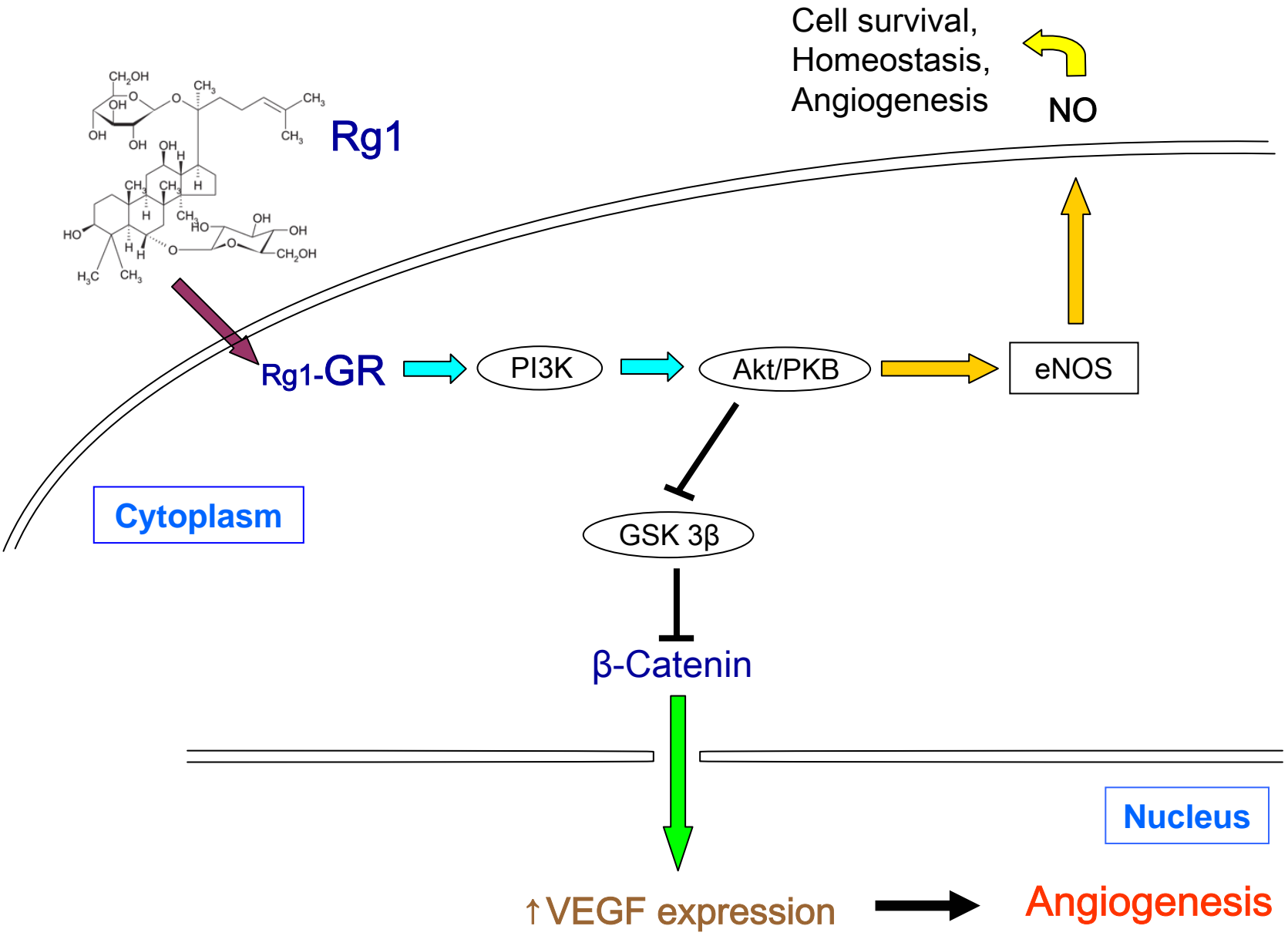


Cytomechanics - cadherin-mediated cell-cell adhesion

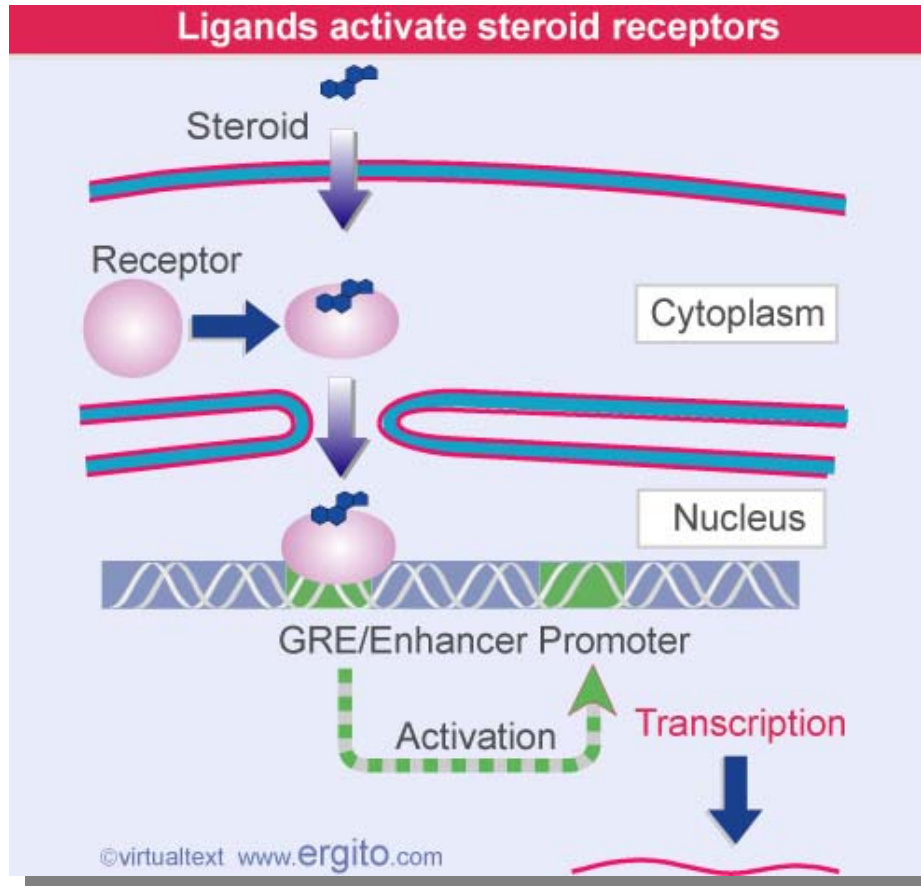


- Activated Rac1/Cdc42 increase
- Inhibit IQGAP1 link with β -ctn
- β -ctn, α -ctn link with E-cadherin to F-actin
- E-cadherin- β ctn- IQGAP1 complex
- weaken cell-cell adhesion

- CALM2 bind with IQGAP1
- Interrupts its association with Cdc42 and F-actin
- Interrupts the cadherin-catenin complex
- weaken cell-cell adhesion



Steroid Receptors



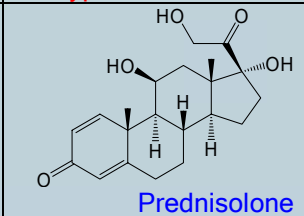
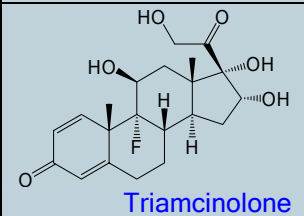
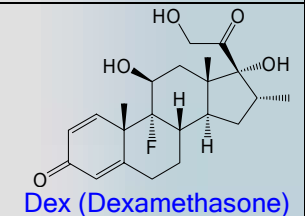
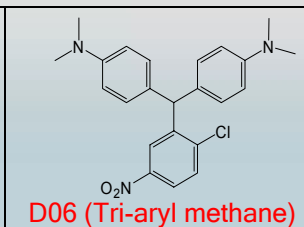
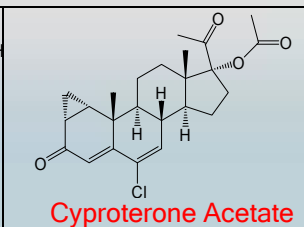
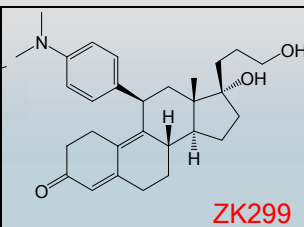
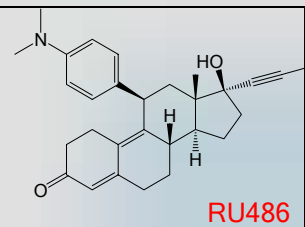
- Glucocorticoid
- Estrogen
- Progesterone
- Mineralocorticoid
- Testosterone

More than 30 ginsenosides have been identified.

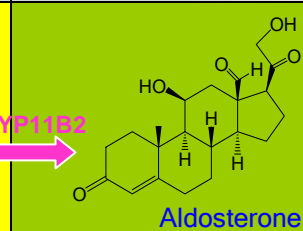
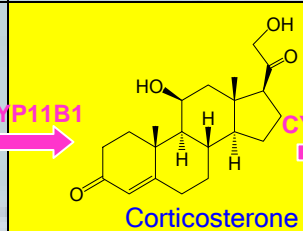
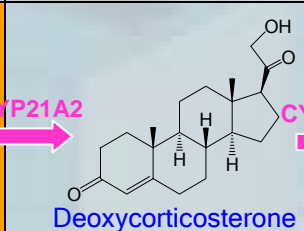
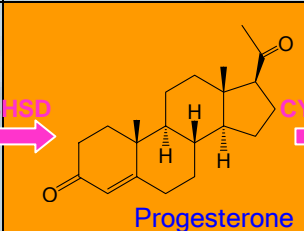
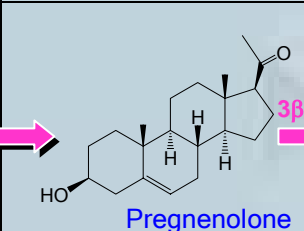
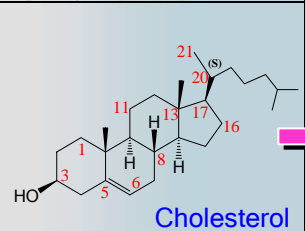
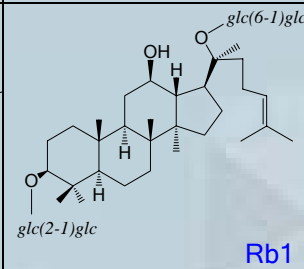
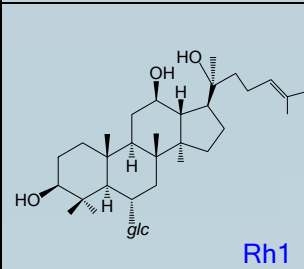
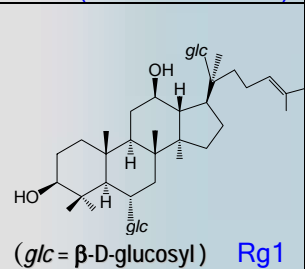
Molecular Modeling of Ginsenoside-Nuclear Receptor Interaction



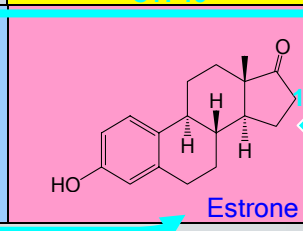
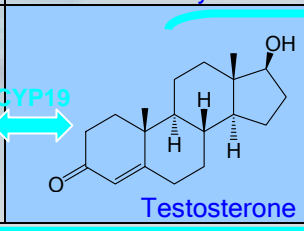
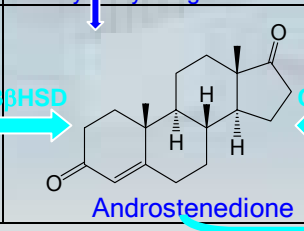
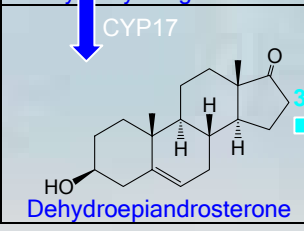
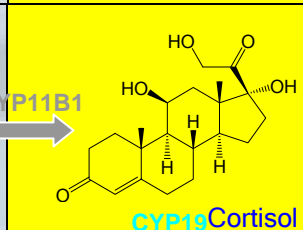
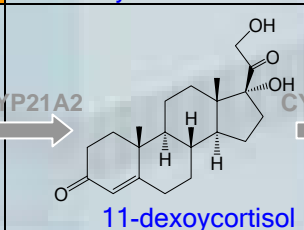
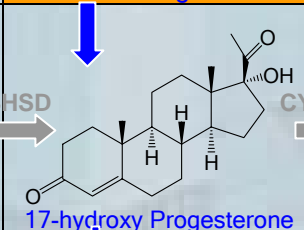
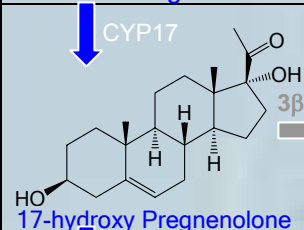
12 September 2005



Biosynthesis of Steroids



major progestagen
major mineralocorticoid
major glucocorticoid
major gonadal estrogens
major gonadal androgen

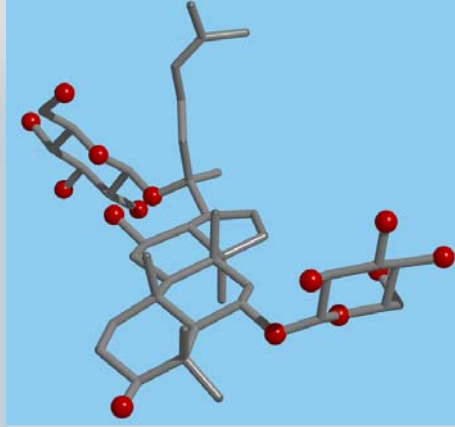


CYP19

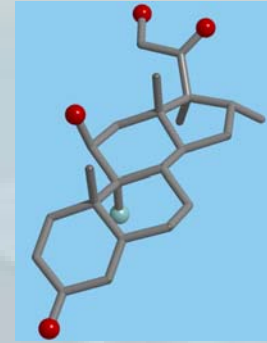
3D Schematics

red balls = O or OH

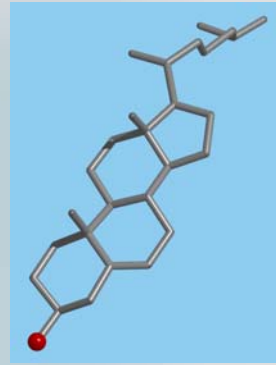
blue ball = F



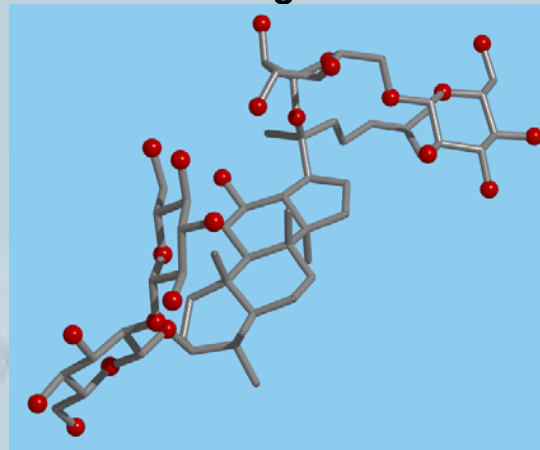
Rg1



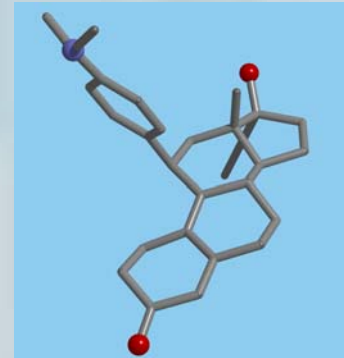
Dex



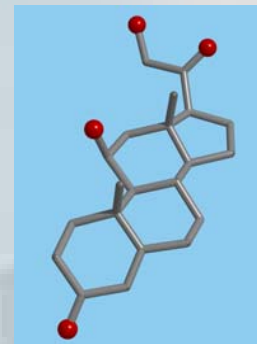
Cholesterol



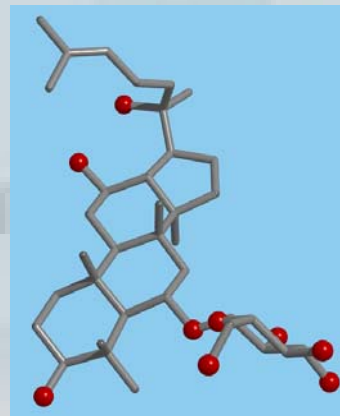
Rb1



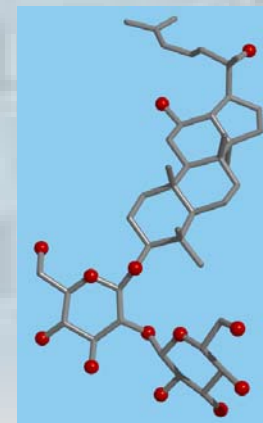
RU486



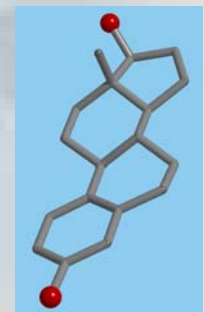
Corticosterone



Rh1



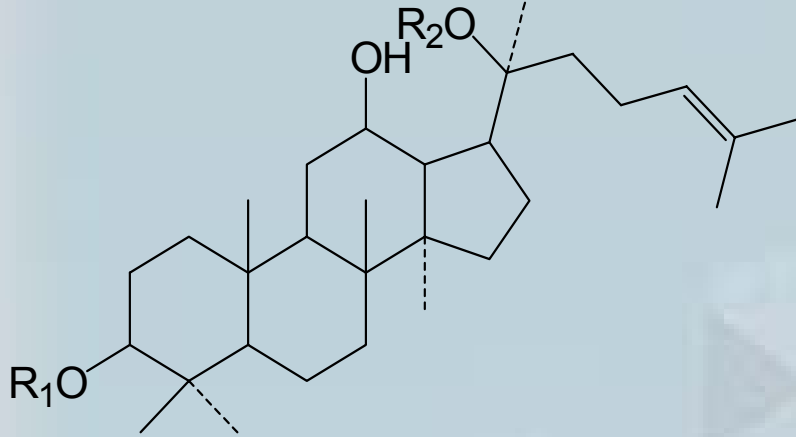
Rg3



Estradiol **56**

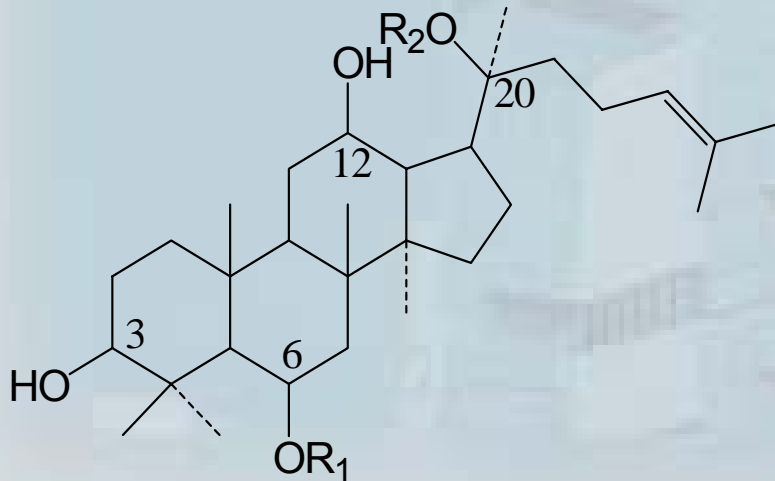
Steroidal Skeleton of Ginsenosides (GS's)

20(S)-Protopanaxadiols (PPD)



Ginsenoside	R ₁	R ₂
Rb₁	<i>disac</i>	<i>disac</i>
Rb₂	<i>disac</i>	<i>disac</i>
Rc	<i>disac</i>	<i>disac</i>
Rd	<i>disac</i>	<i>msac</i>
Rg₃	<i>disac</i>	H
Rh₂	<i>msac</i>	H
Rh₃	<i>msac</i>	

20(S)-Protopanaxatriols (PPT)



Ginsenoside	R ₁	R ₂
Re	<i>disac</i>	<i>msac</i>
Rf	<i>disac</i>	H
Rg₁	<i>msac</i>	<i>msac</i>
Rg₂	<i>disac</i>	H
Rh₁	<i>disac</i>	H

Glucocorticoid Receptor (GR) Dexamethasone

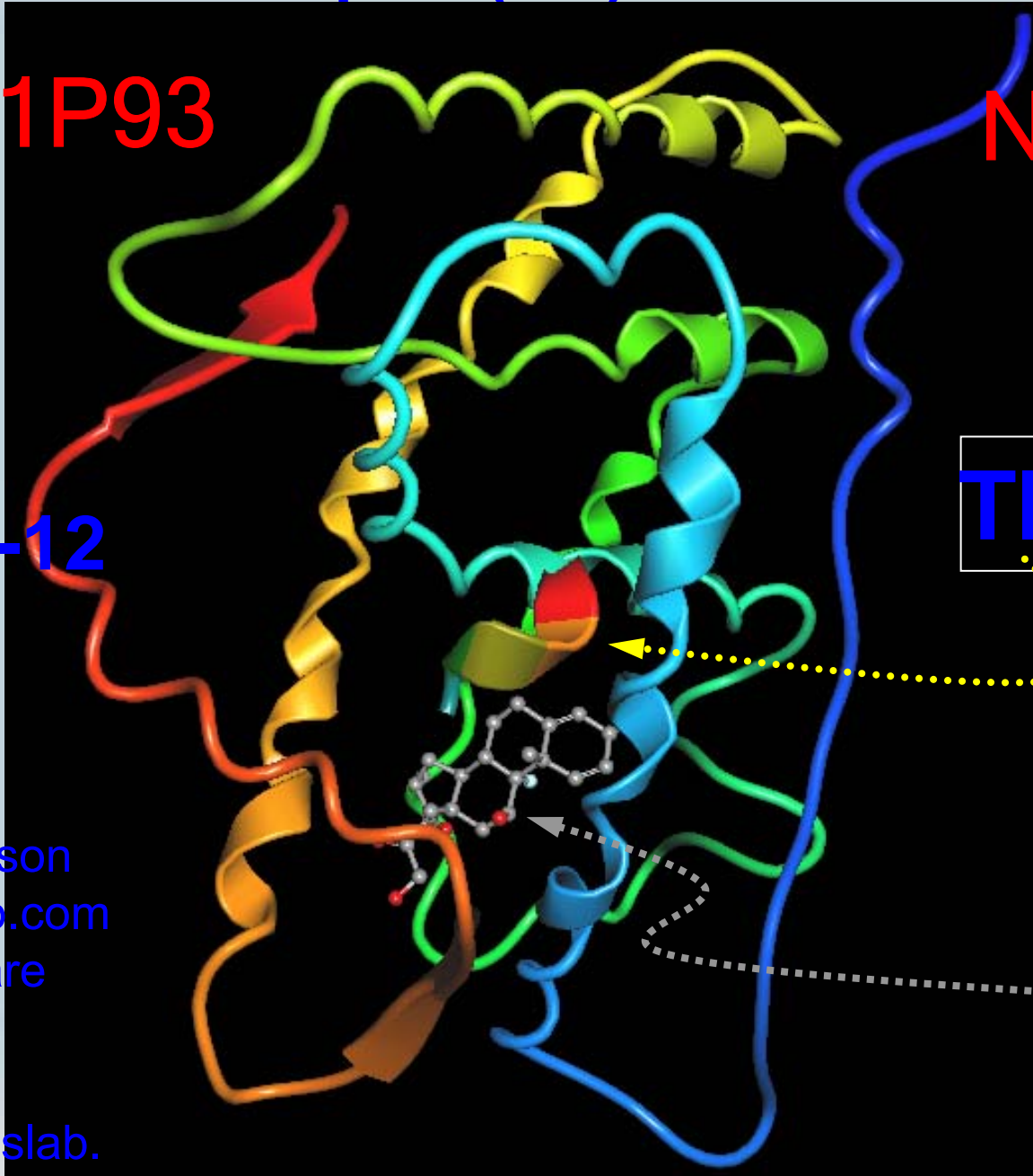
PDB: 1P93

N-Ter

Helix 11-12

TIF

Dex

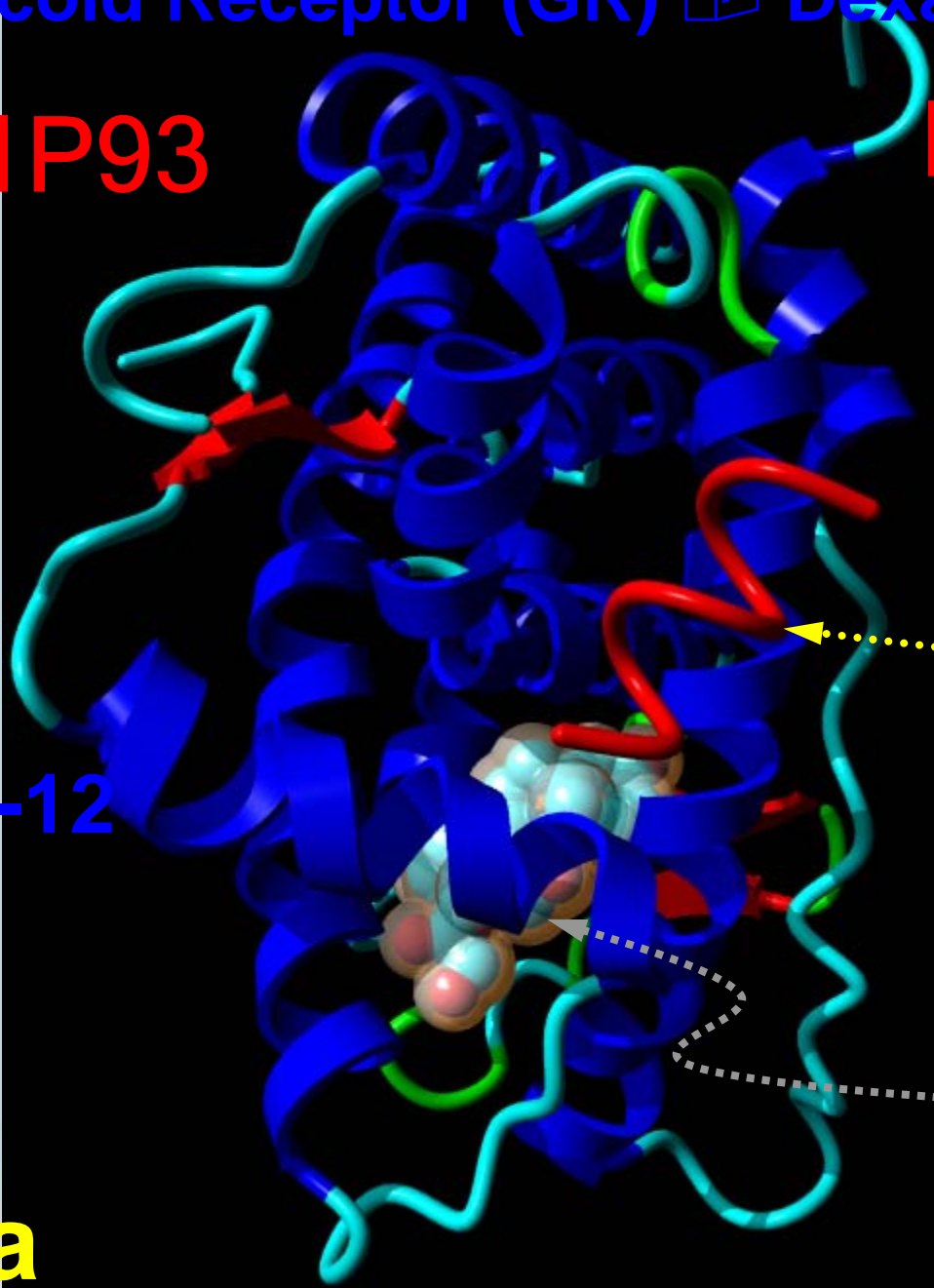


ArgusLab 4.0.1
Mark A. Thompson
mark@arguslab.com
Planaria Software
LLC,
Seattle, WA
<http://www.arguslab.com>

Glucocorticoid Receptor (GR) Dexamehasone

PDB: 1P93

N-Ter



TIF

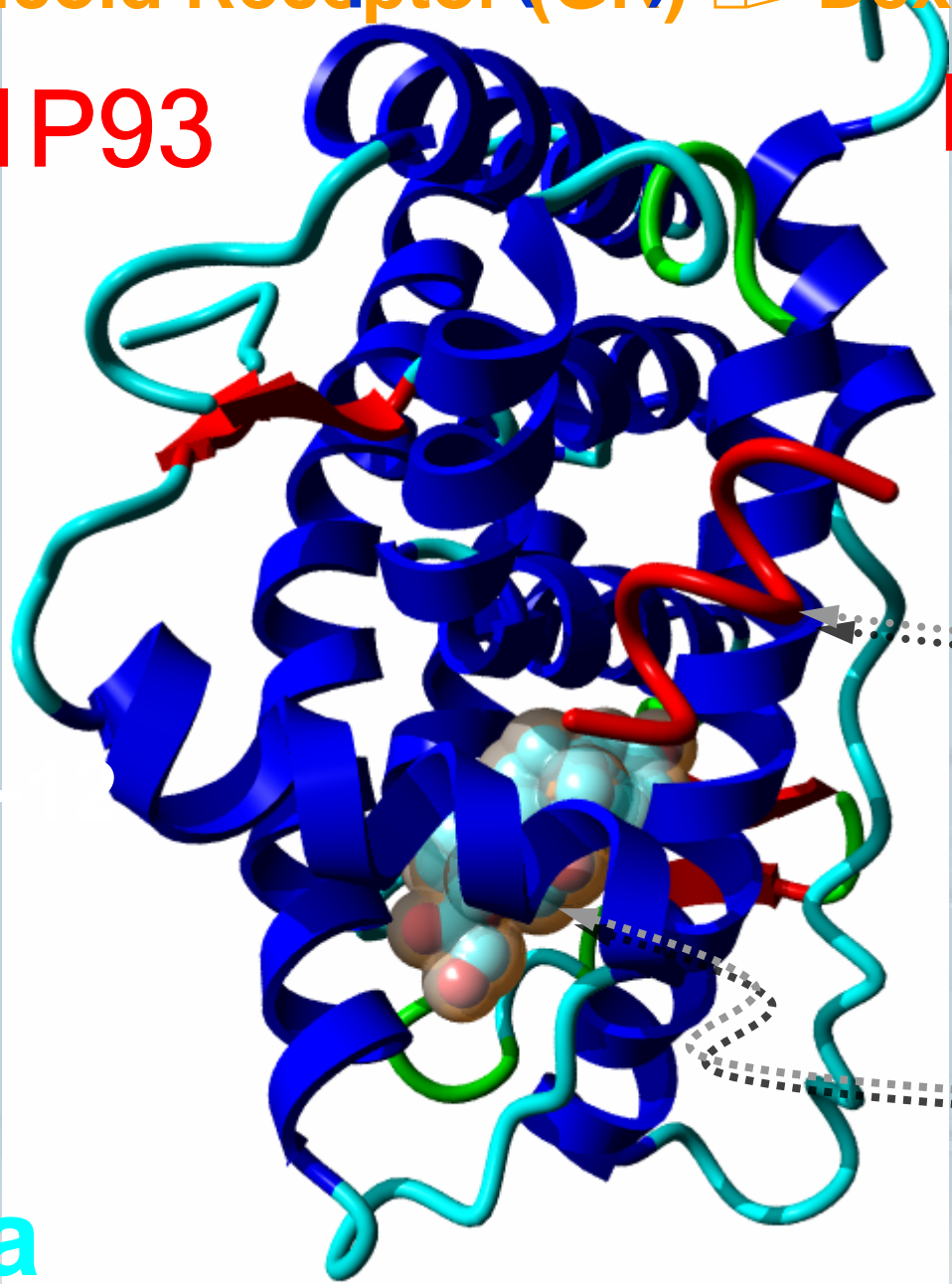
Helix 11-12

Dex

Glucocorticoid Receptor (GR) Dexamethasone

PDB: 1P93

N-Ter



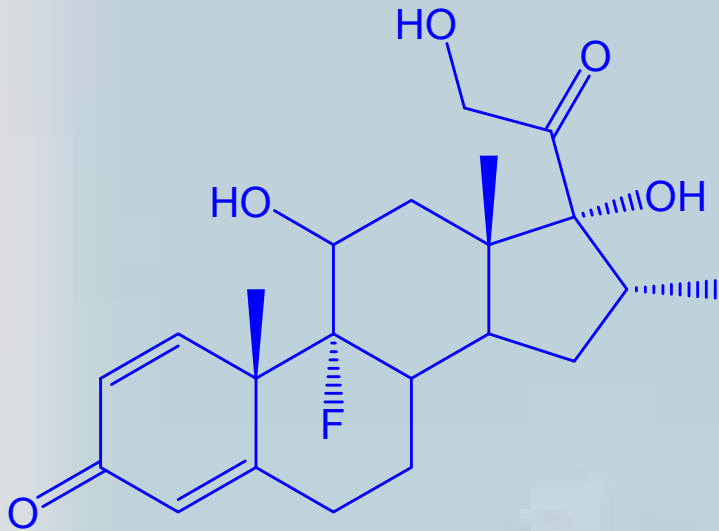
TIF

Helix 11

Dex

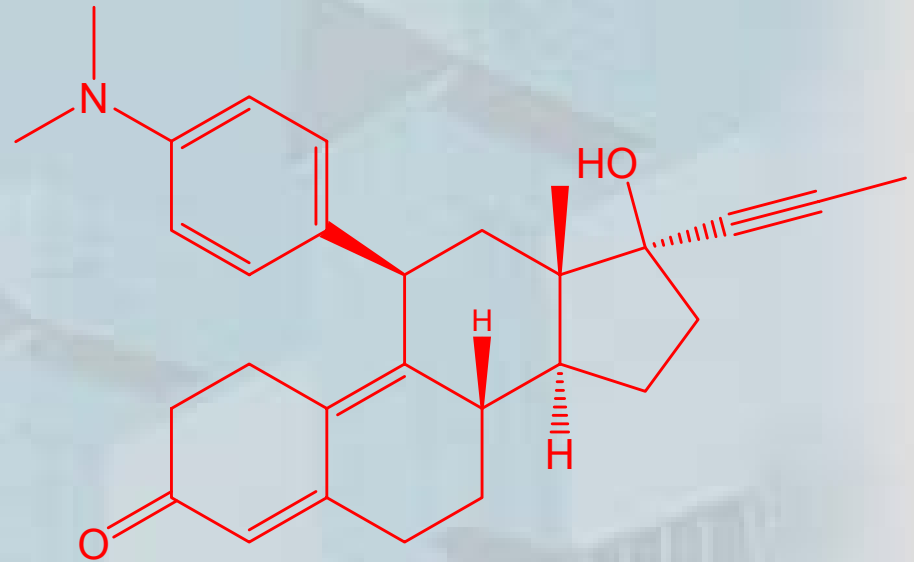
by Yasara

Agonist vs Antagonist of Glucocorticoid Receptor (GR)

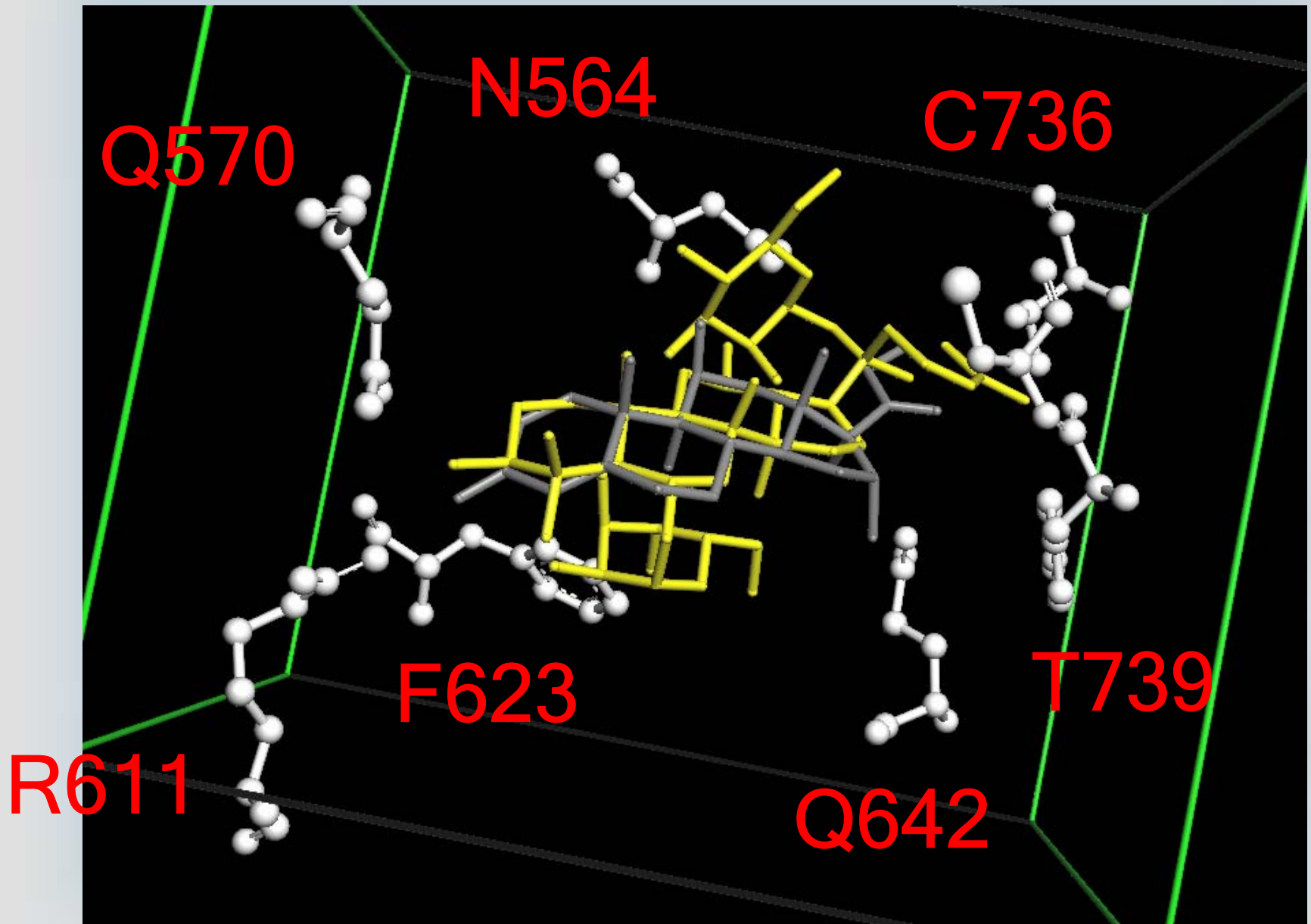


dexamethasone

RU-486

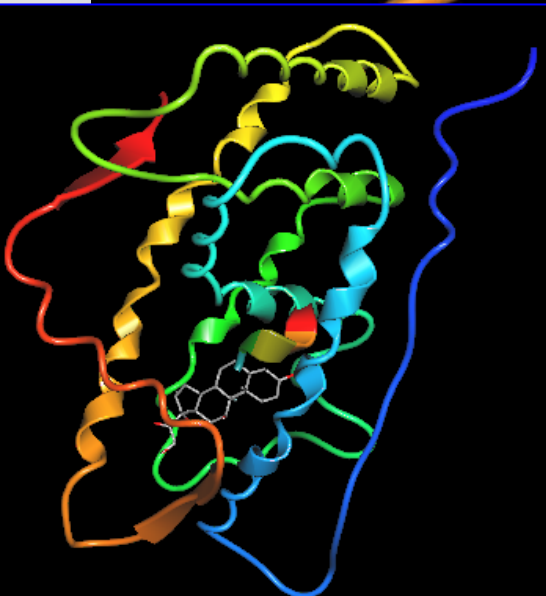
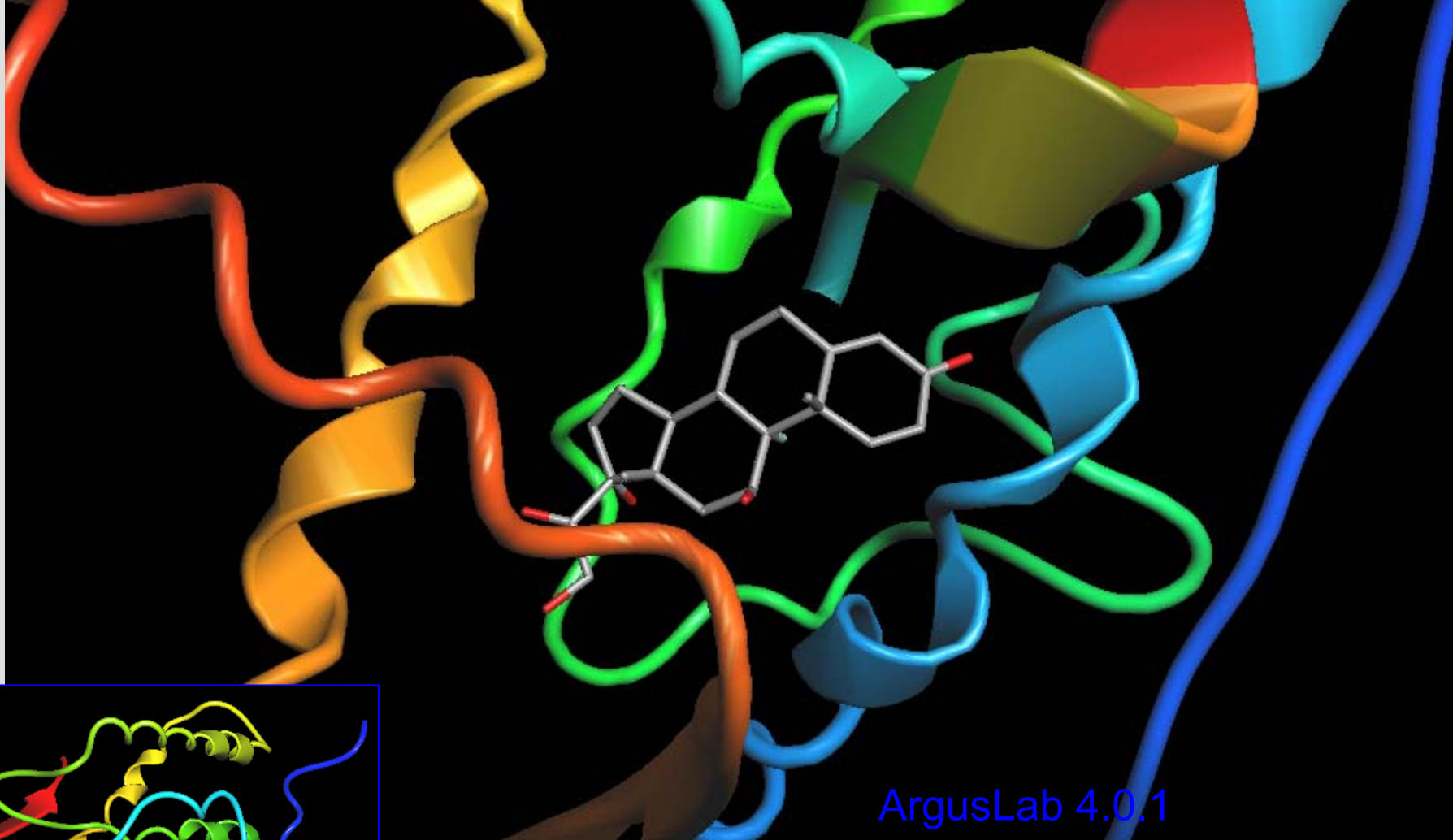


Important Interactions in the LBD



Superposition





ArgusLab 4.0.1

Mark A. Thompson

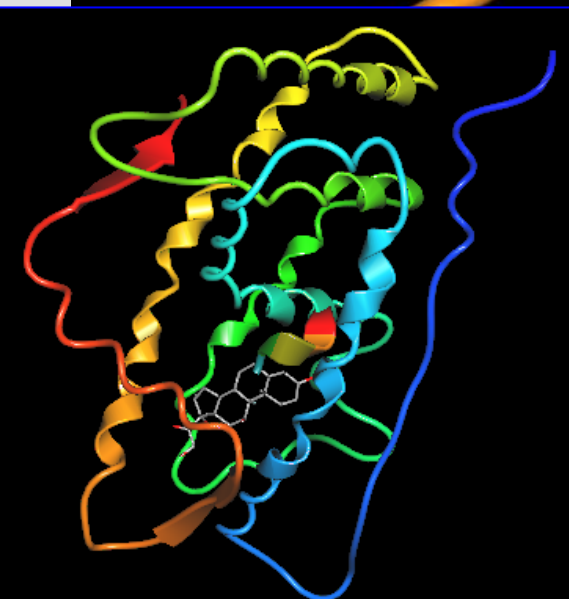
mark@arguslab.com

Planaria Software LLC,

Seattle, WA

<http://www.arguslab.com>

GR-Dex



GR-Dex+Rg1

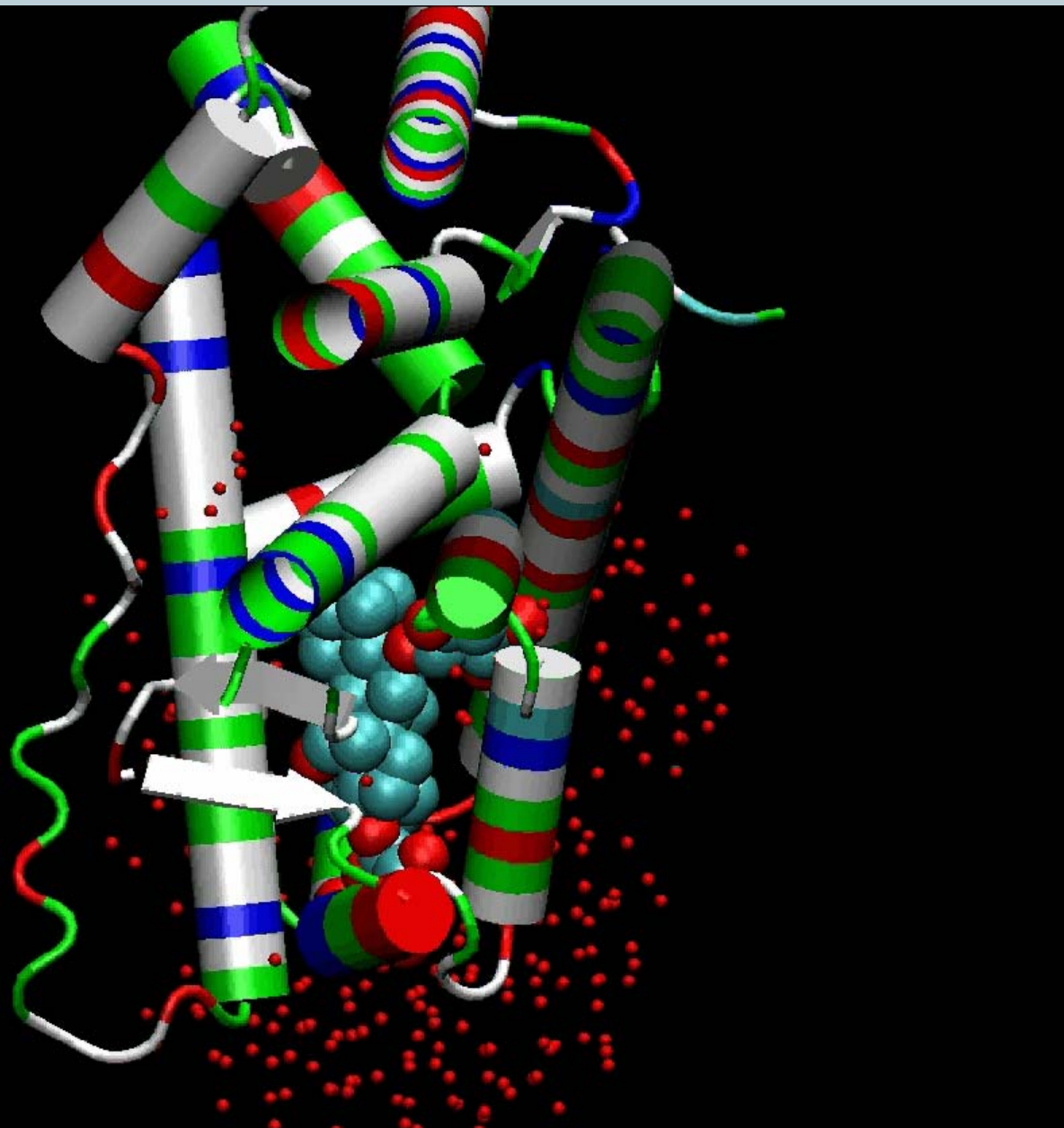
ArgusLab 4.0.1
Mark A. Thompson
mark@arguslab.com
Planaria Software
LLC,
Seattle, WA
<http://www.arguslab.com>



ArgusLab 4.0.1
Mark A. Thompson
mark@arguslab.com
Planaria Software
LLC,
Seattle, WA
<http://www.arguslab.com>

GR-Dex+Rg1+Rb1

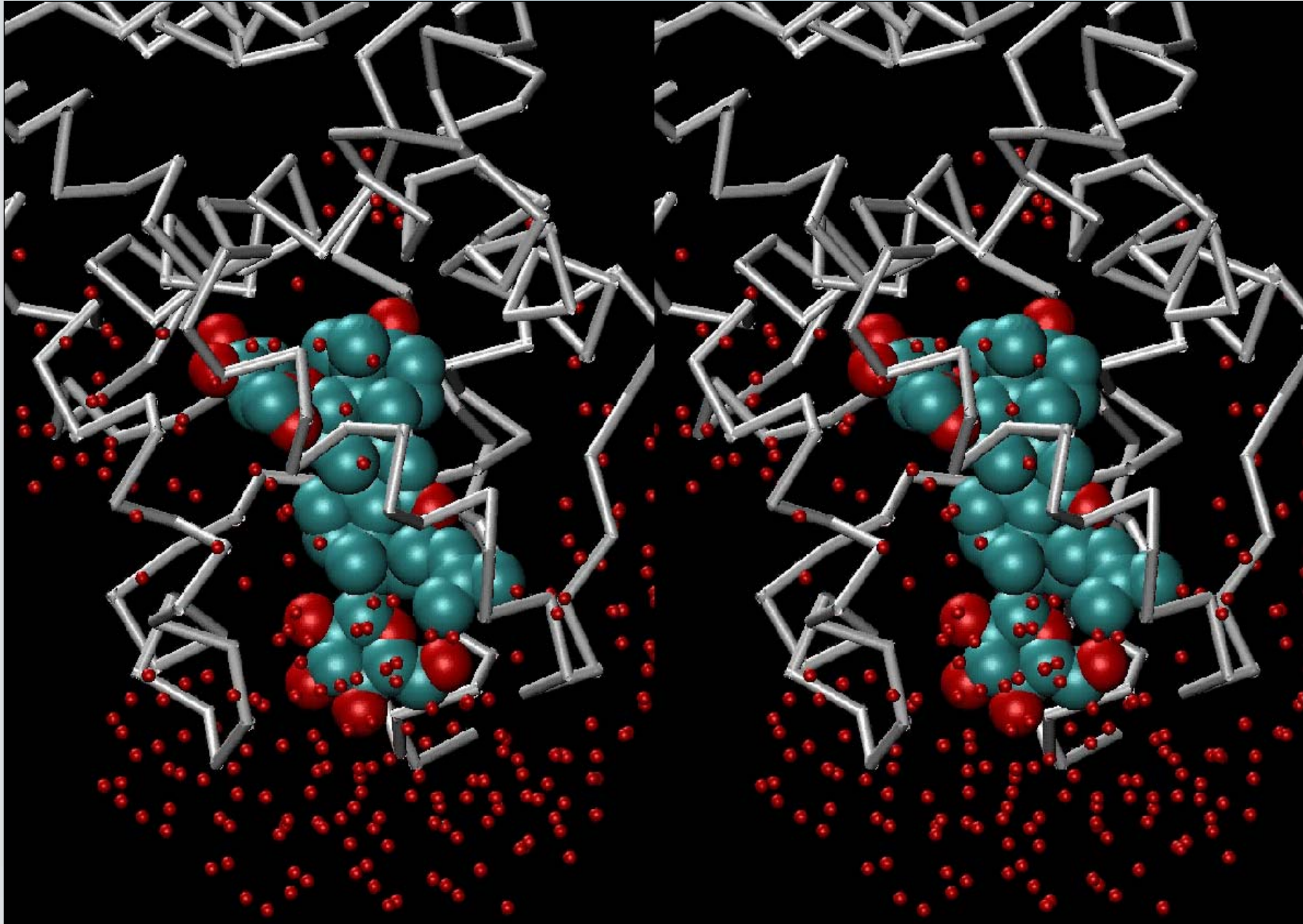
An Molecular Dynamics Trajectory



**GR + Rg1 +
H₂O** (only those
w/in several Å are
shown)

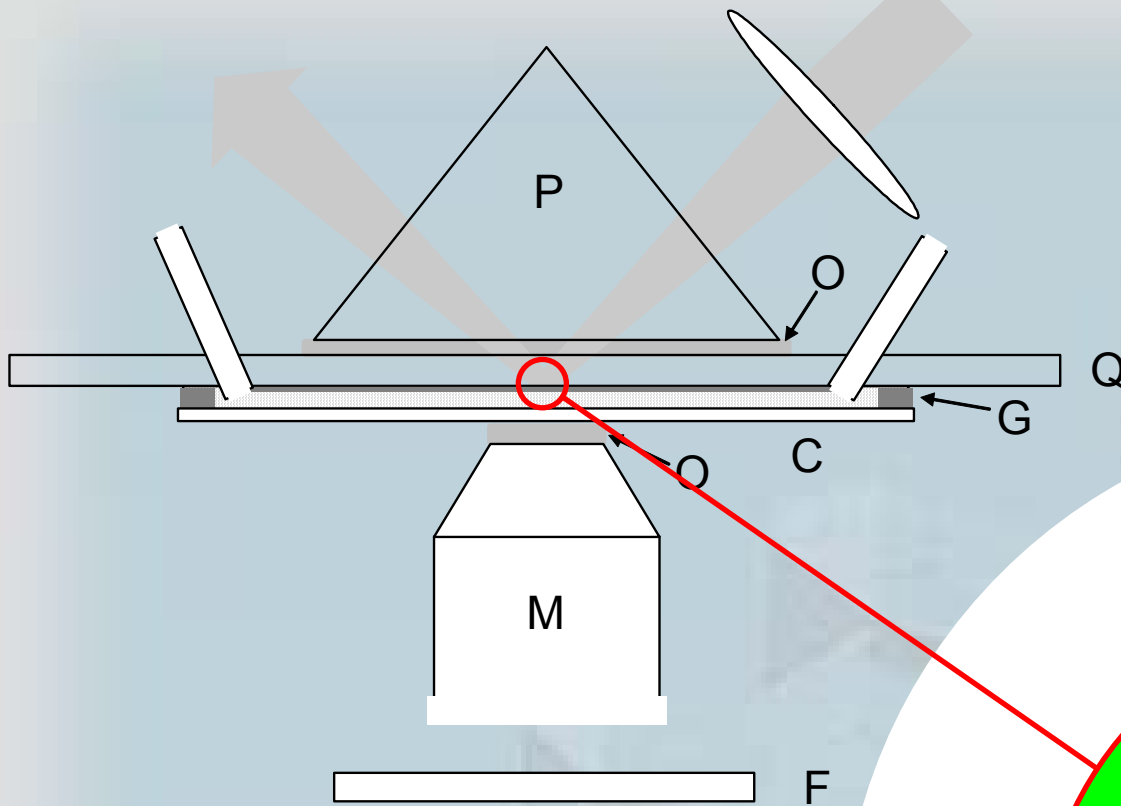
~ 2 ns

3D – well, if you can superimpose using naked eye!

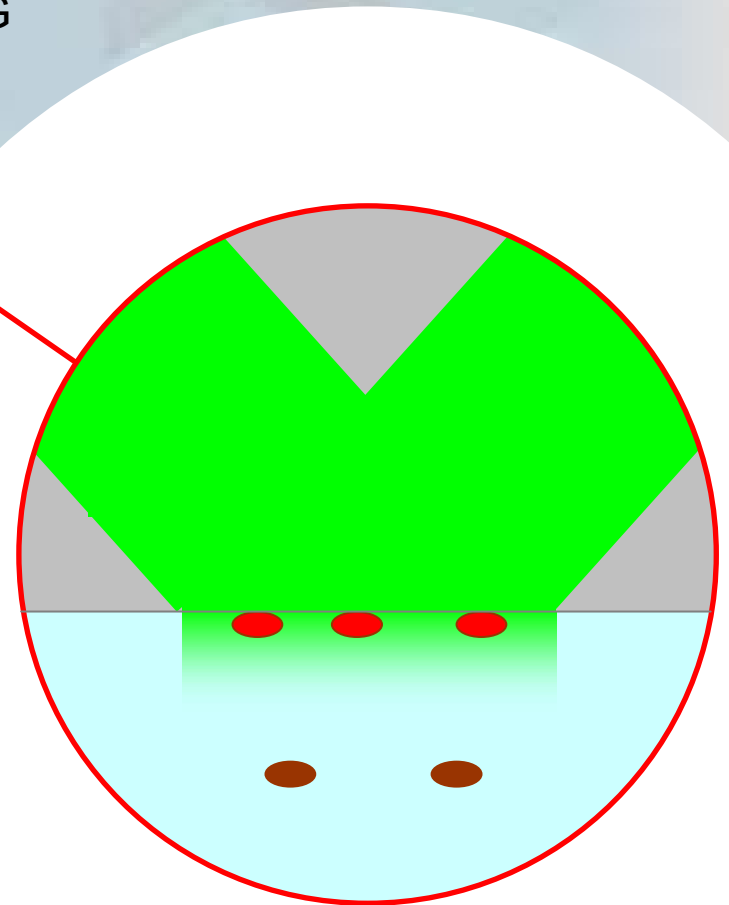


TIRFM

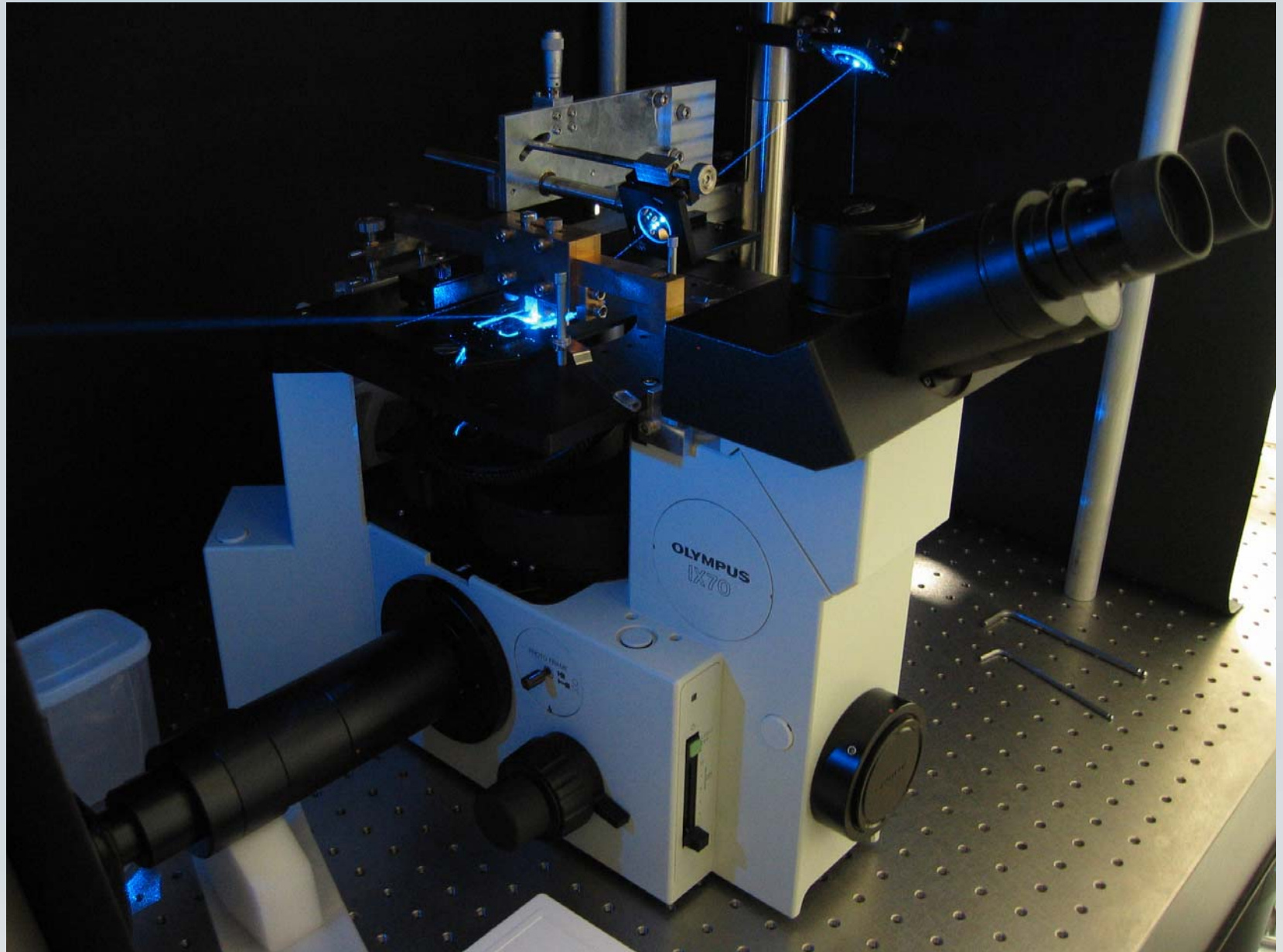
**Total-internal reflection
fluorescence microscopy**



The TIRFM setup was built around an inverted microscope. The sample cell was made from a quartz slide **Q** coated with GR on the underside, filled with PBS buffer, sandwiched with a cover glass **C** and sealed with vacuum grease **G**. Liquid inlet and outlet were inserted from the top. The cell was clamped onto the traveling stage and coupled via immersion oil **O** to a prism **P** fixed relative to the microscope stand. The cell could freely translate without moving the prism. A 532-nm laser beam was incident through the prism on the quartz-water interface at 69° and was totally reflected internally. Fluorescence signal was collected by a $60\times$ NA 1.4 objective **M** oil-coupled to the coverslip, band-filtered **F** and imaged onto an intensified CCD camera mounted at one of the microscope exit ports.



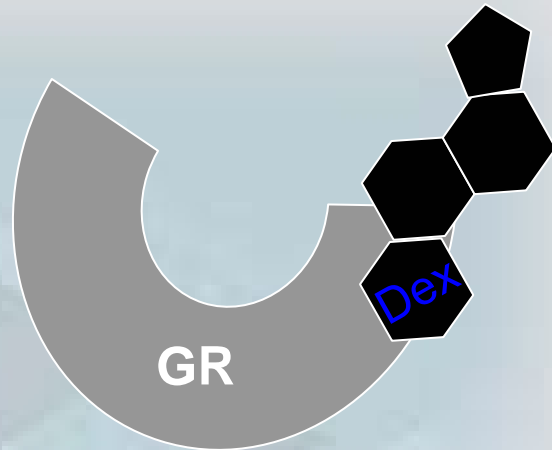
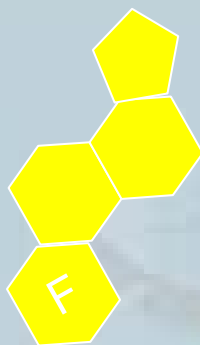
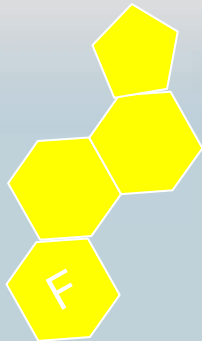
A snapshot of the real thing



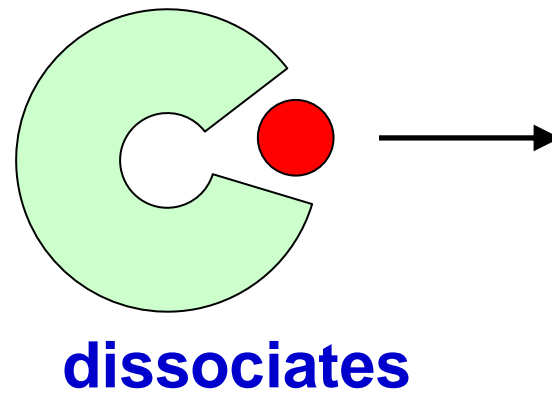
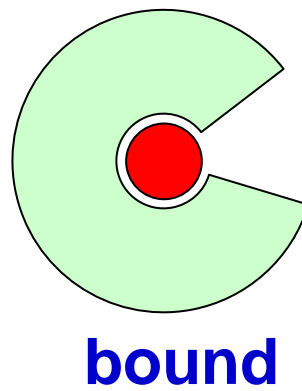
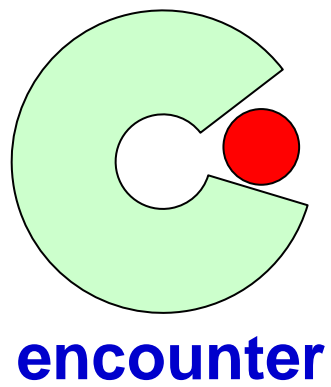
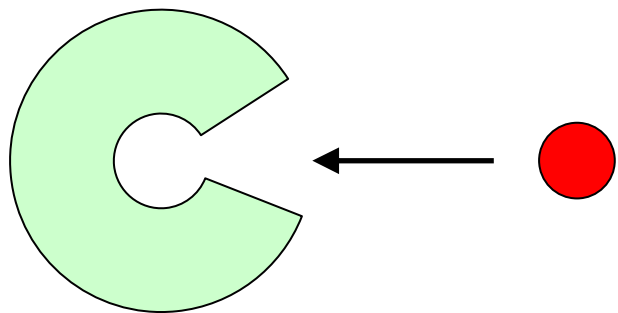
12 September 2005

Project Aim

To study the **binding kinetics** between **ligands** (ginsenosides) and **protein receptors** (nuclear hormone receptors) in order to understand the associated pharmacological effects at the molecular and cellular level.

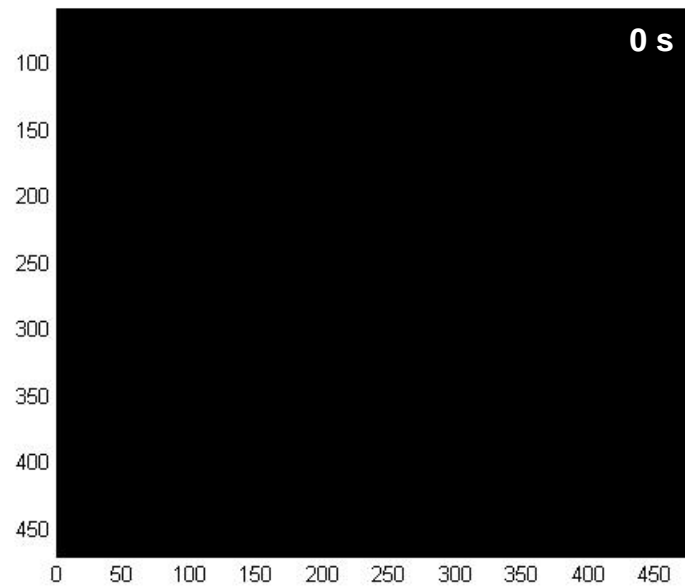


transport

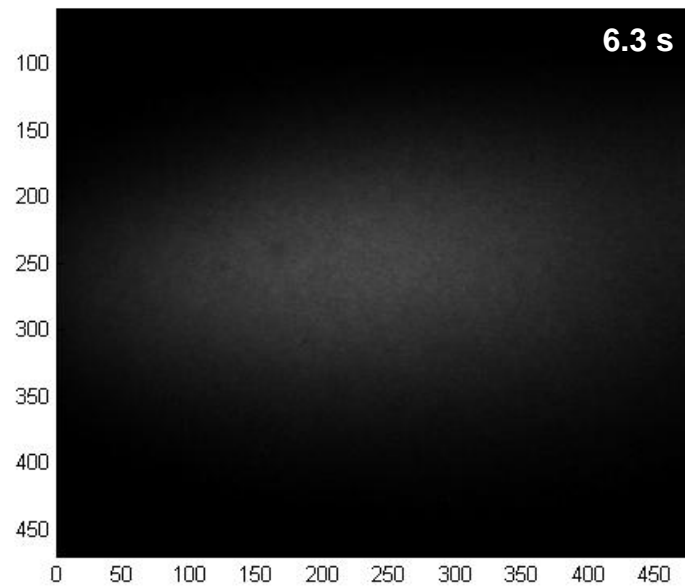


Episode 1:

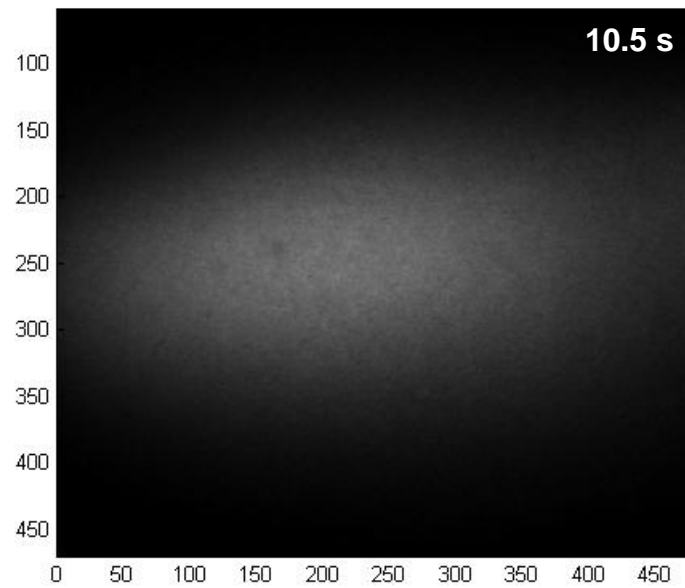
Fluormone (2 *nM*) TIRFM images at various time points



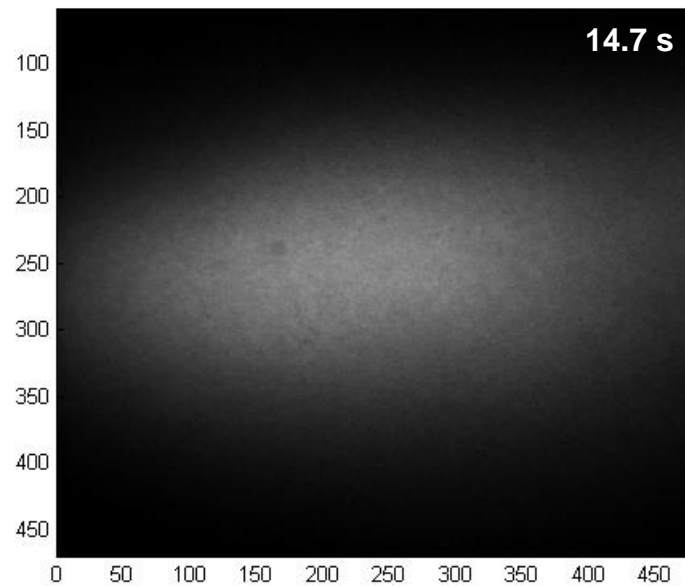
Grey scale is 50-1500



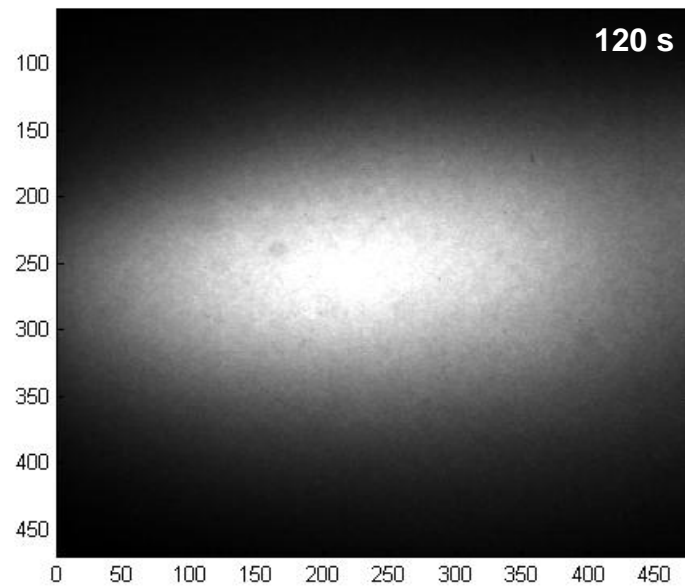
Grey scale is 50-1500



Grey scale is 50-1500

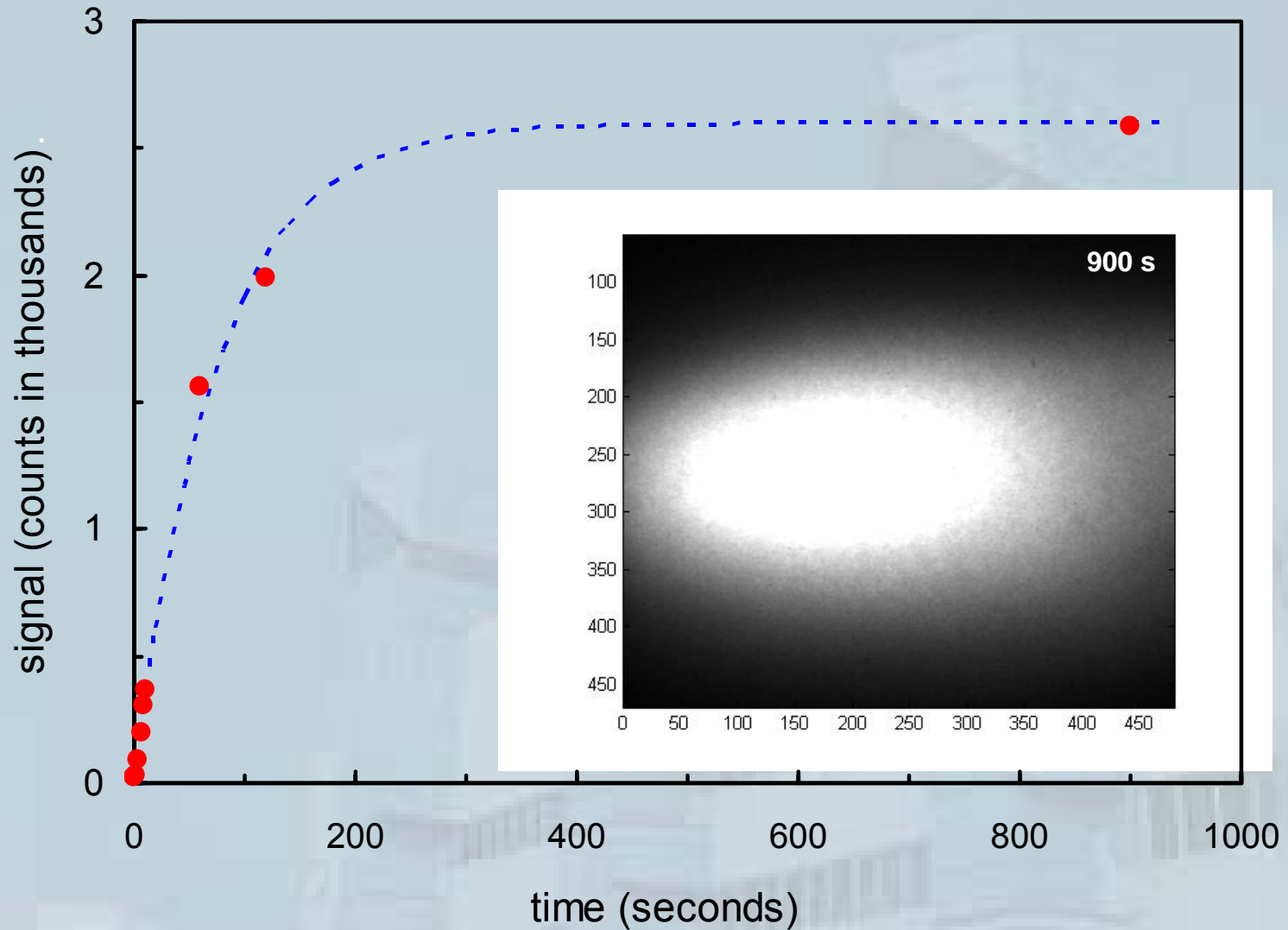


Grey scale is 50-1500



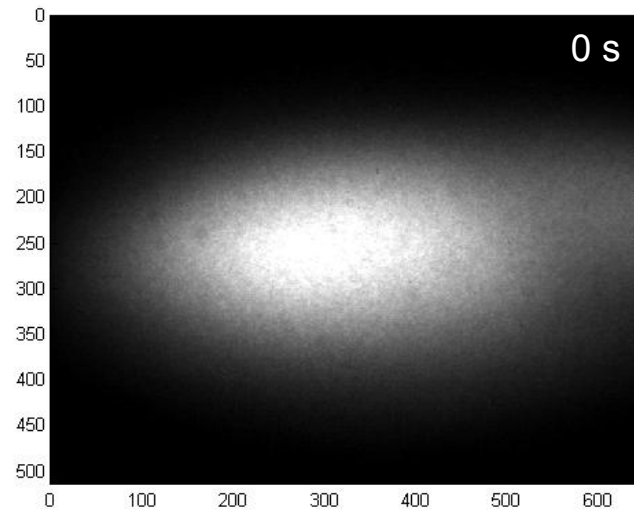
Grey scale is 50-1500

How about all in one?

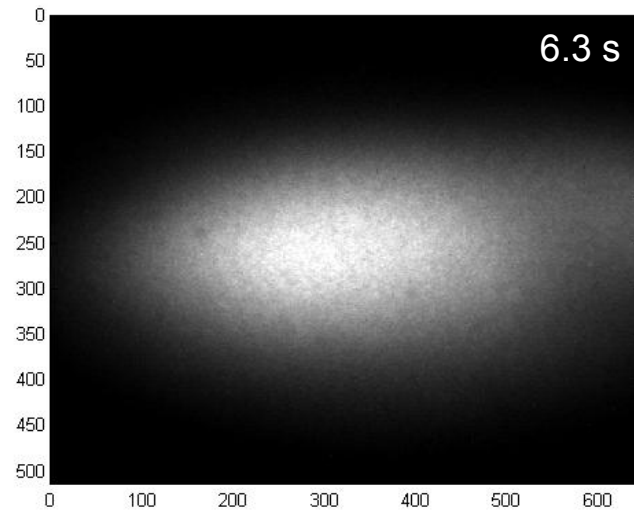


Episode 2:

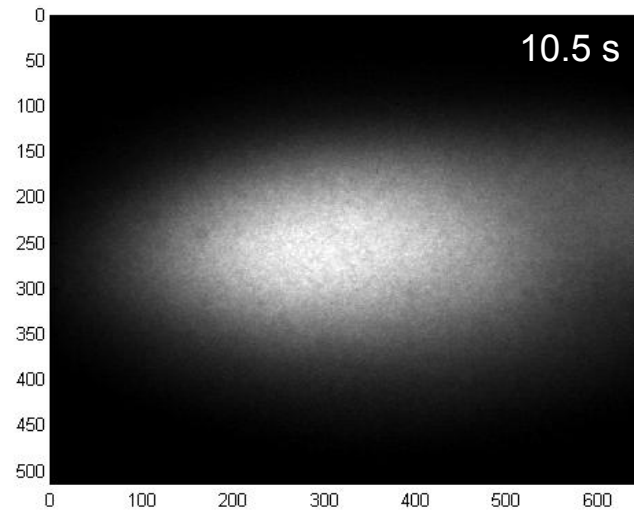
Dexamethasone (1 *mM*) TIRFM images at various time points



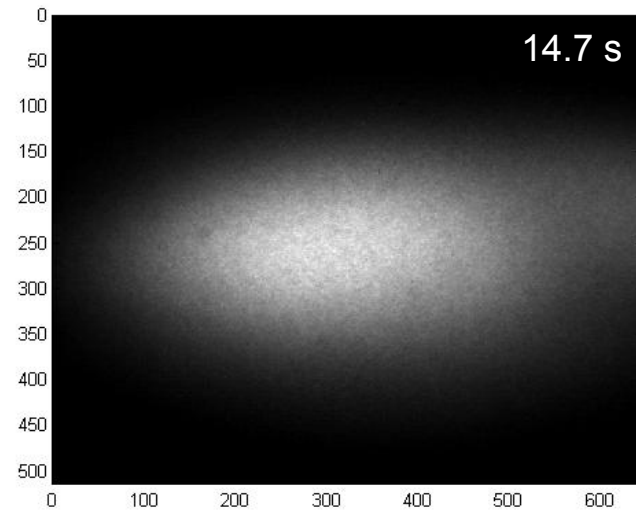
Grey scale is 50-800



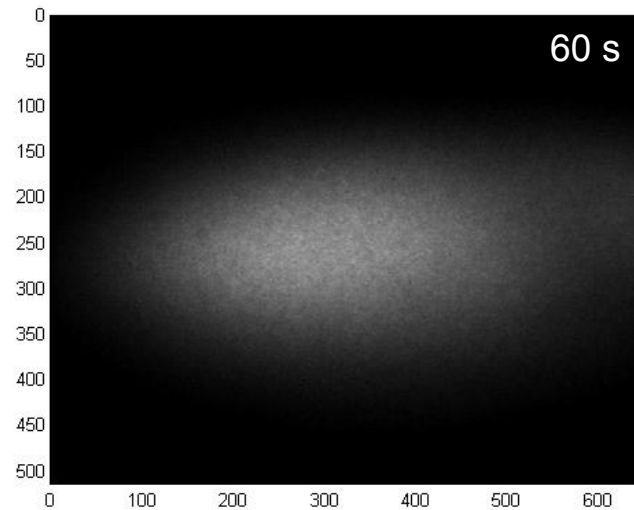
Grey scale is 50-800



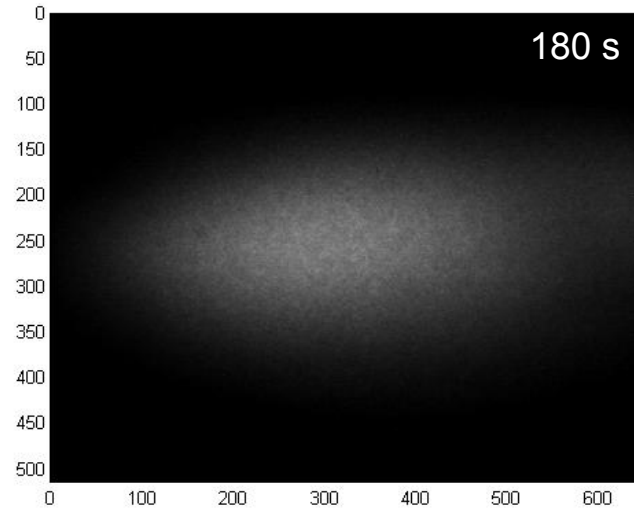
Grey scale is 50-800



Grey scale is 50-800

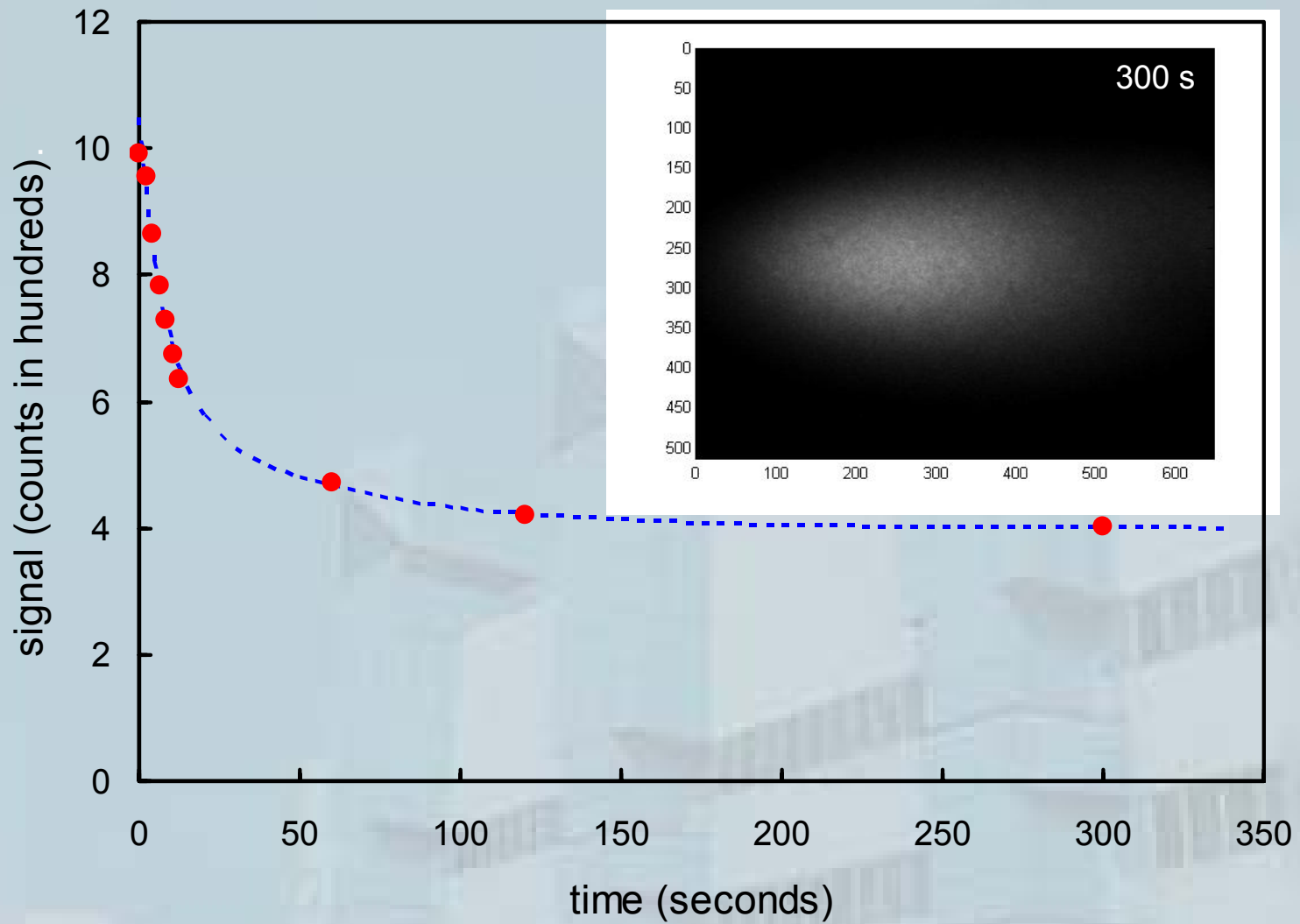


Grey scale is 50-800

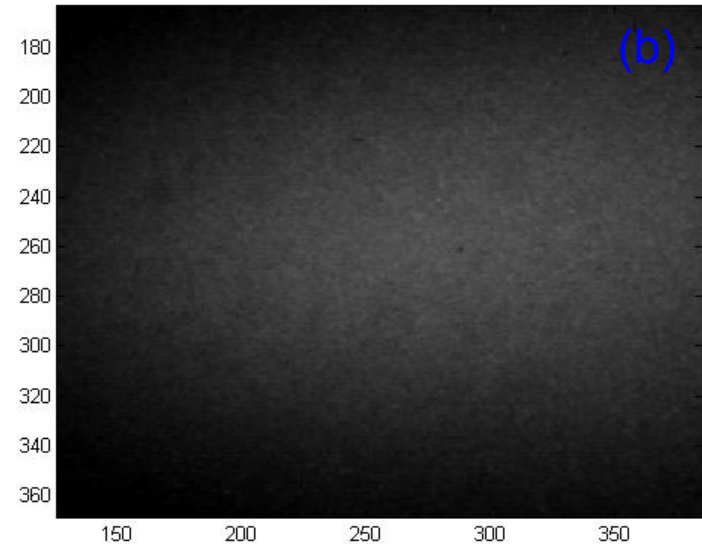
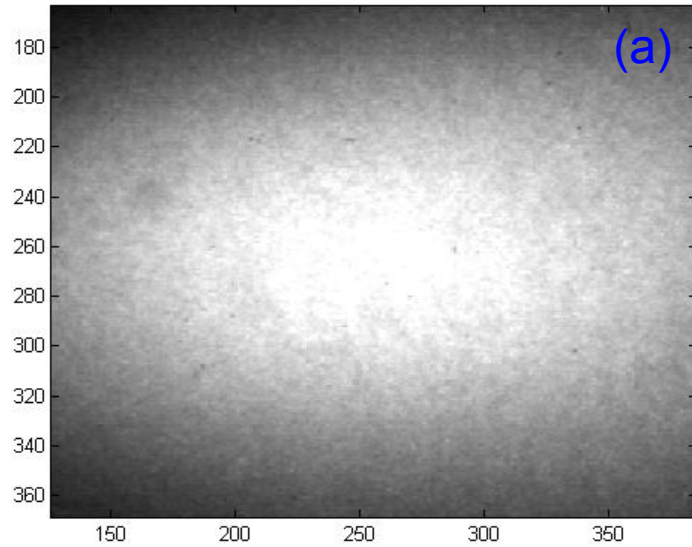


Grey scale is 50-800

Putting together



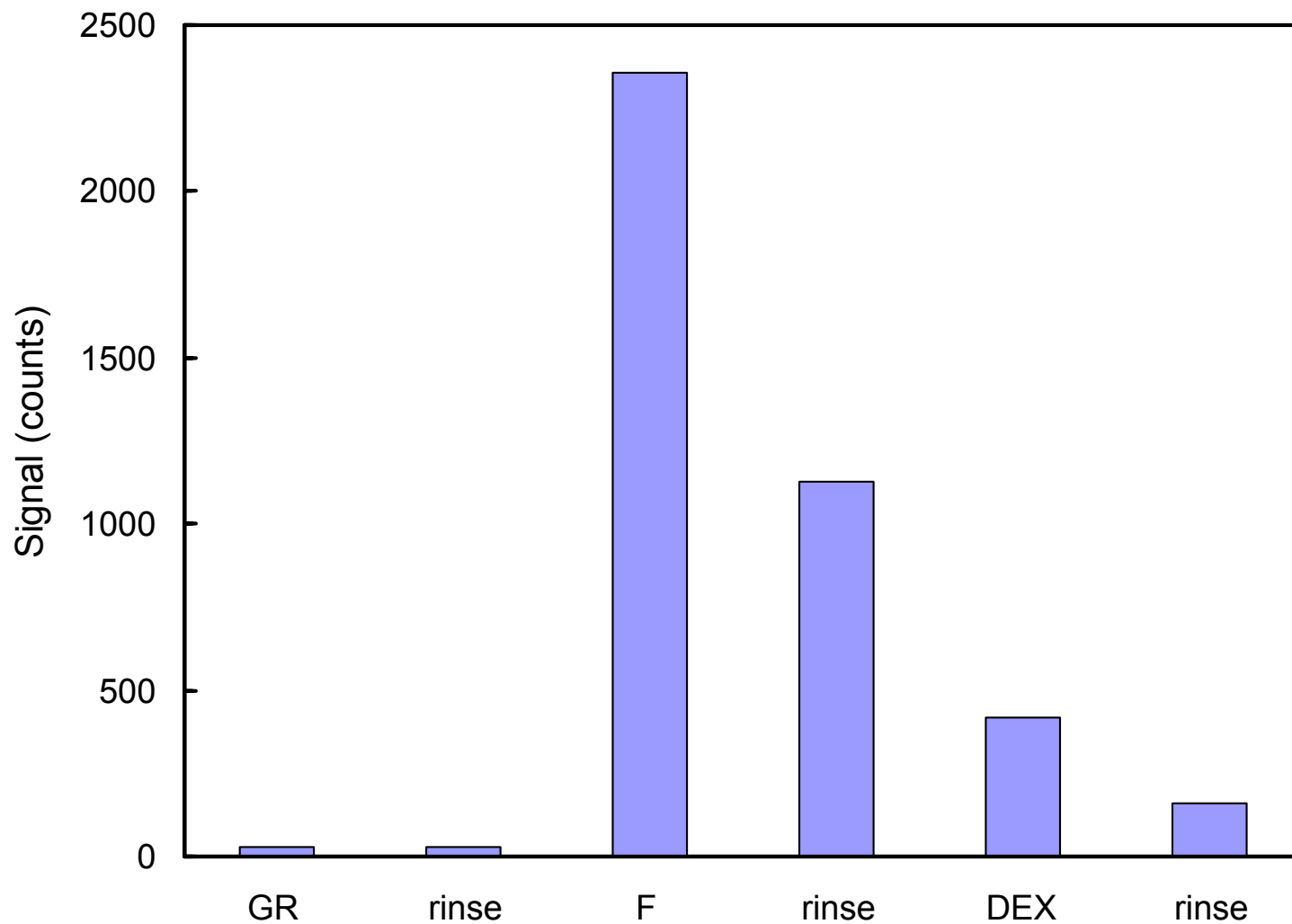
(a) TIRFM image after Dex displacement for > 130 s



(b) and after further rinsing with blank buffer

Grey scale is 50-450

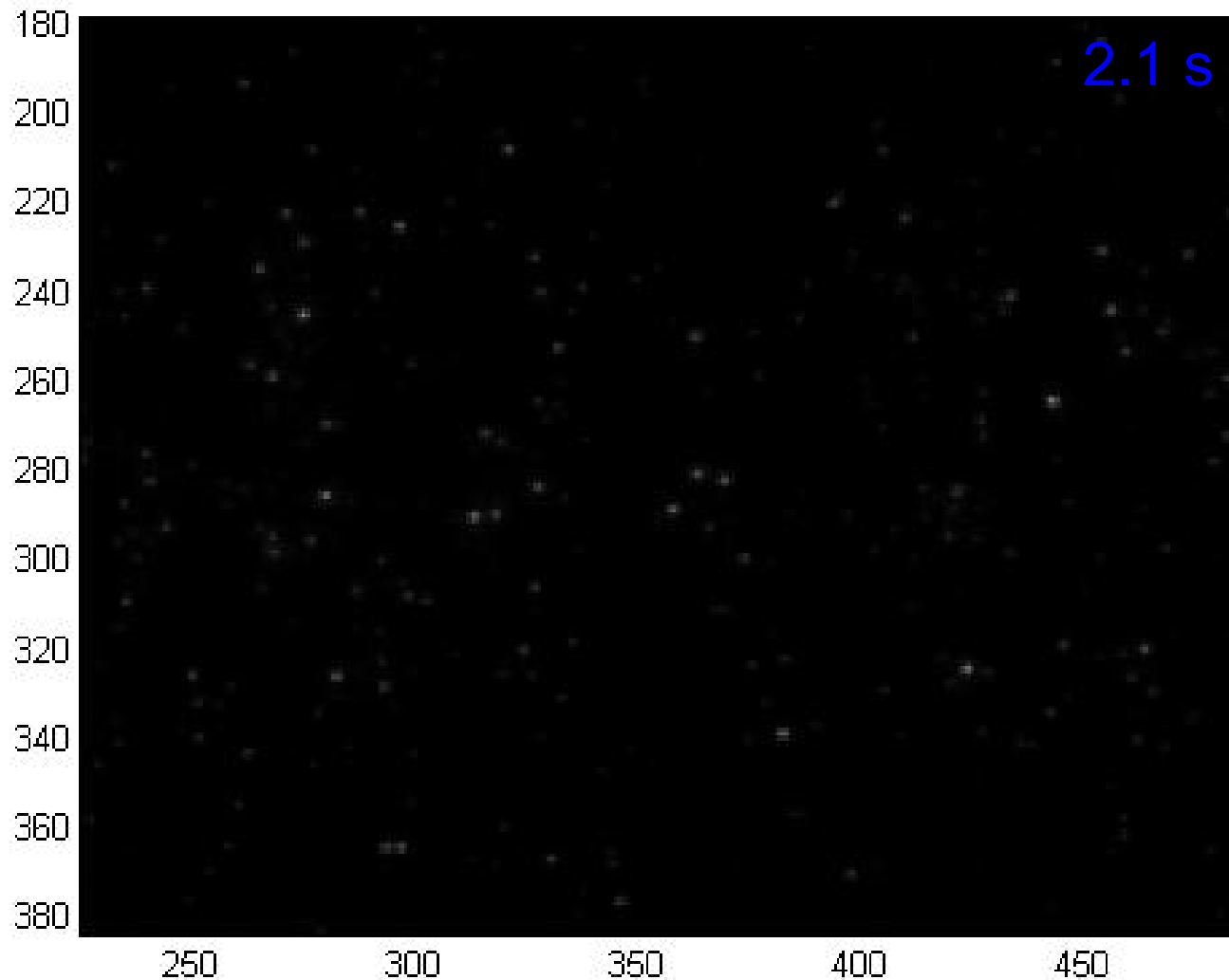
So ...



Episode 3:

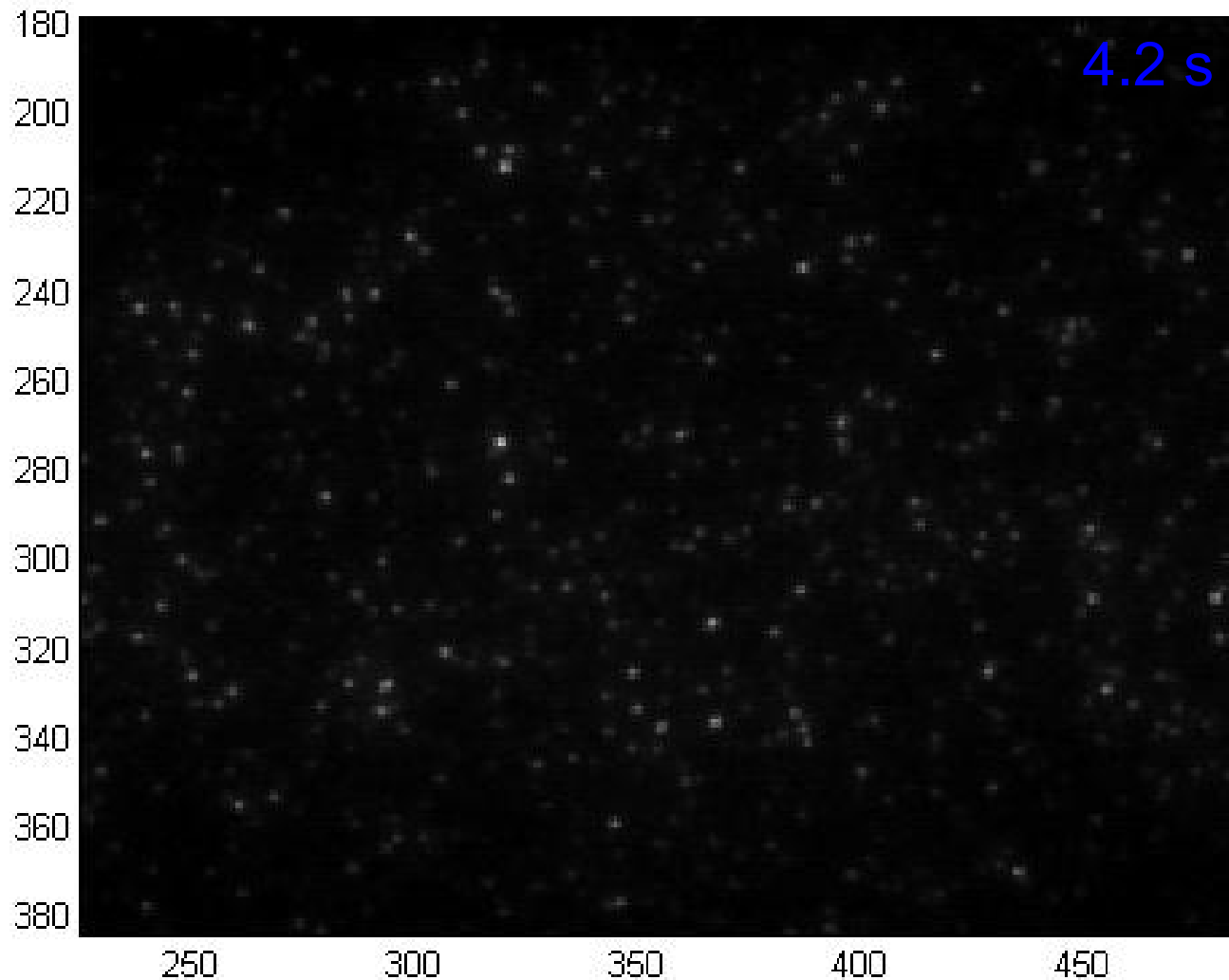
Now! GR with Fluromone (40 μ M)

Grey scale is 50-800



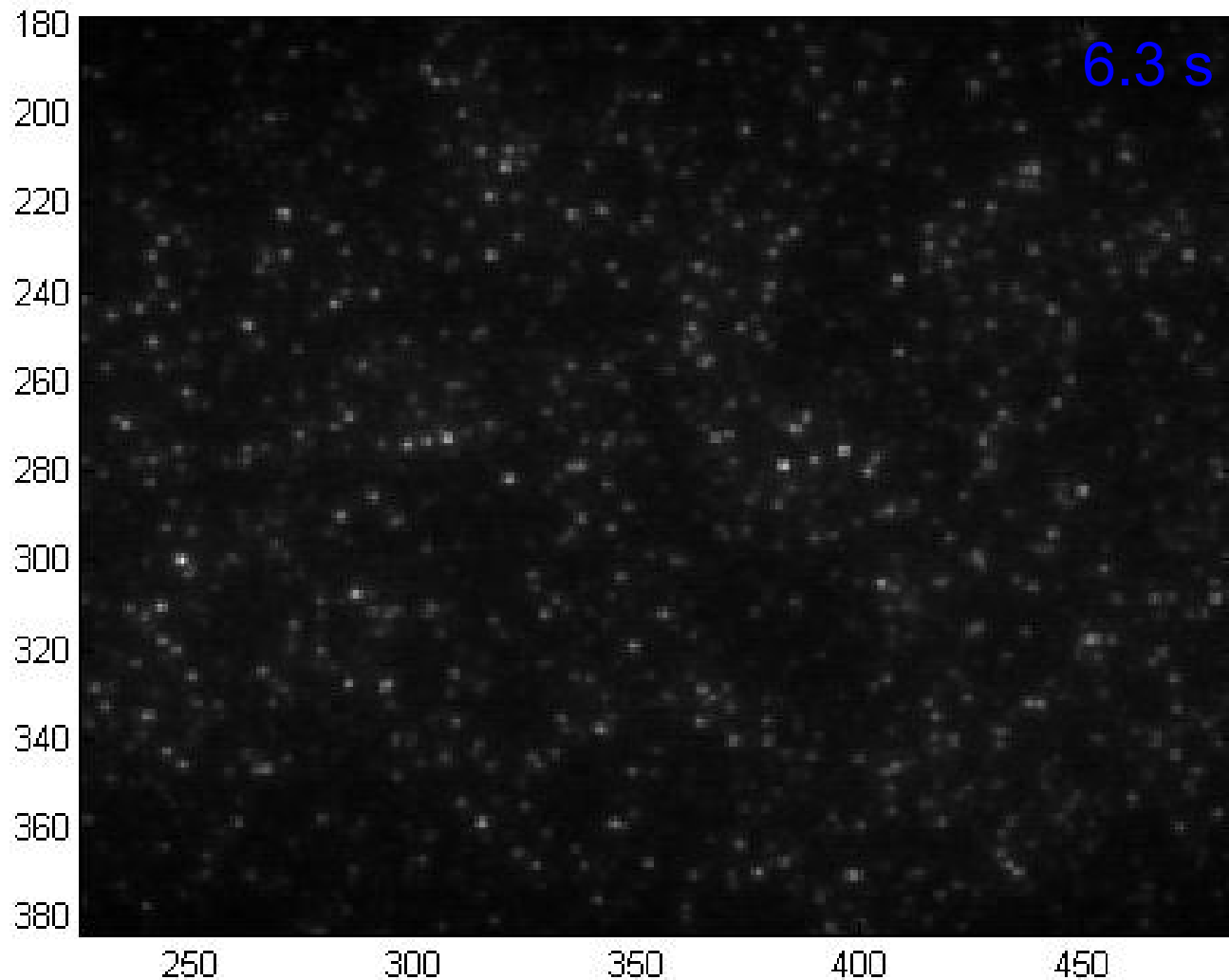
Time sequence of images of GR binding with F , at *time indicated*. A 40 pM F solution was flowed into the cell at $t = 0$. Each bright dot was one F molecule bound to surface-immobilized GR. Grey scale: 50-800.

Grey scale is 50-800



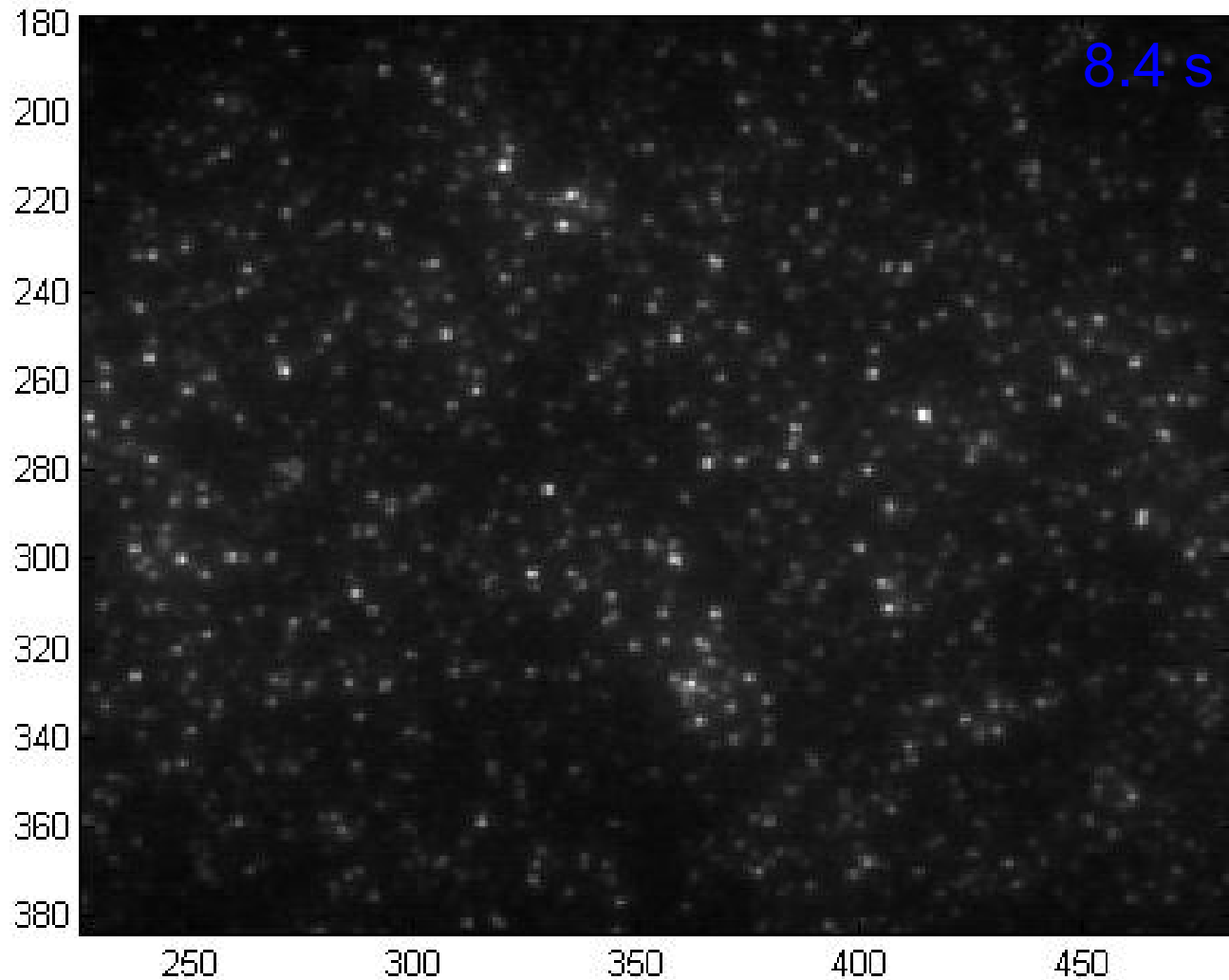
Time sequence of images of GR binding with F , at *time indicated*. A 40 pM F solution was flowed into the cell at $t = 0$. Each bright dot was one F molecule bound to surface-immobilized GR. Grey scale: 50-800.

Grey scale is 50-800



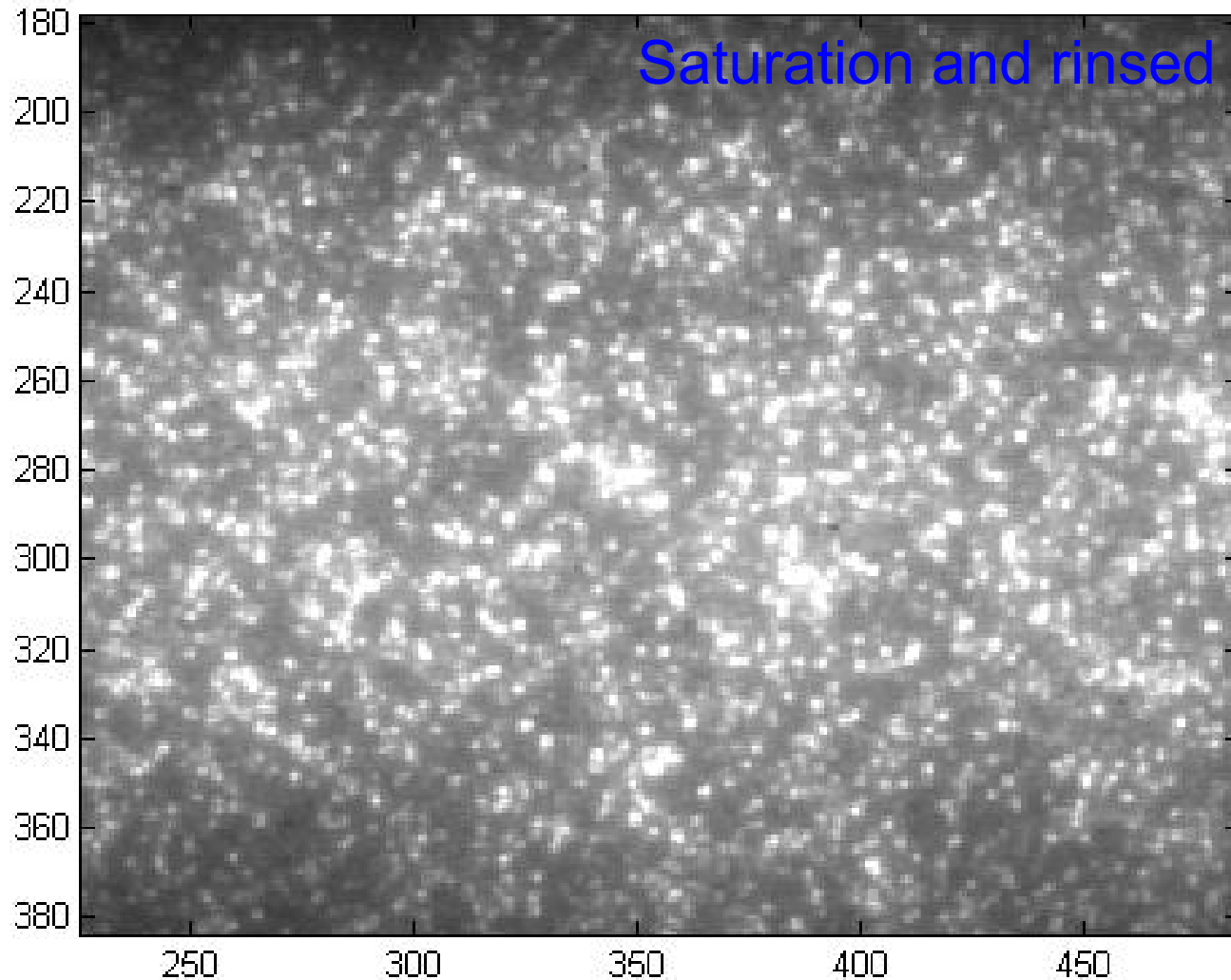
Time sequence of images of GR binding with F , at *time indicated*. A 40 pM F solution was flowed into the cell at $t = 0$. Each bright dot was one F molecule bound to surface-immobilized GR. Grey scale: 50-800.

Grey scale is 50-800



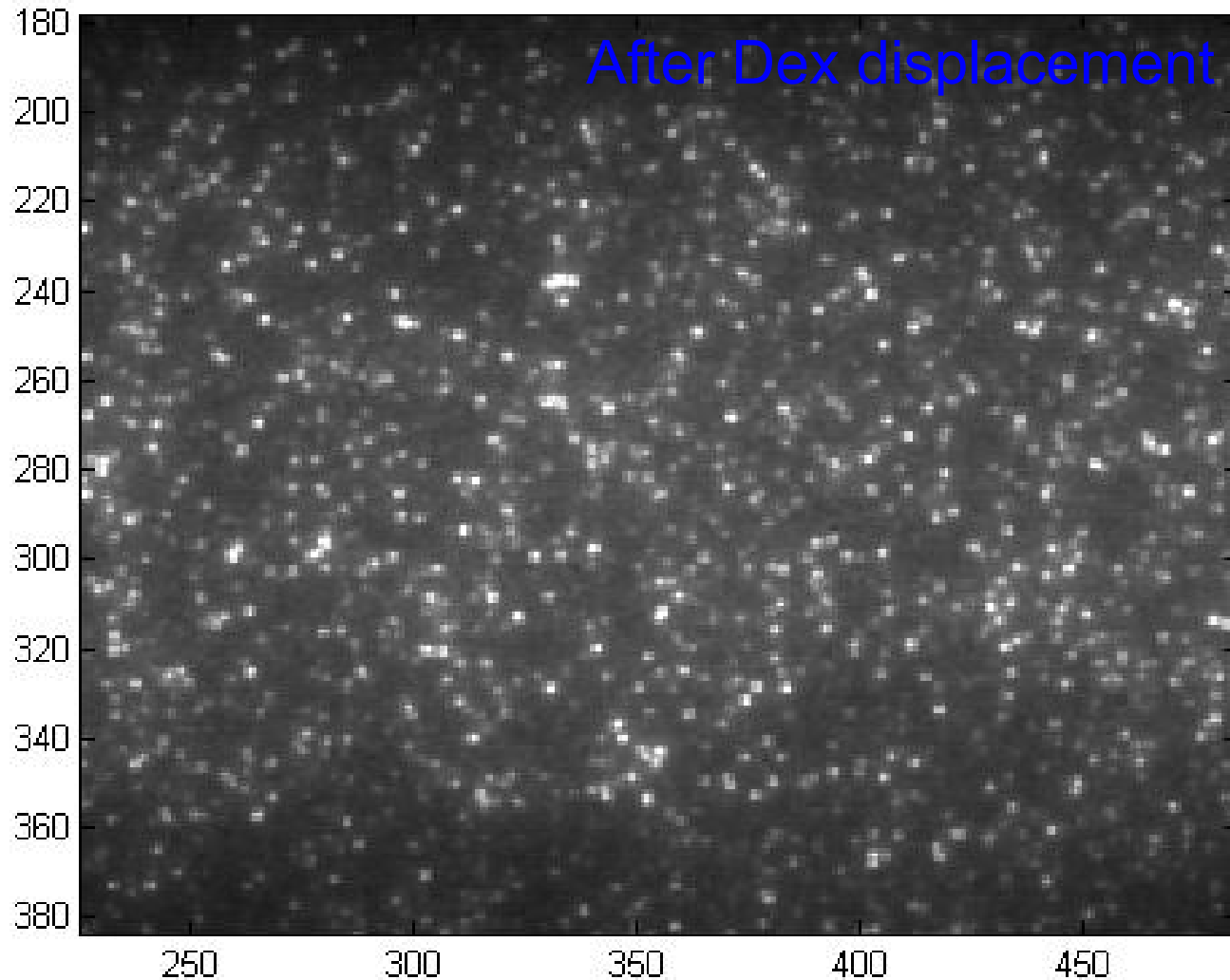
Time sequence of images of GR binding with F , at *time indicated*. A 40 pM F solution was flowed into the cell at $t = 0$. Each bright dot was one F molecule bound to surface-immobilized GR. Grey scale: 50-800.

Grey scale is 50-800

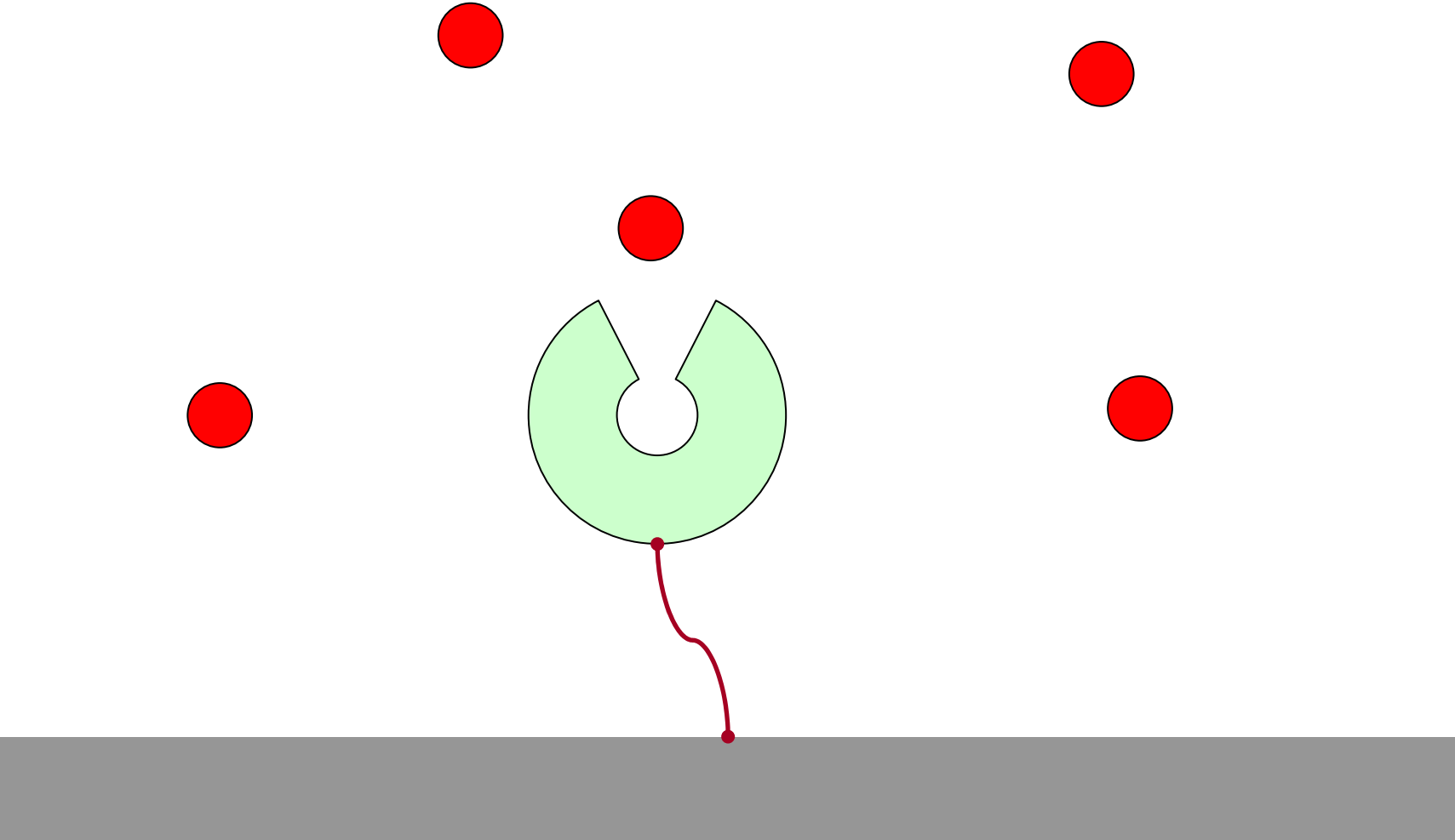


Images of GR-F before Dex displacement, at the level of single F molecules. Grey scale is 50-800.

Grey scale is 50-800

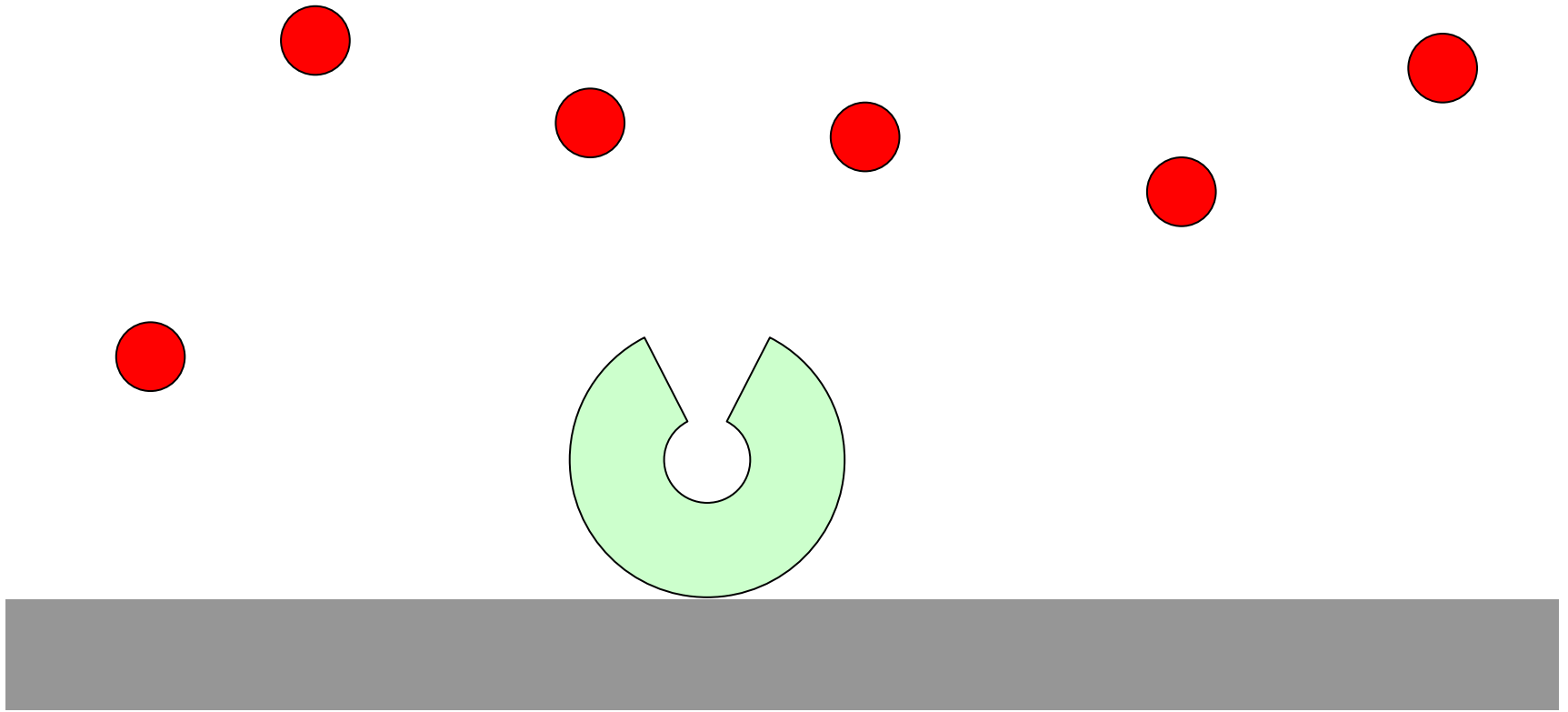


Images of GR-F after Dex displacement, at the level of single F molecules. Grey scale is 50-800.



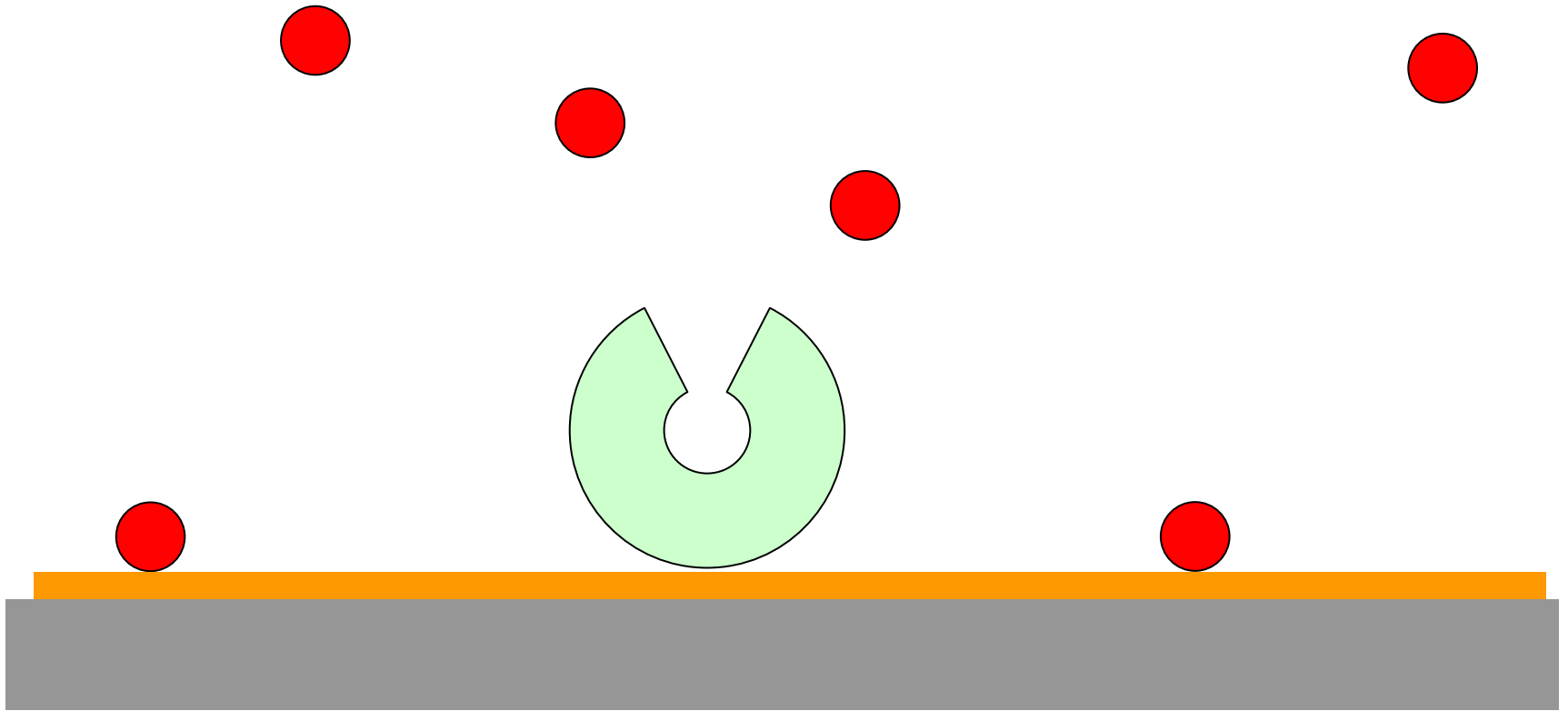
Need immobilization
of
functional receptors
on a substrate

Nontrivial, took us one year!



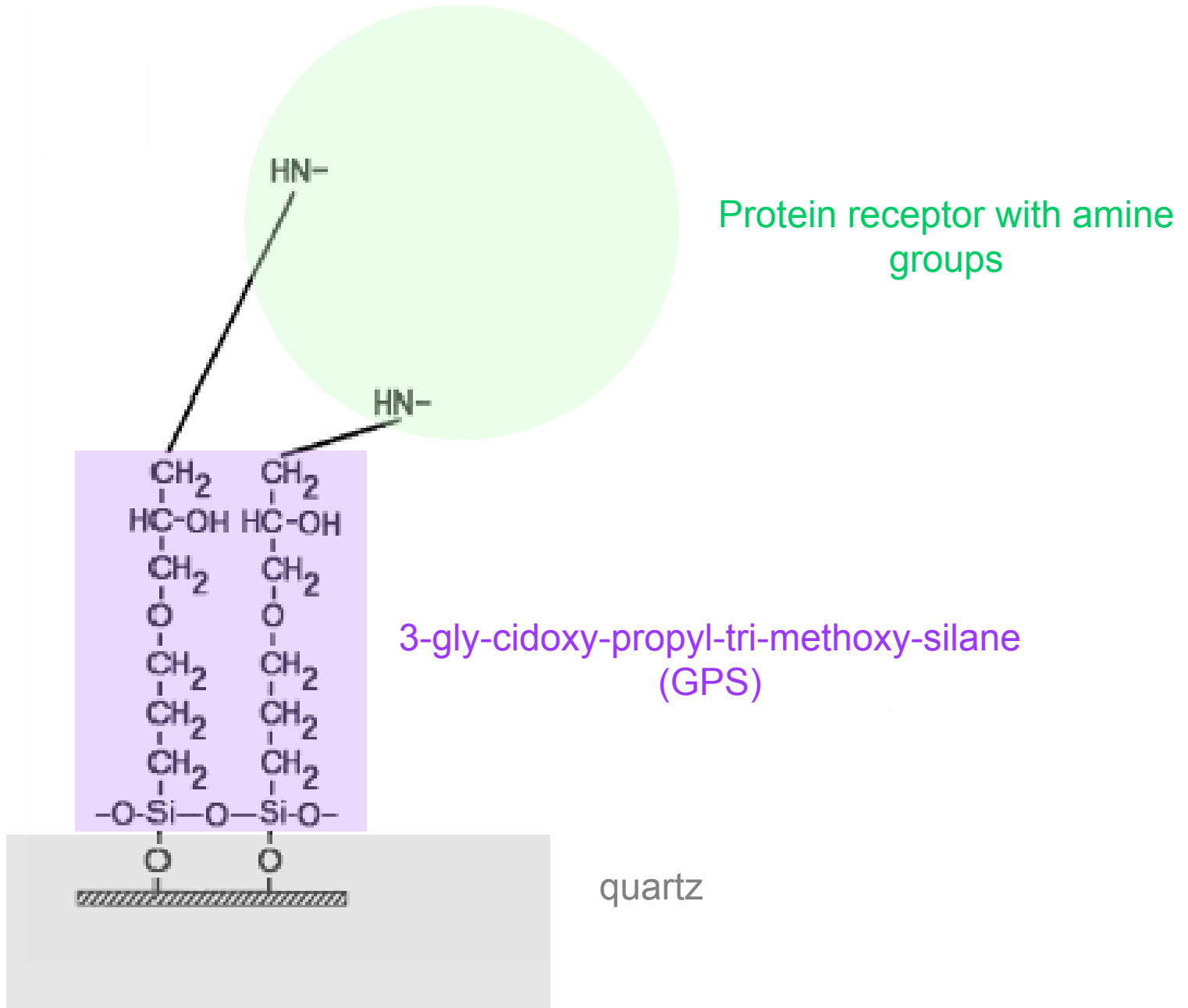
Physi-sorb on bare quartz

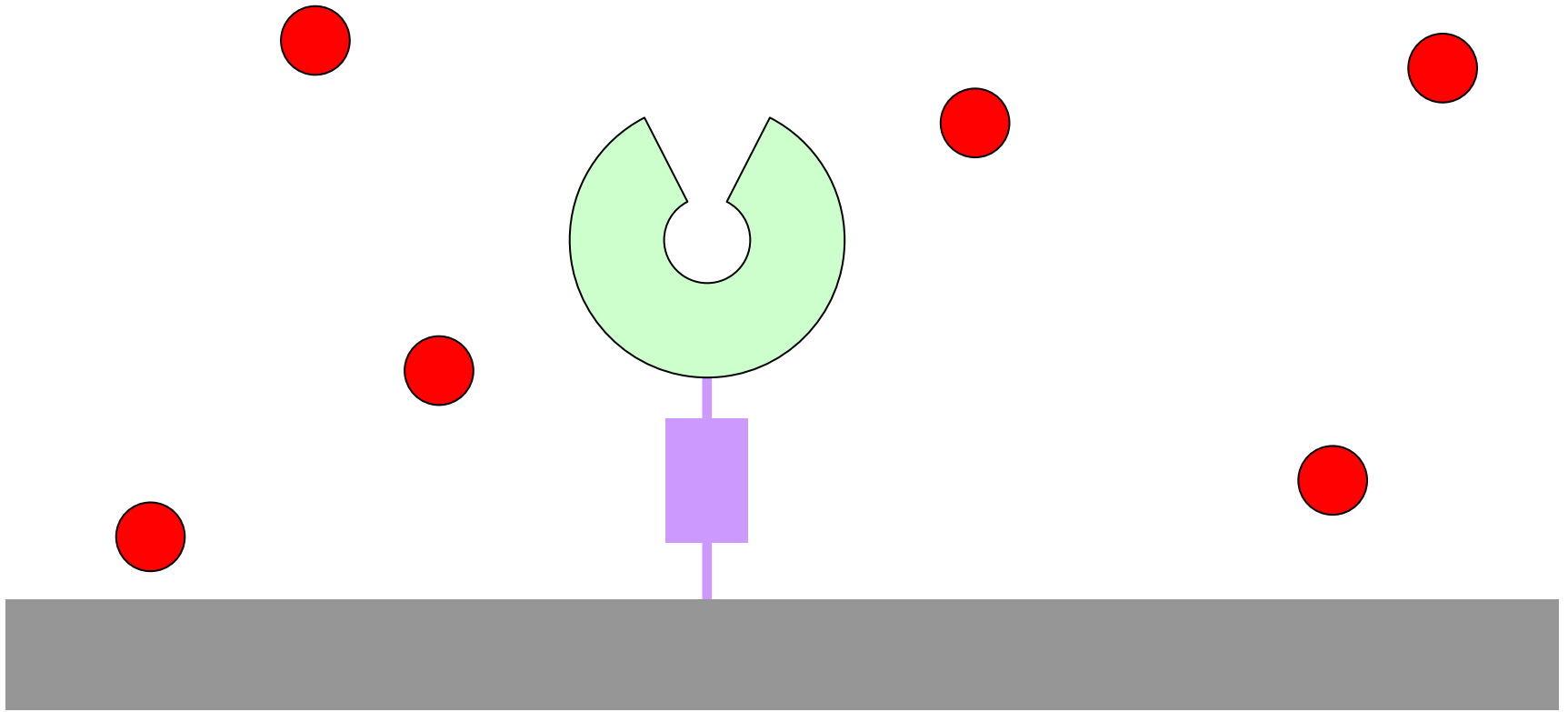
Not secure enough



Physi-sorb on octyl-tri-chloro-silane (OTS):

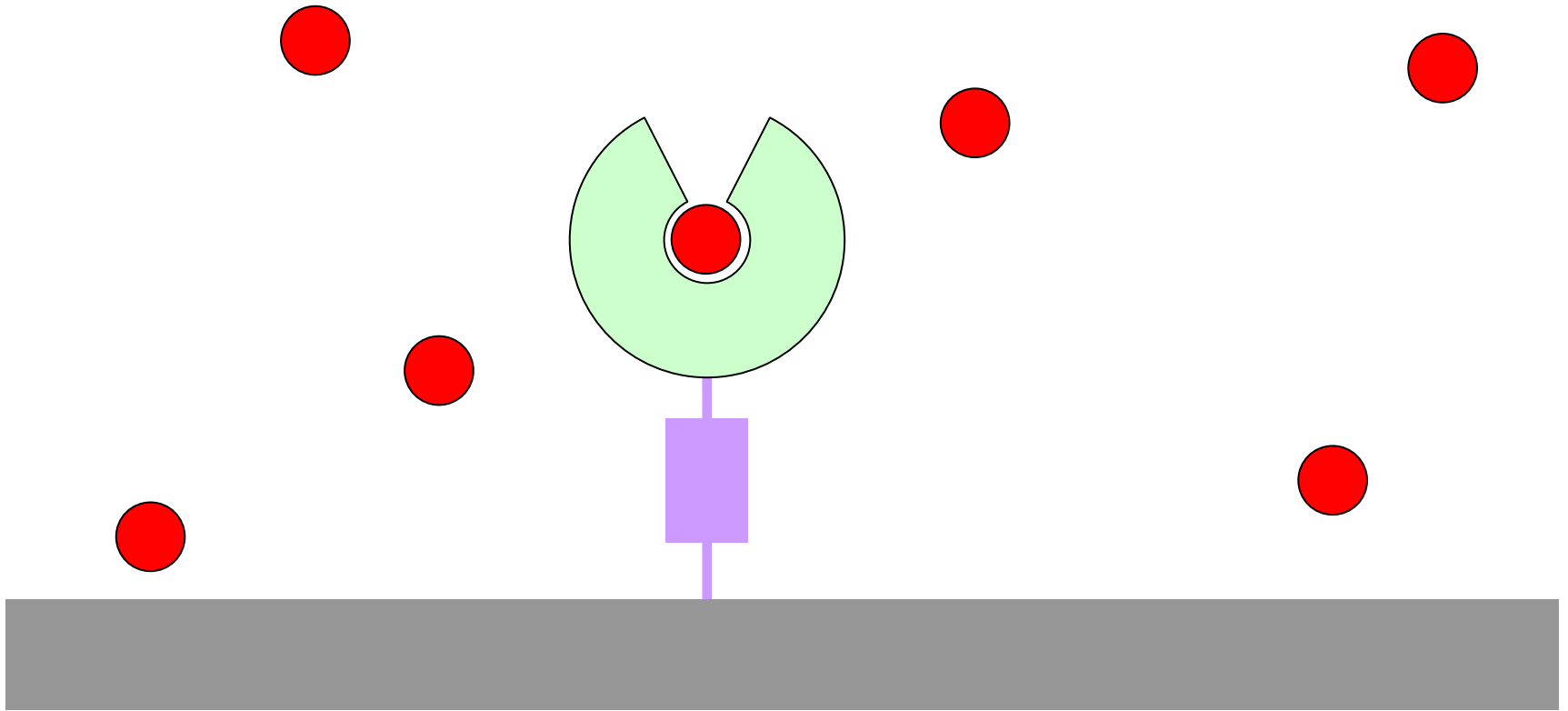
Too much non-specific binding





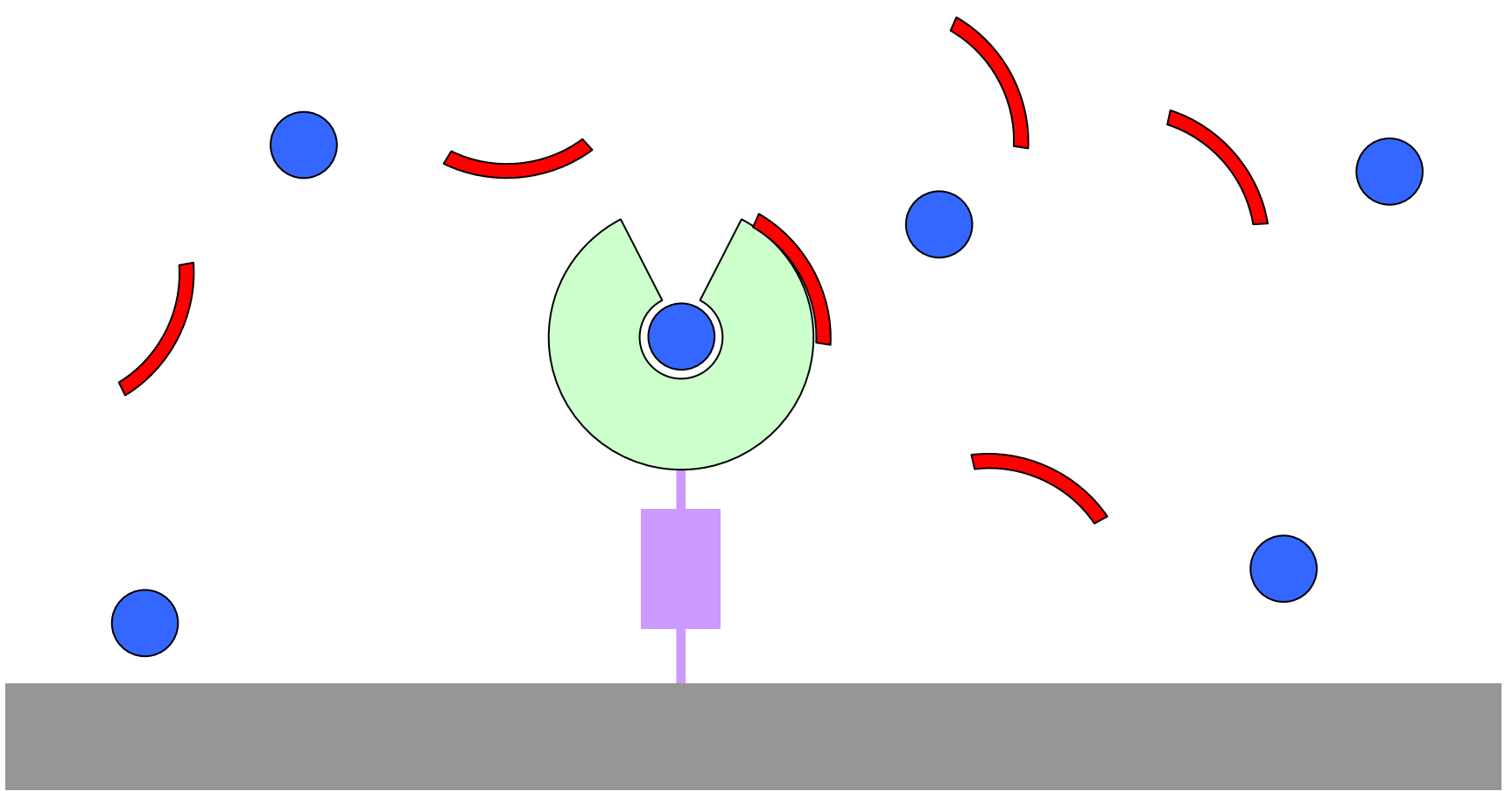
GR covalently bonded to quartz:

Ligand will not bind



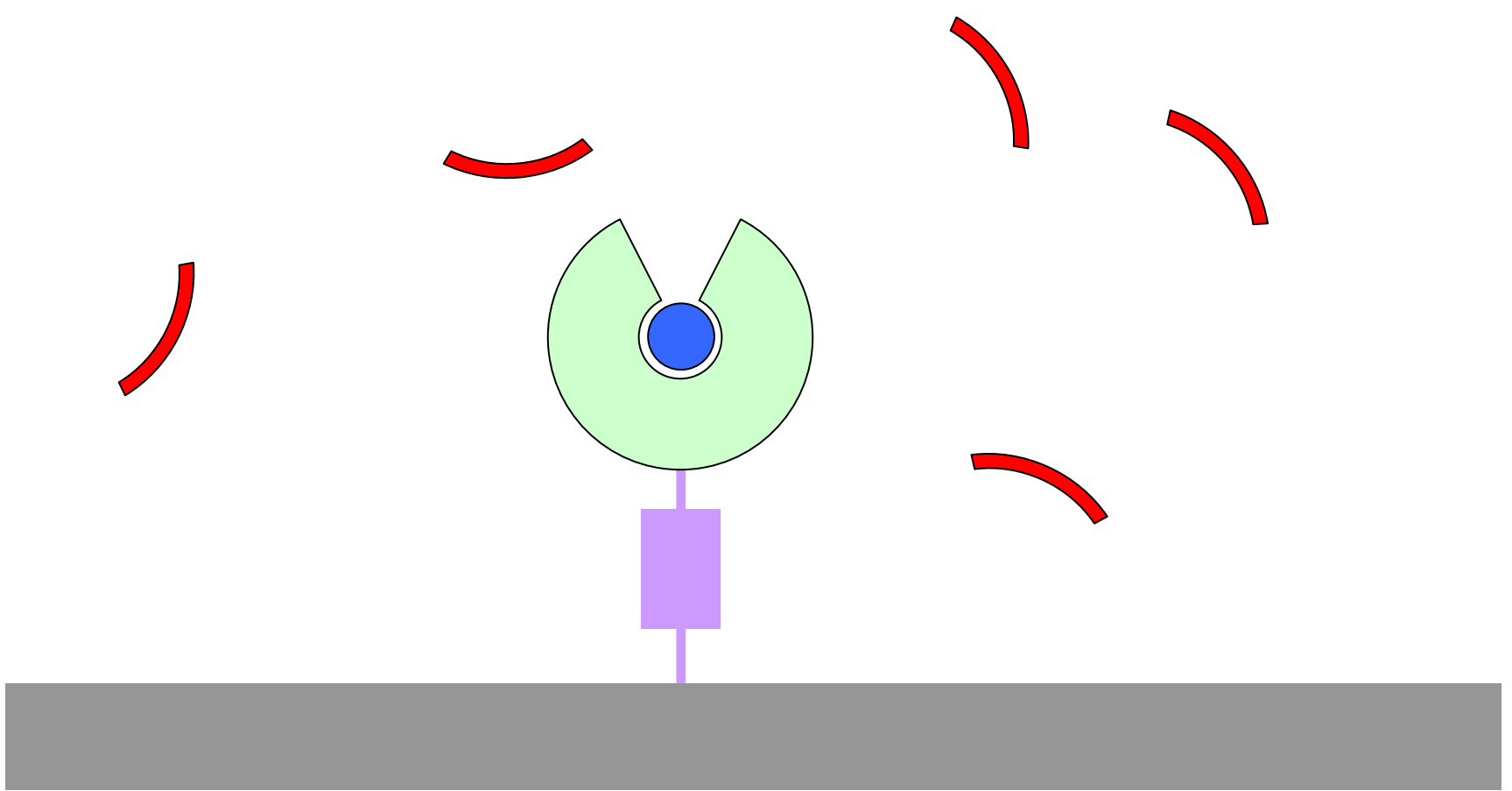
ER β covalently bonded to quartz:

Ligand will bind specifically!



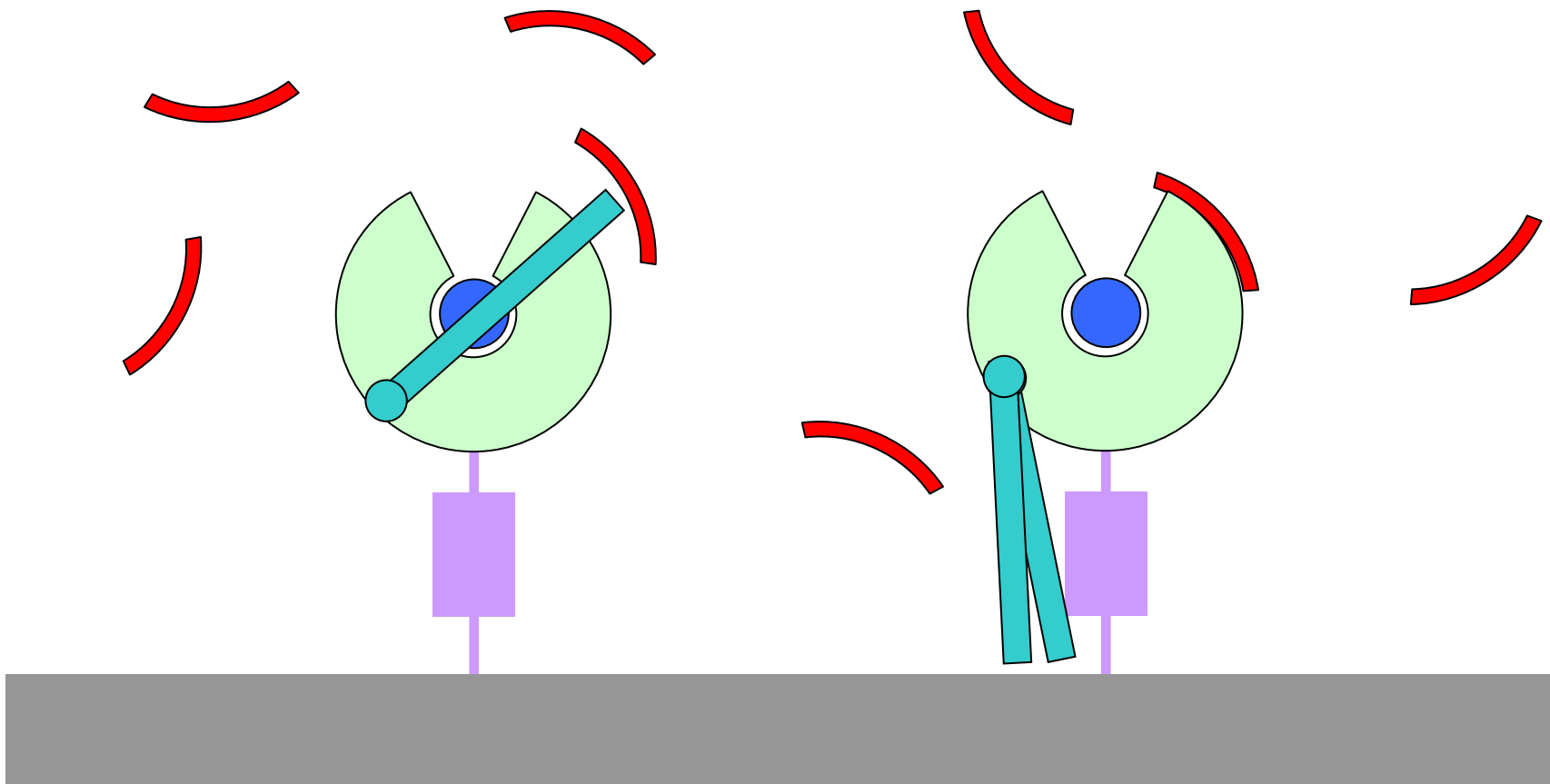
ER β covalently bonded to quartz:

CoA binds to ER β -antagonist complex!



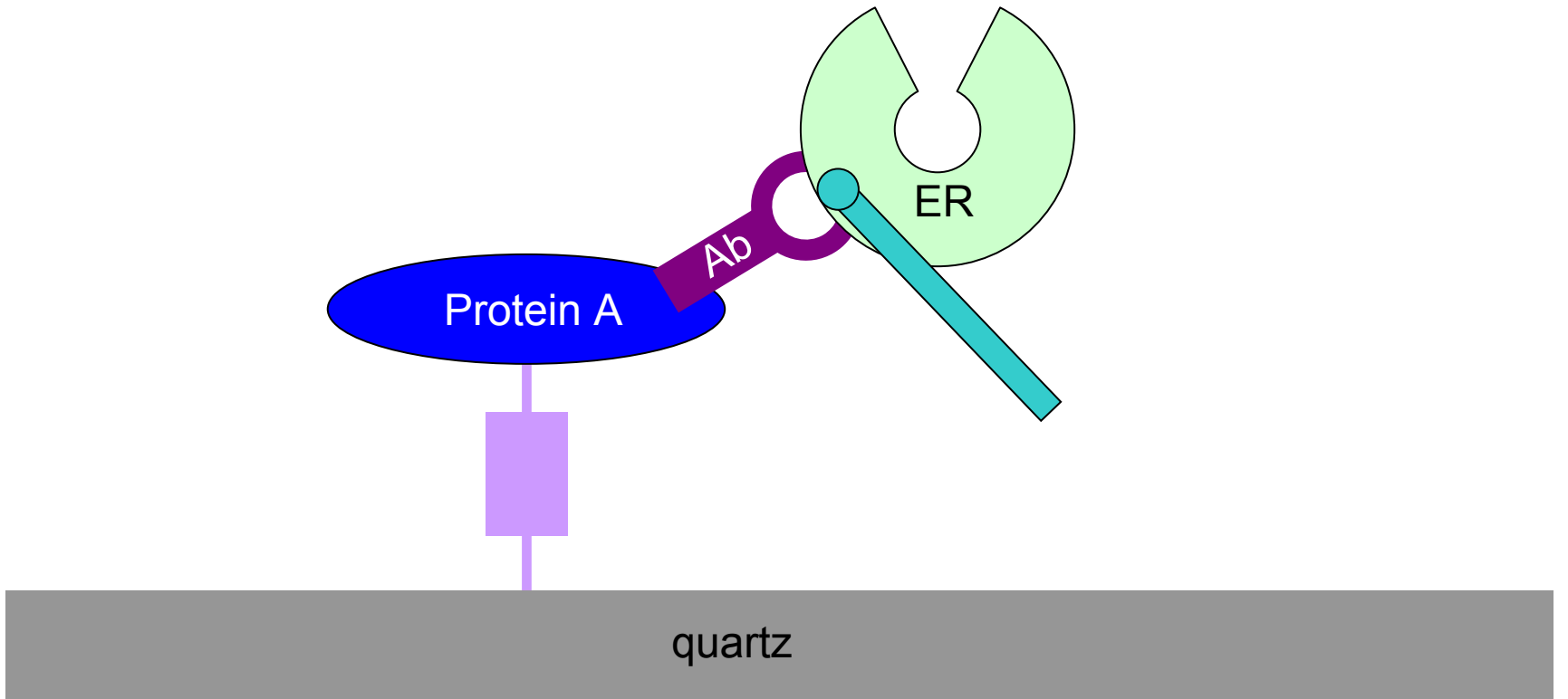
ER β -L covalently bonded to quartz:

CoA blocked from pre-complexed ER β -
antagonist

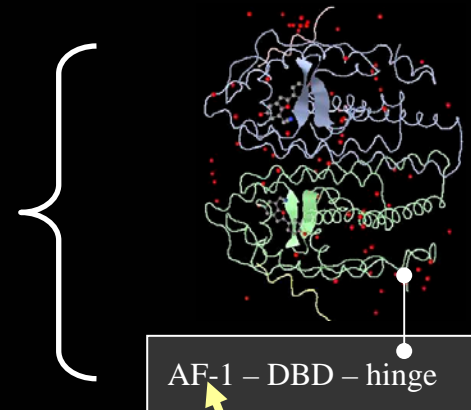


Pre-complex

Post-complex

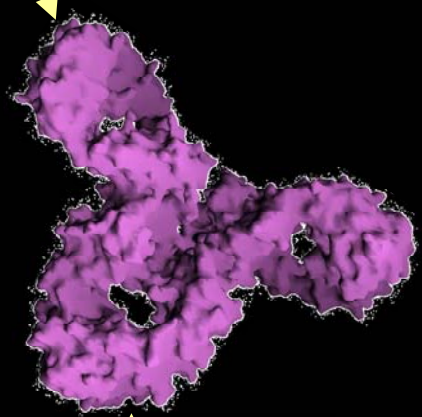


**Estrogen Receptor
Alpha**



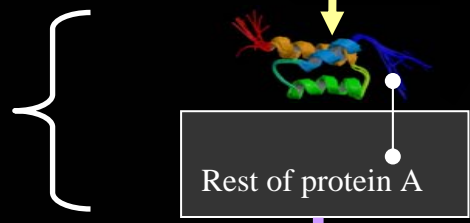
AF-1 – DBD – hinge

Immunoglobulin G



~ 6 nm

Protein A



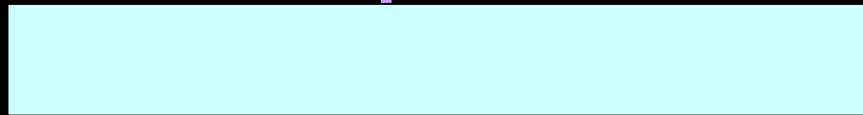
Rest of protein A

~ 5 nm

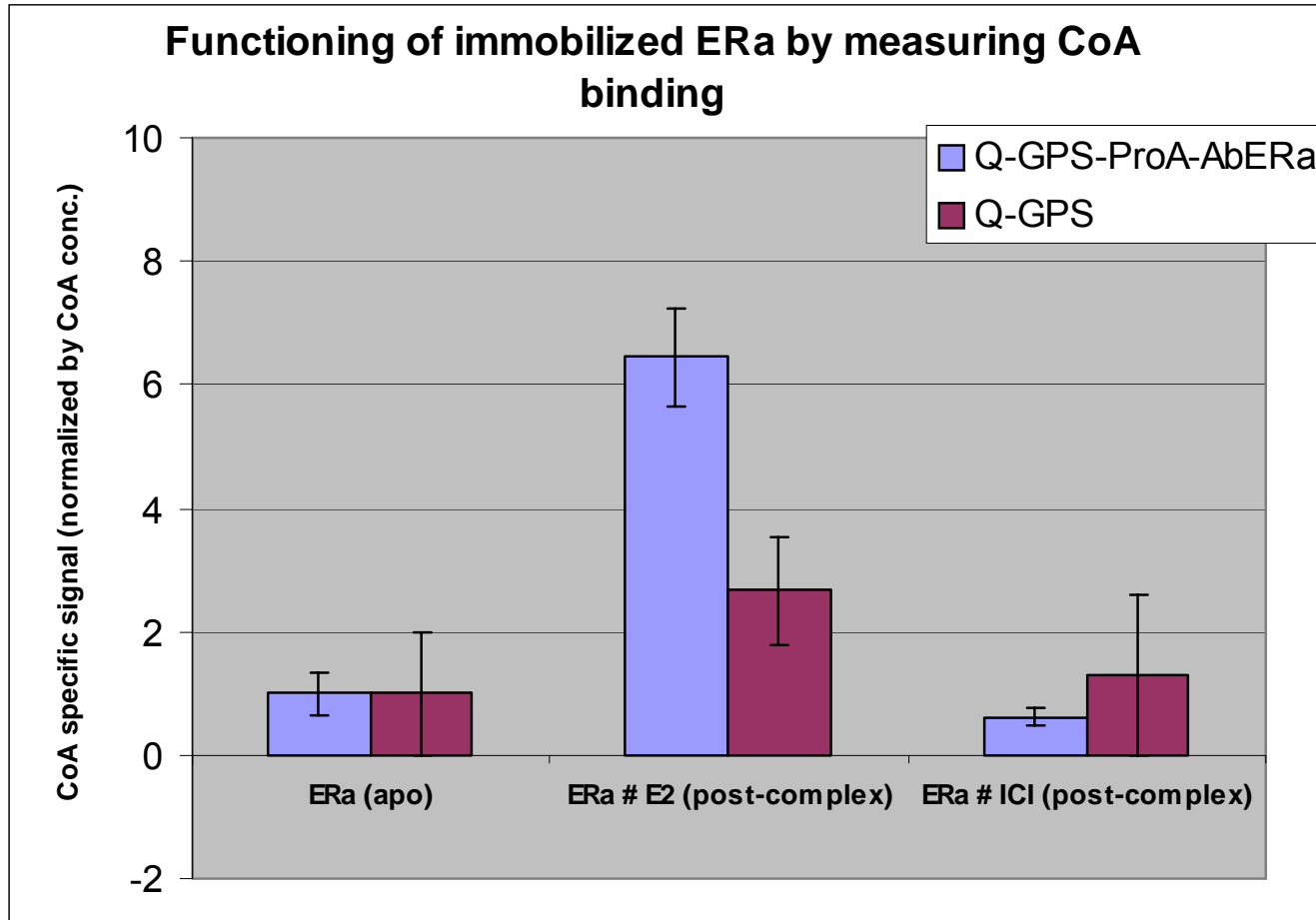
GPS (epoxy)



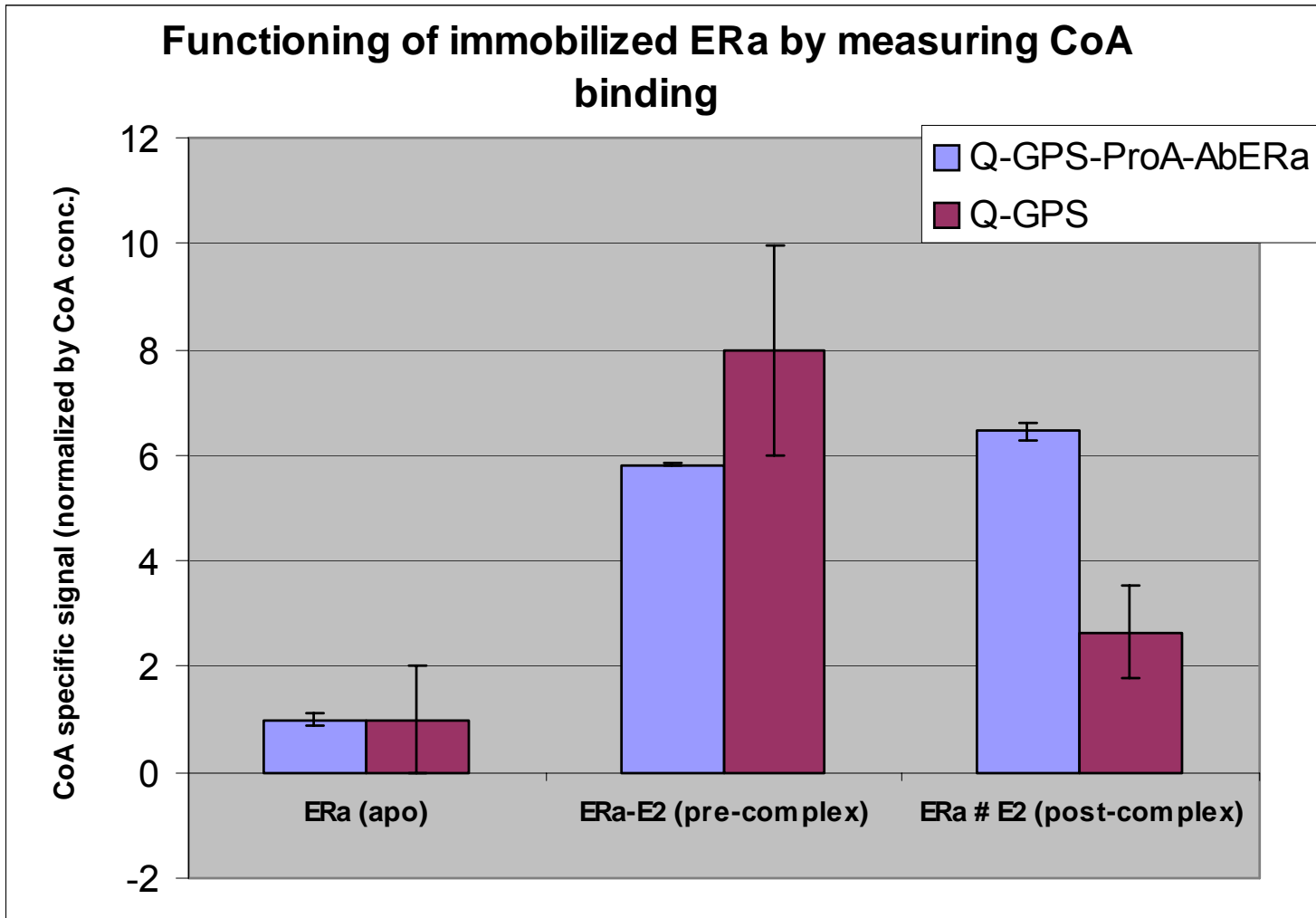
Quartz



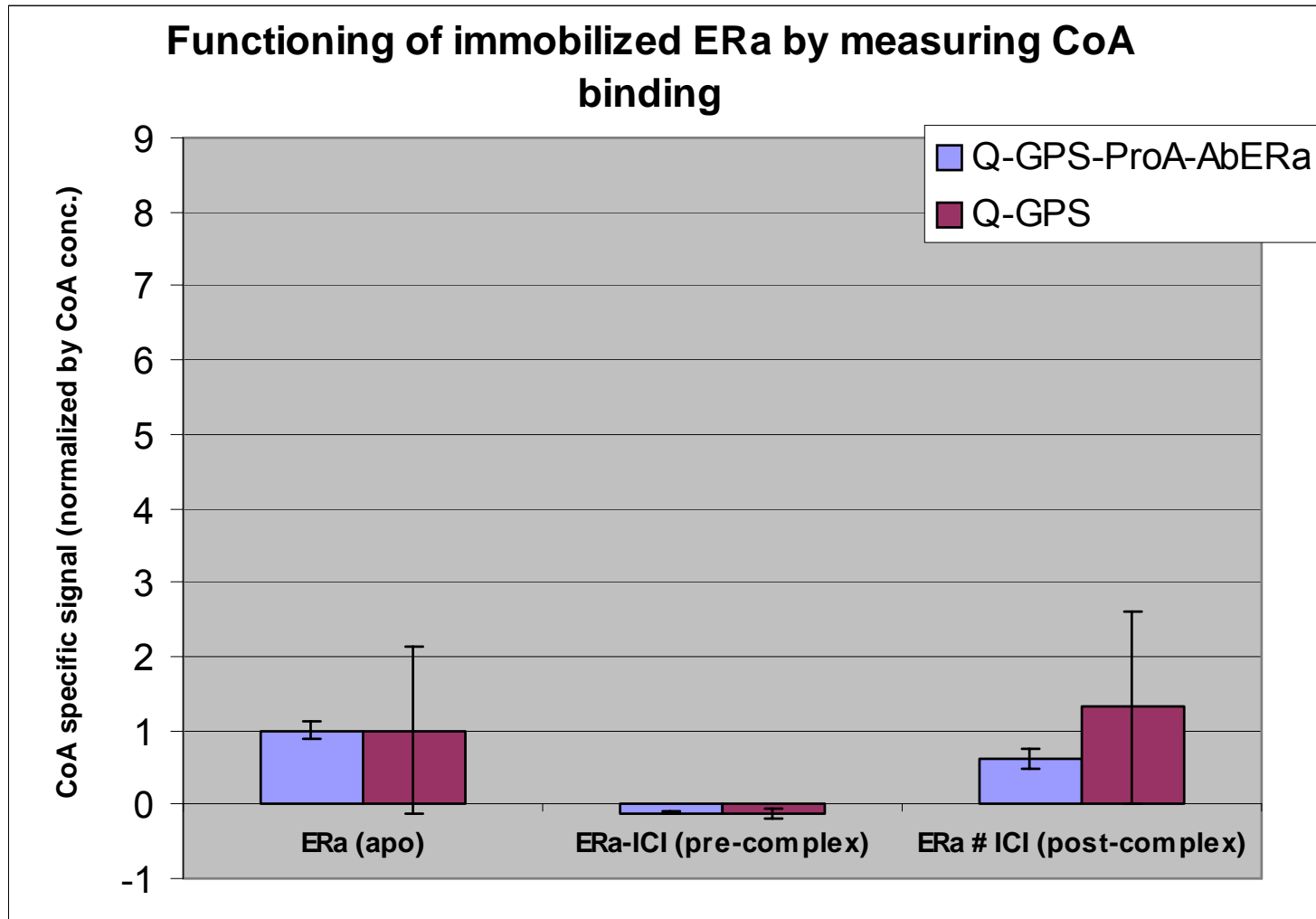
CoA recruitment: Apo, agonist, and antagonist complexed ER α



Agonist complex: pre vs post



Antagonist complex: pre vs post



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- Prof. N.K. Mak
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- Dr Alice S.T. Wong (HKU)

~ Thank you ~

The END