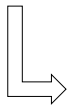
Practical laboratory experience

Study of intracellular Ca^{2+} signals

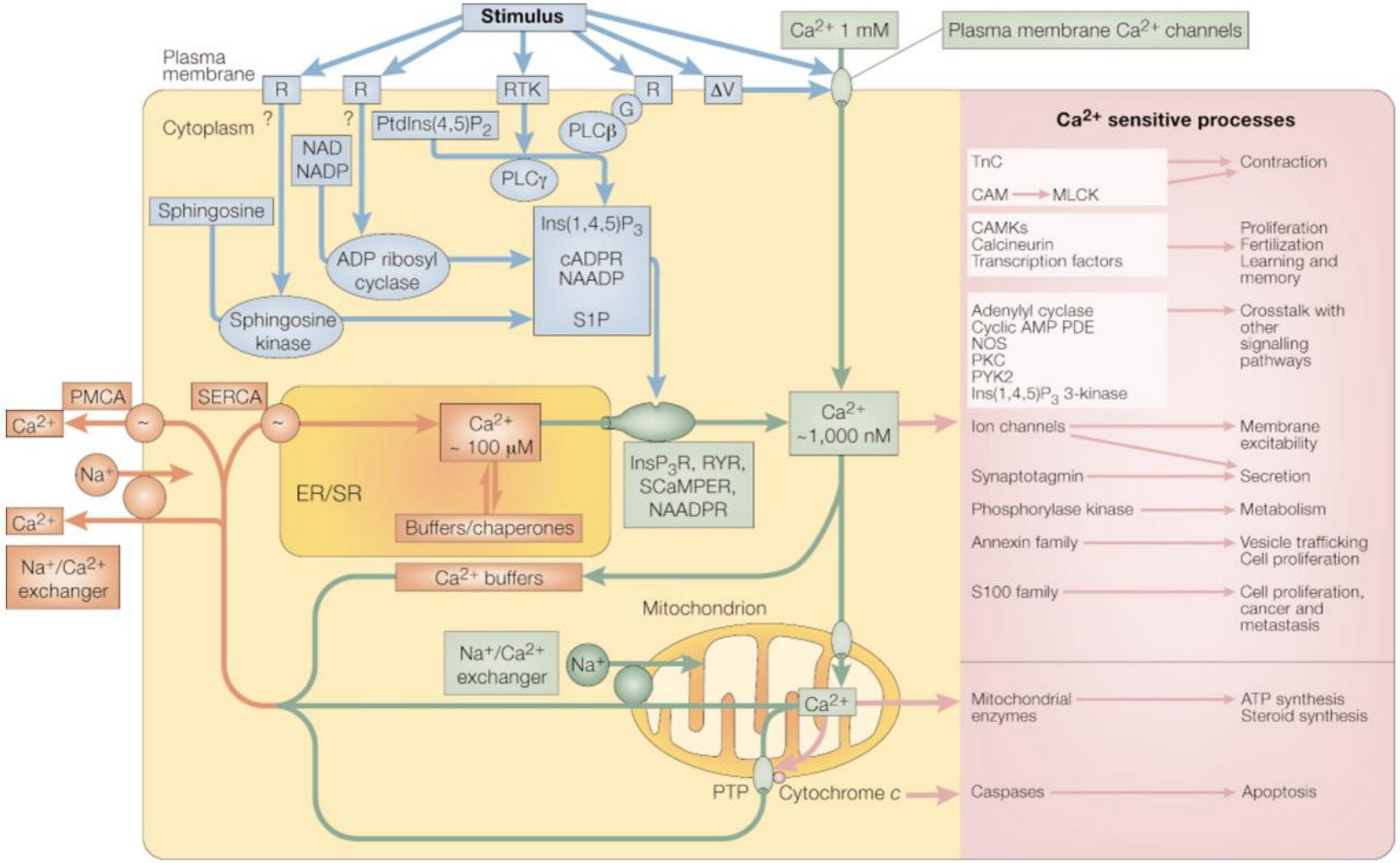
1. Introduction to Ca^{2+} signaling



see theoretical lesson

2. Experimental procedure

The versatility of Ca^{2+} signaling



One messenger

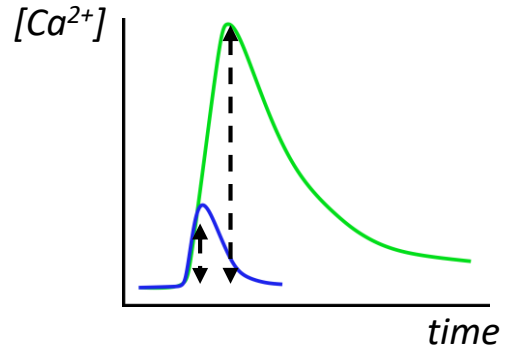
⇩

multiple effects

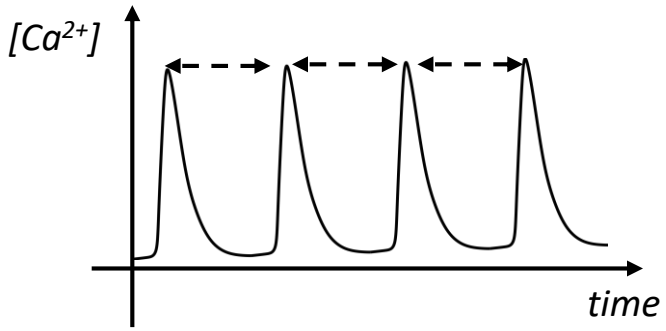
Berridge, Lipp, & Bootman, Nature Reviews 2000

Decodification of Ca^{2+} signals

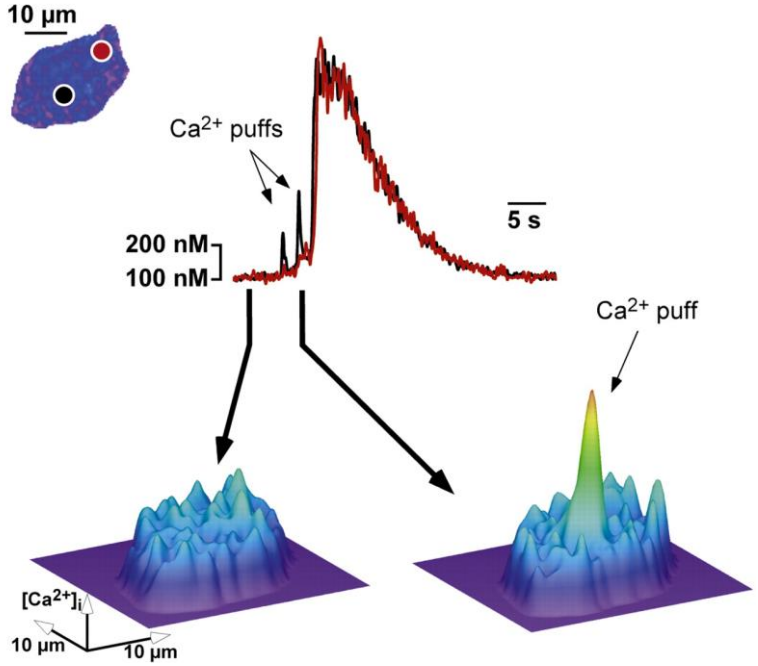
1. AMPLITUDE



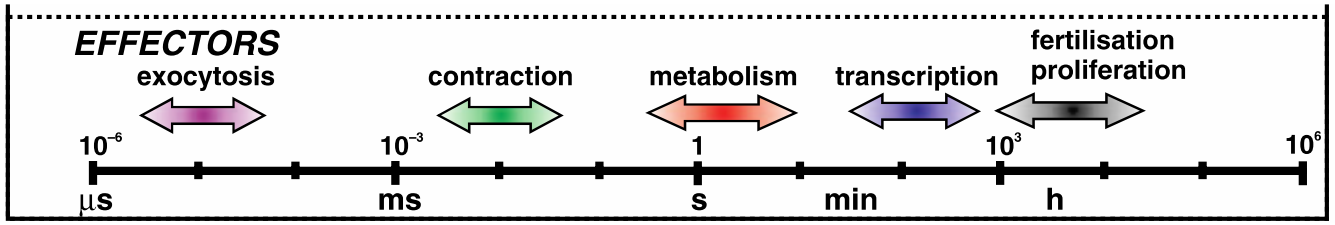
2. FREQUENCY



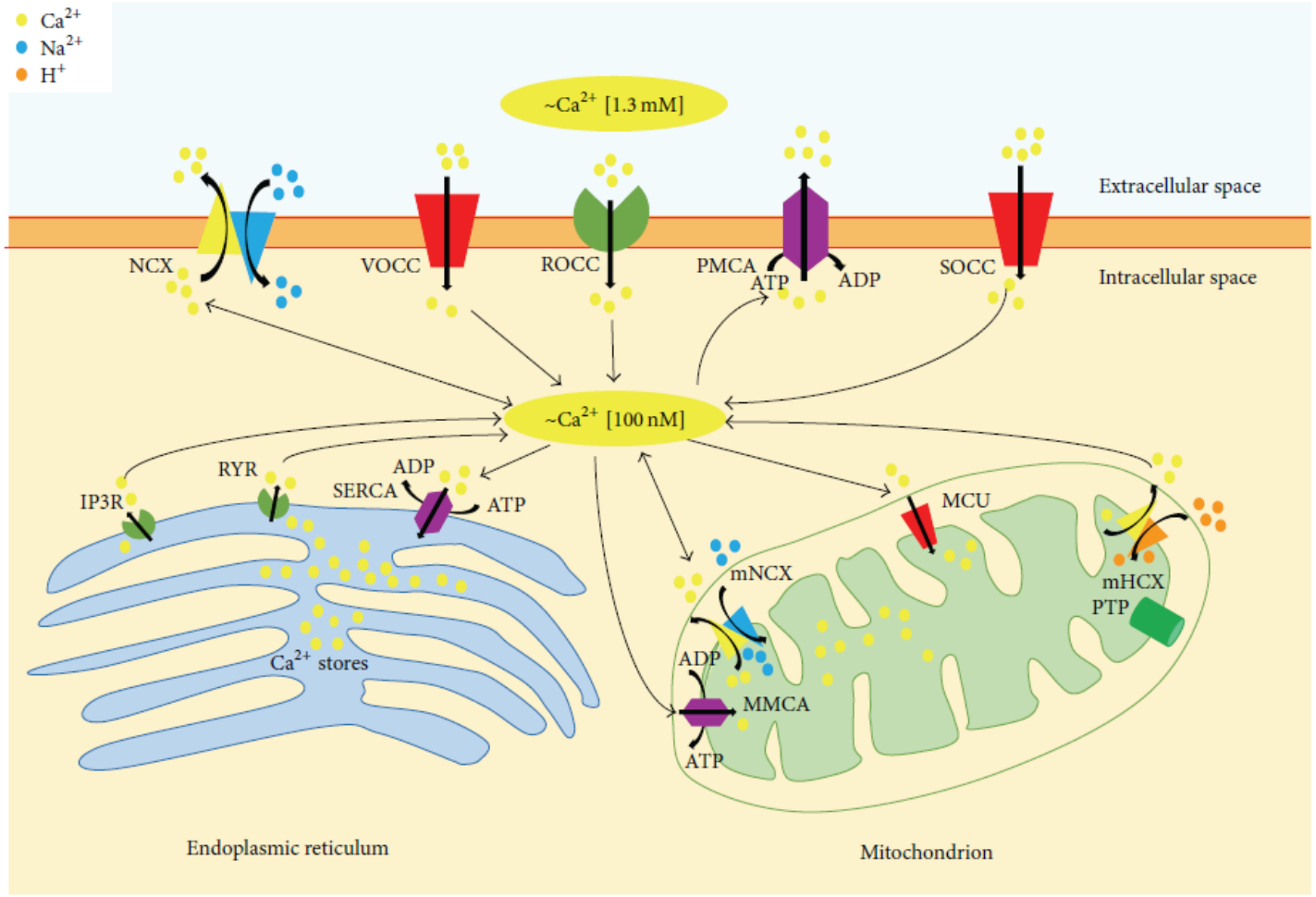
3. LOCALIZATION



4. TIME



Intracellular Ca^{2+} homeostasis

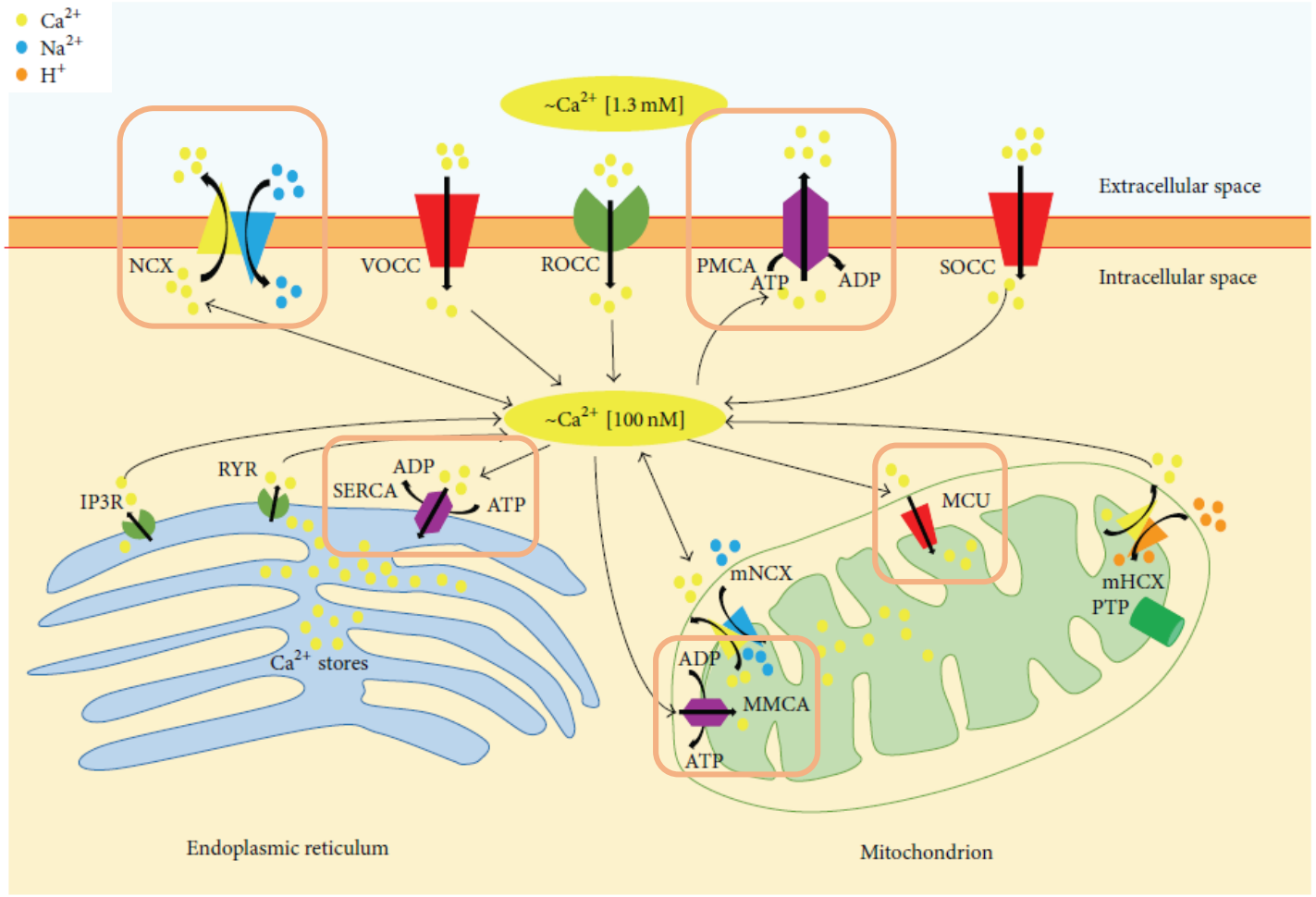


Magi et al., Biomed Res Int 2016

Intracellular calcium levels are tightly regulated within a narrow physiological range

by the so-called "Ca^{2+} signaling toolkit"

Intracellular Ca^{2+} homeostasis



OFF MECHANISMS

- **PMCA** pump
- Na^+/Ca^{2+} exchanger (**NCX**)

Plasma membrane

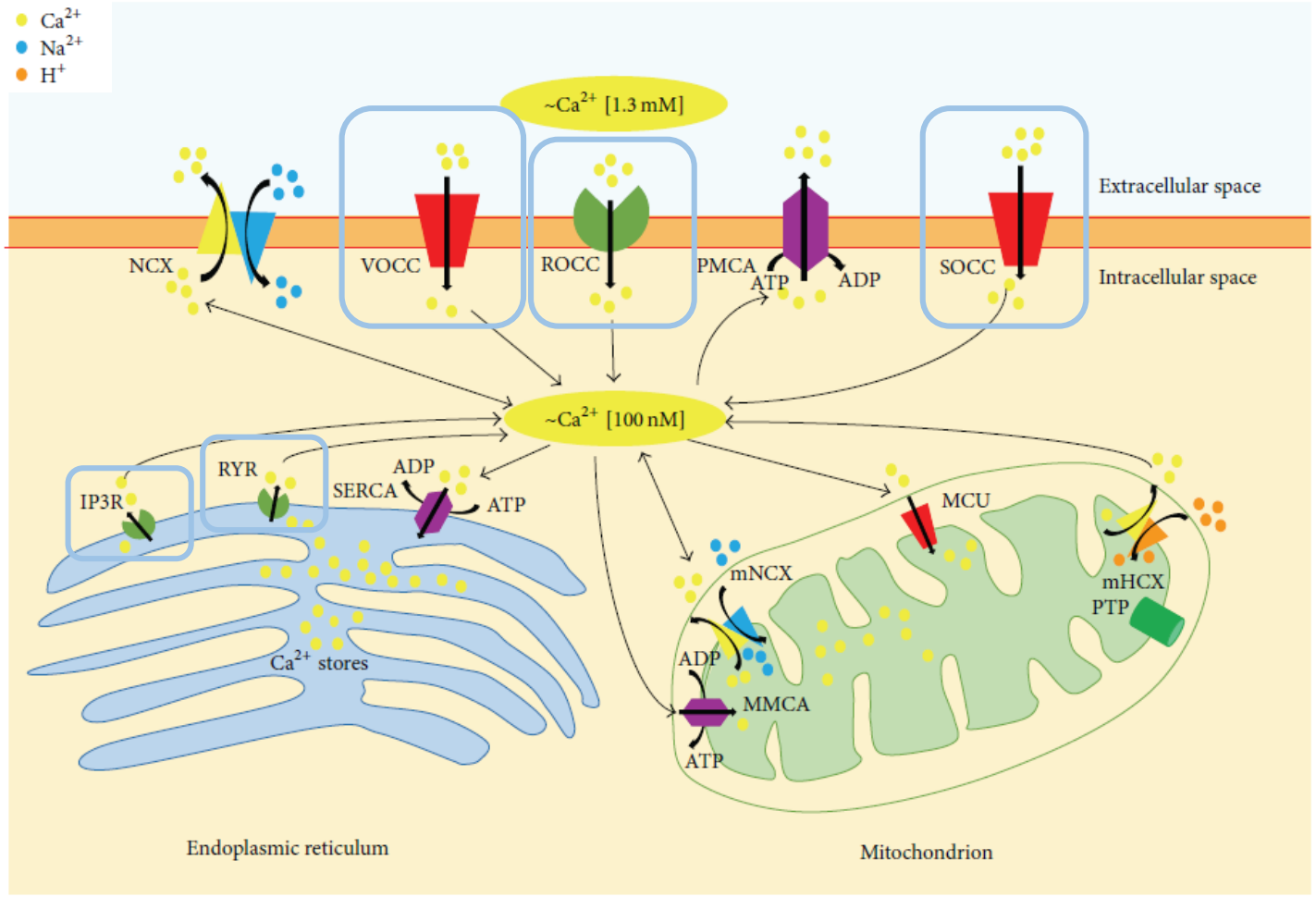
- **SERCA** pump → ER

- **MCU**
- **MMCA** pump

Mitochondria

Magi et al., Biomed Res Int 2016

Intracellular Ca^{2+} signals



Magi et al., Biomed Res Int 2016

ON MECHANISMS

- Plasma membrane Ca^{2+} channels
  - Voltage-operated (**VOOC**)
  - Receptor-operated (**ROOC**)
  - Store-operated (**SOOC**)

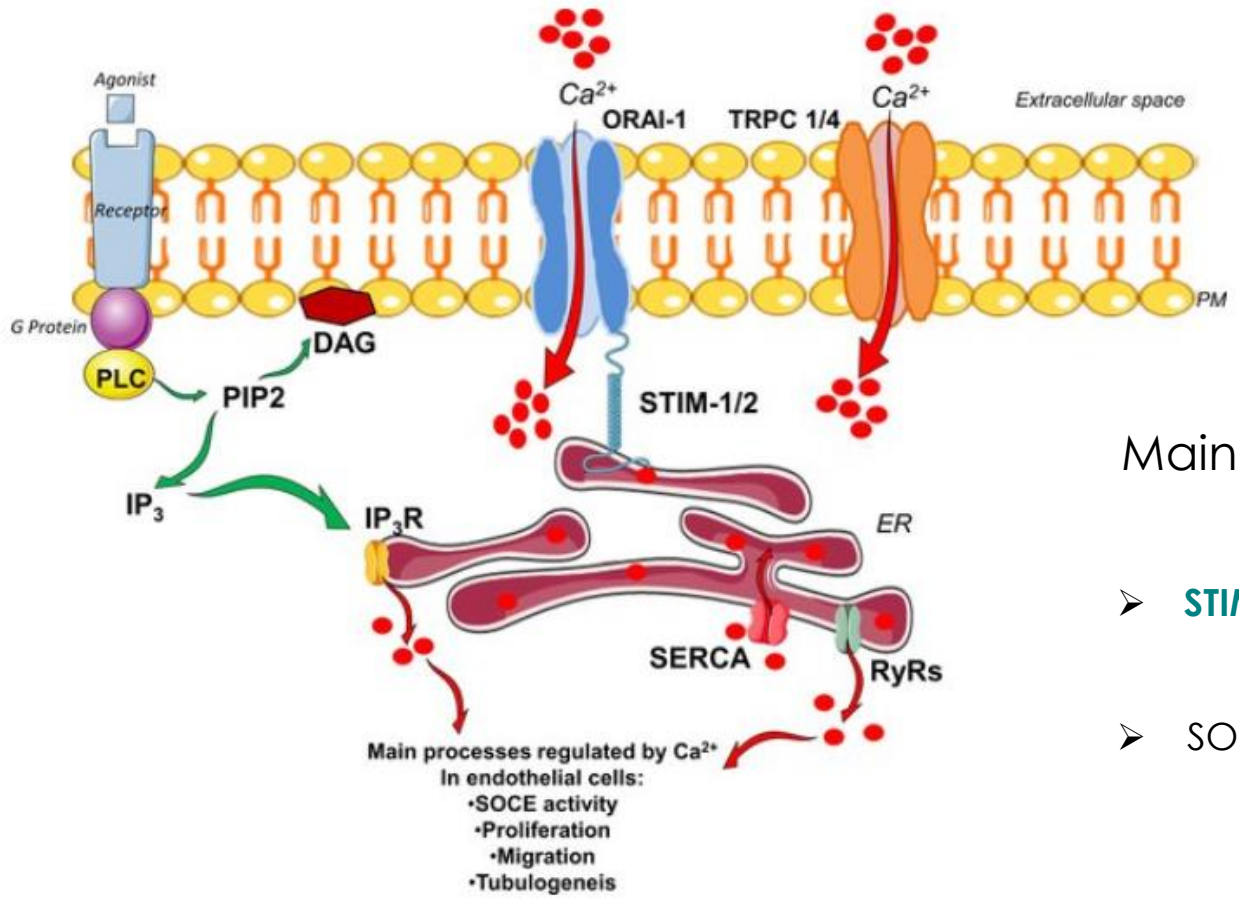
Plasma membrane

- **IP_3R**
- **RyR**

ER

- **SOCE** mechanism

Store-Operated Ca^{2+} Entry (SOCE)

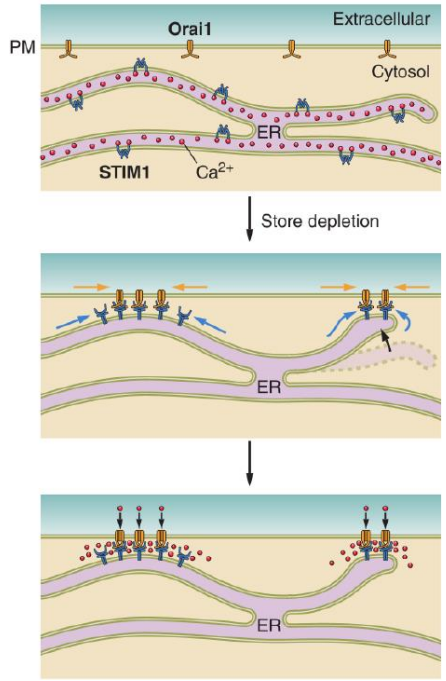


Stanzione et al., Pflugers Arch 2022

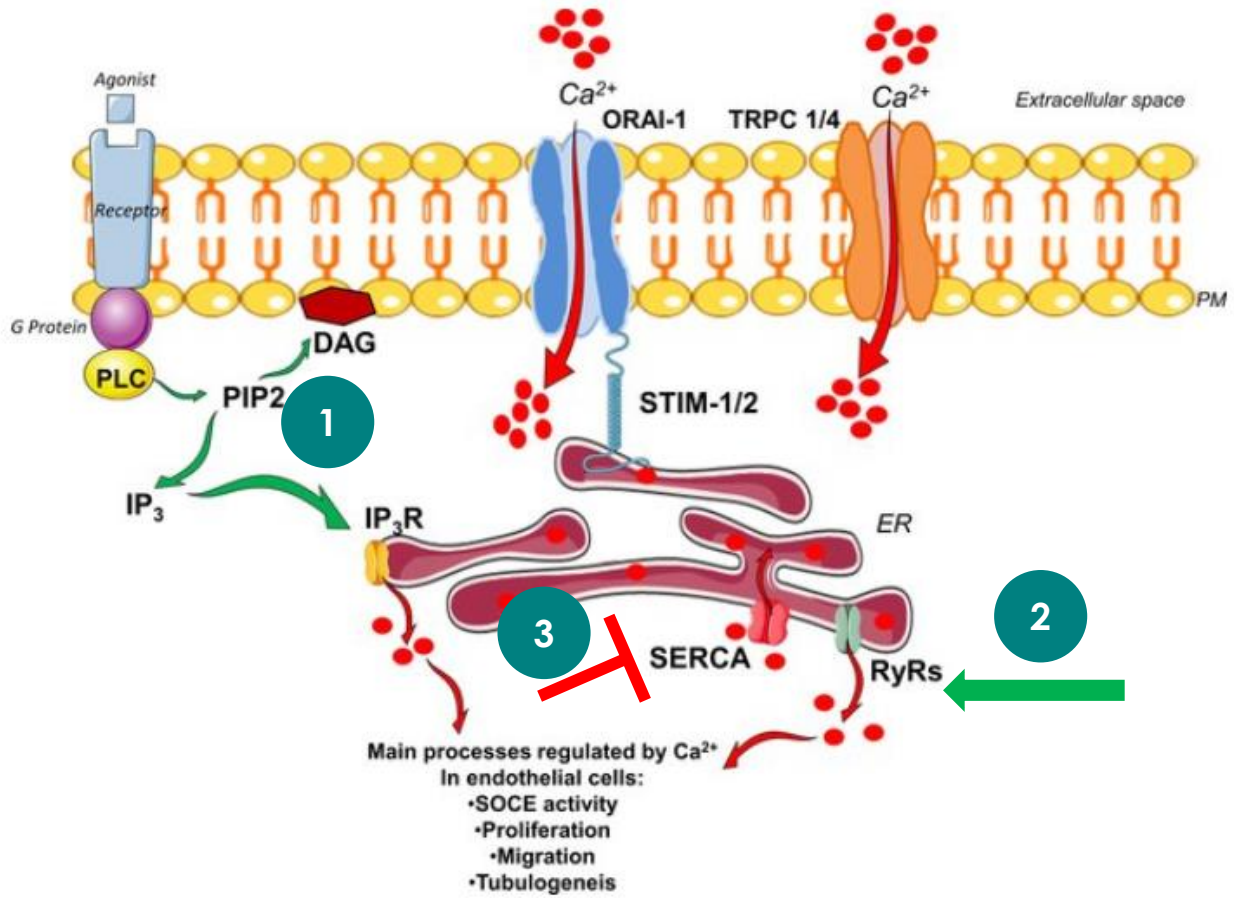
= extracellular Ca^{2+} entry induced by ER Ca^{2+} release
(aimed at restoring intracellular Ca^{2+} homeostasis)

Main actors:

- **STIM** → ER Ca^{2+} sensor
- SOOC (Store-Operated Ca^{2+} Channels)
  - **ORAI-1**
  - **TRPC1/4**



Store-Operated Ca^{2+} Entry (SOCE)

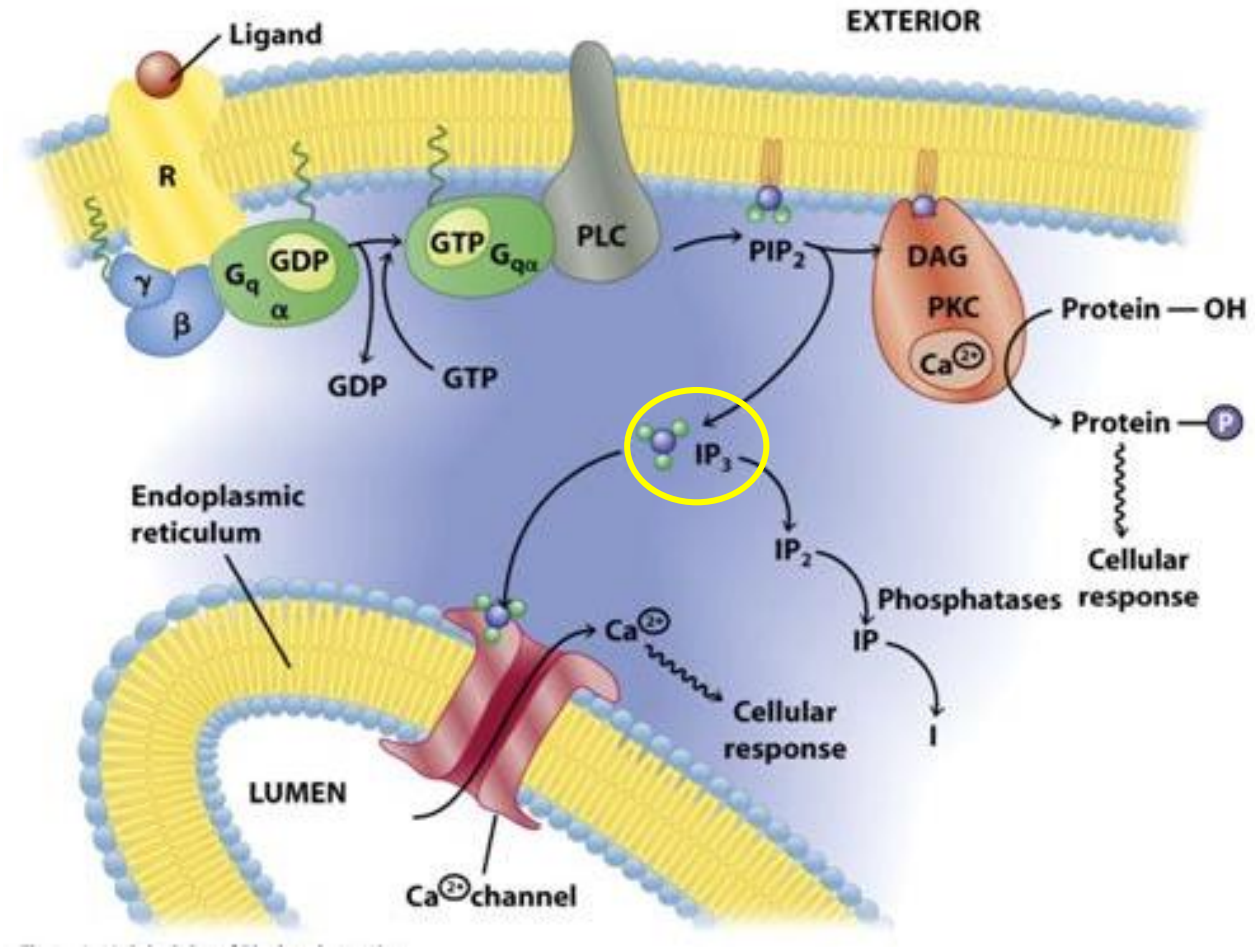


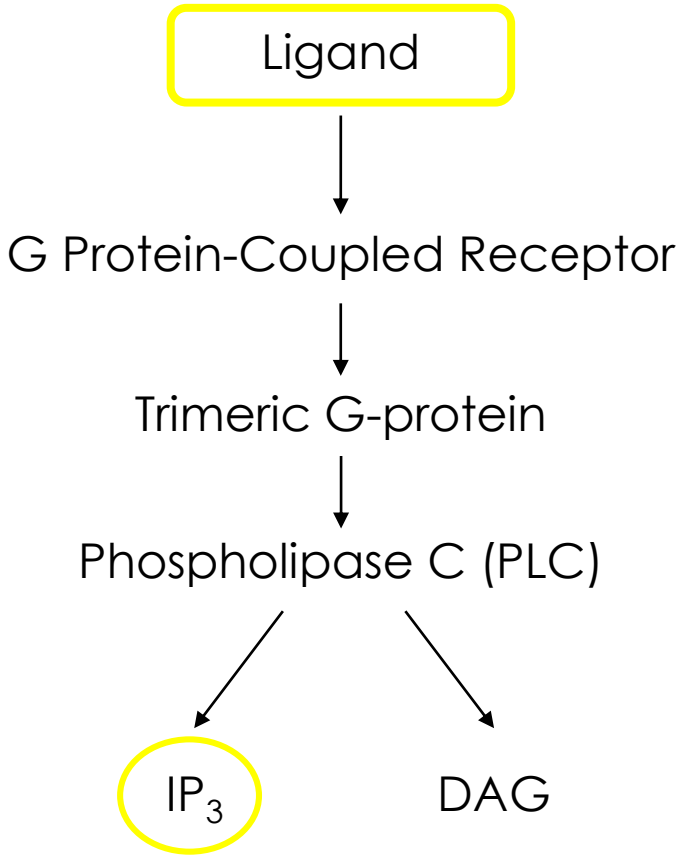
Stanzione et al., Pflugers Arch 2022

ER Ca^{2+}-release can be triggered by:

1. **IP_3R** activation
2. **RYRs** activation
3. **SERCA** Inhibition

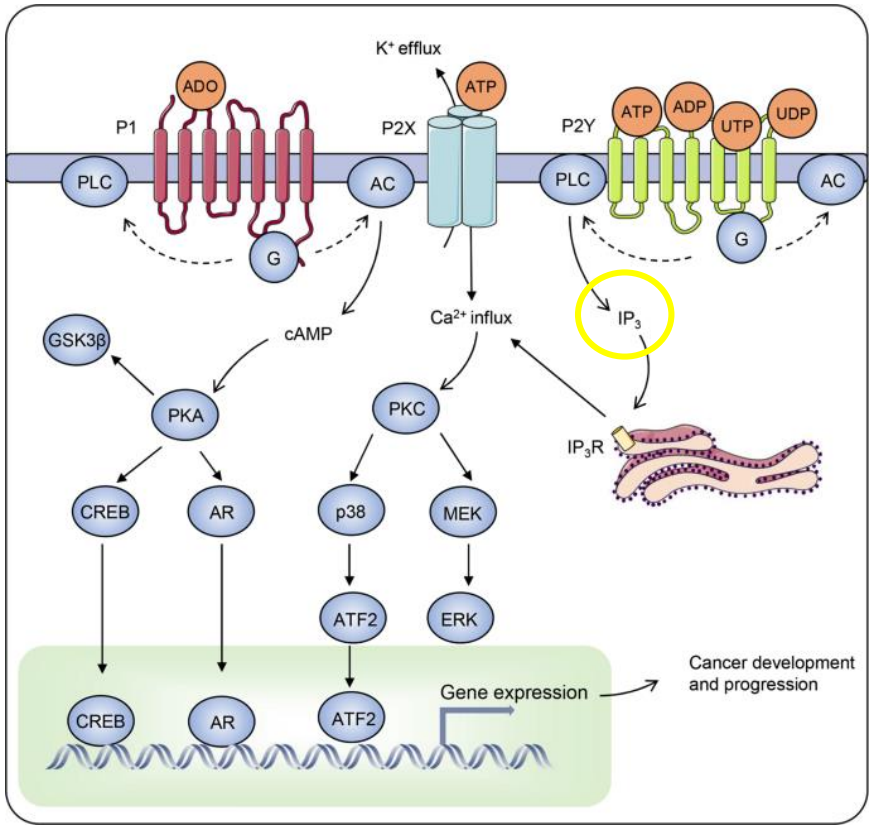
IP_3 pathway


Ligand
EXTERIOR
R
GDP
G_q
α
β
γ
GTP
$G_{qα}$
PLC
PIP_2
DAG
PKC
Ca^{2+}
Protein — OH
Protein — P
GDP
GTP
IP_3
Endoplasmic reticulum
IP_2
Phosphatases
IP
I
Cellular response
Ca^{2+}
Cellular response
LUMEN
Ca^{2+} channel


Ligand
G Protein-Coupled Receptor
Trimeric G-protein
Phospholipase C (PLC)
IP_3
DAG

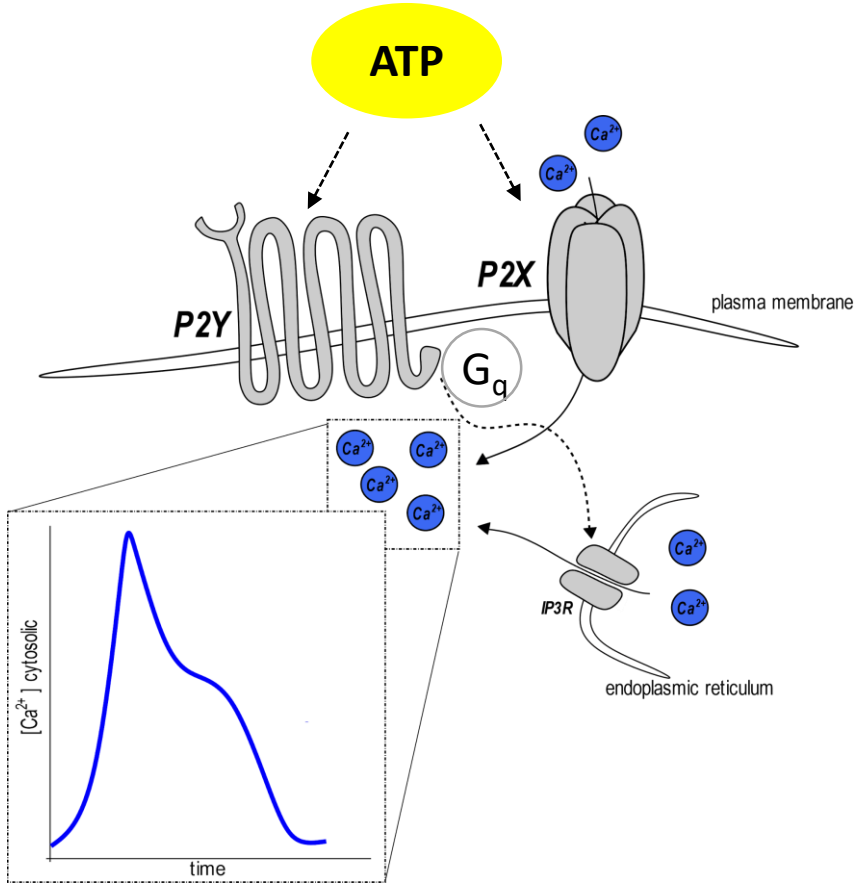
Purinergic signaling

PURINS



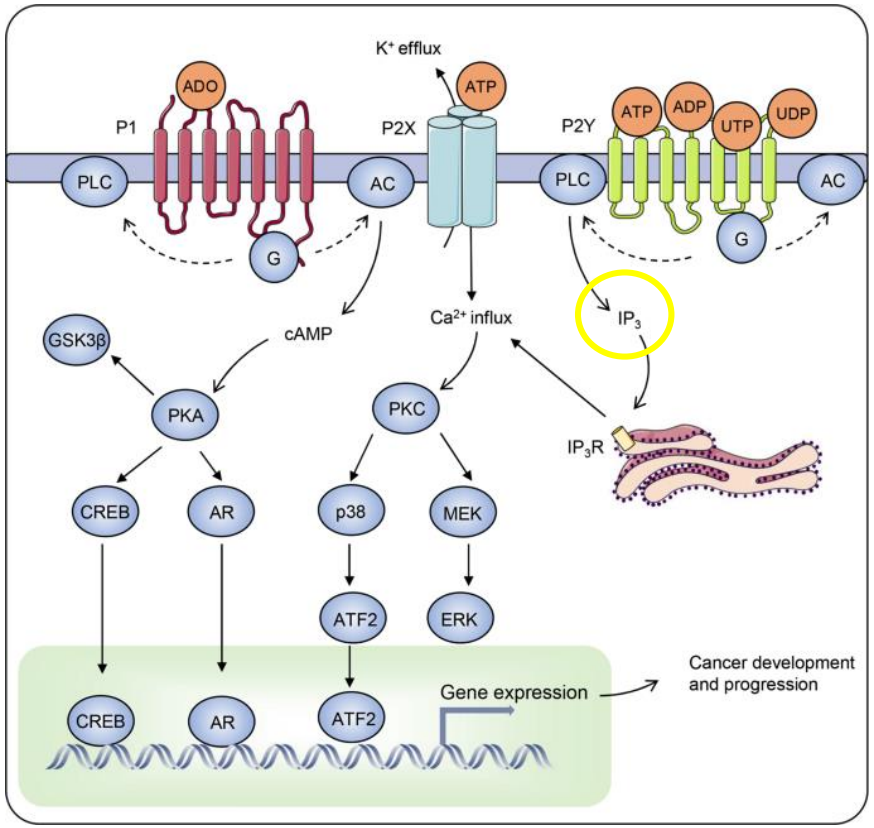
Huang et al., Signal Transduct Target Ther 2021

Purinergic signaling



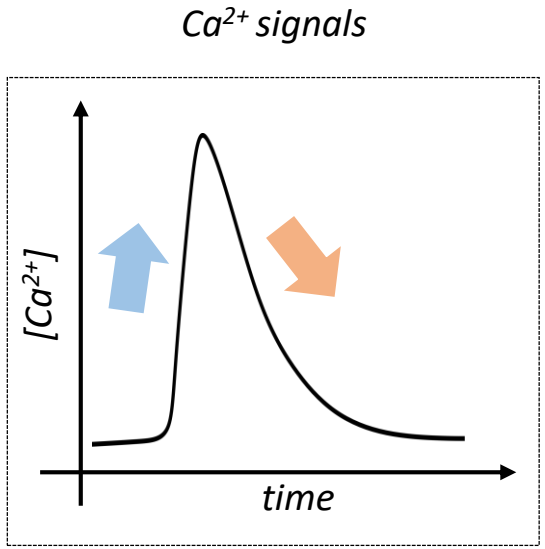
Among others, it plays a crucial role in vascular tone and remodeling

PURINS



Huang et al., Signal Transduct Target Ther 2021

Intracellular Ca^{2+} signals

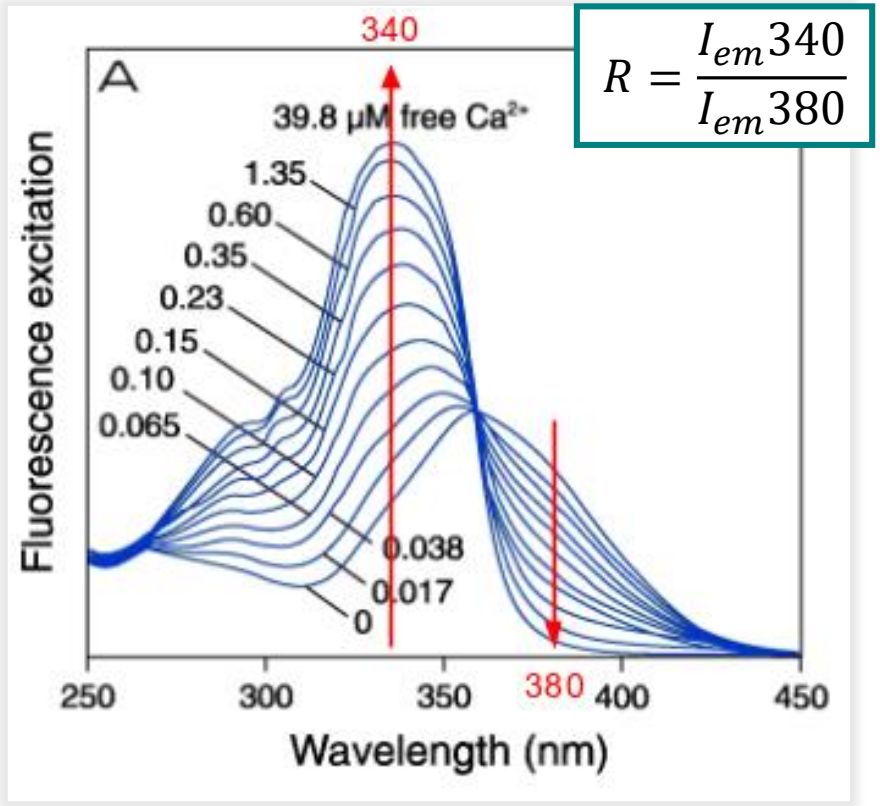


ON MECHANISMS

OFF MECHANISMS

How to detect them?

Intracellular Ca^{2+} indicators:
Fura-2 probe



$$R = \frac{I_{em}340}{I_{em}380}$$

Fura-2 is a fixed emission ratiometric dye used to measure **cytosolic Ca^{2+} signals**

Excitation:
λ_{ex} = 340 nm (bounded form)
λ_{ex} = 380 nm (free form)

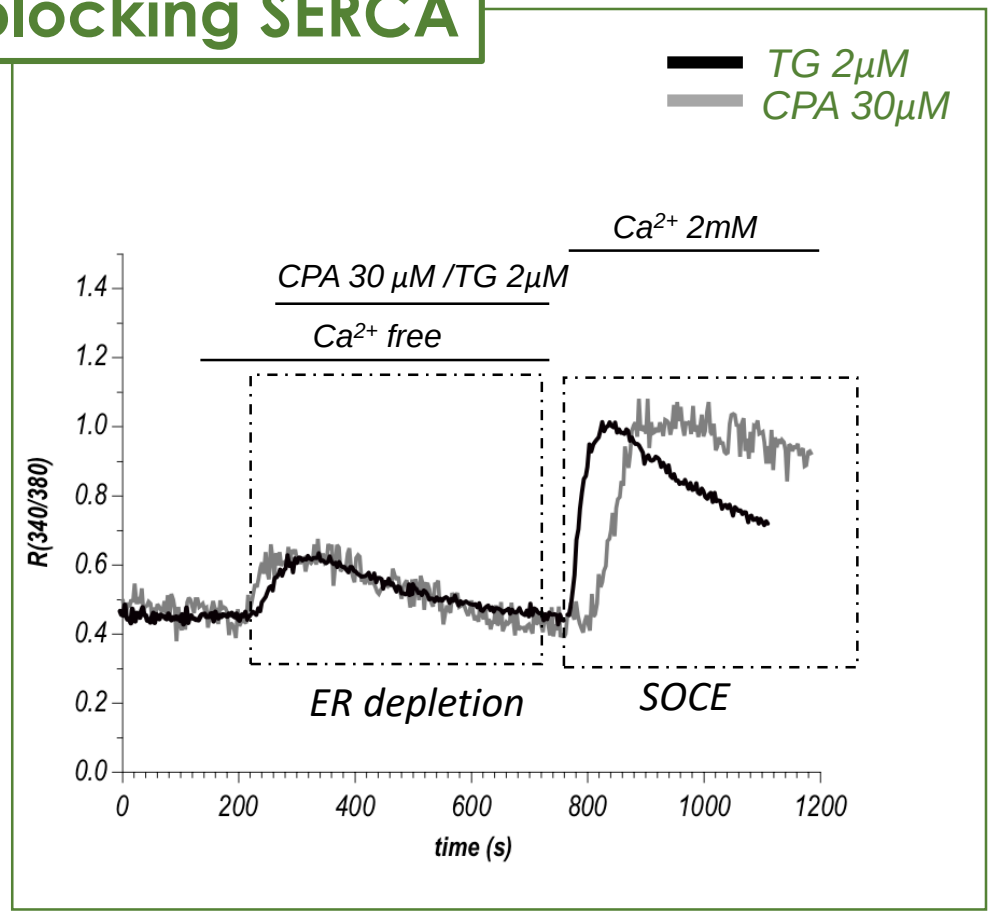
Fixed emission:
λ_{em} = 510 nm

Table 1. Spectroscopic properties and Ca^{2+} dissociation constants for fura-2, indo-1, and their derivatives.

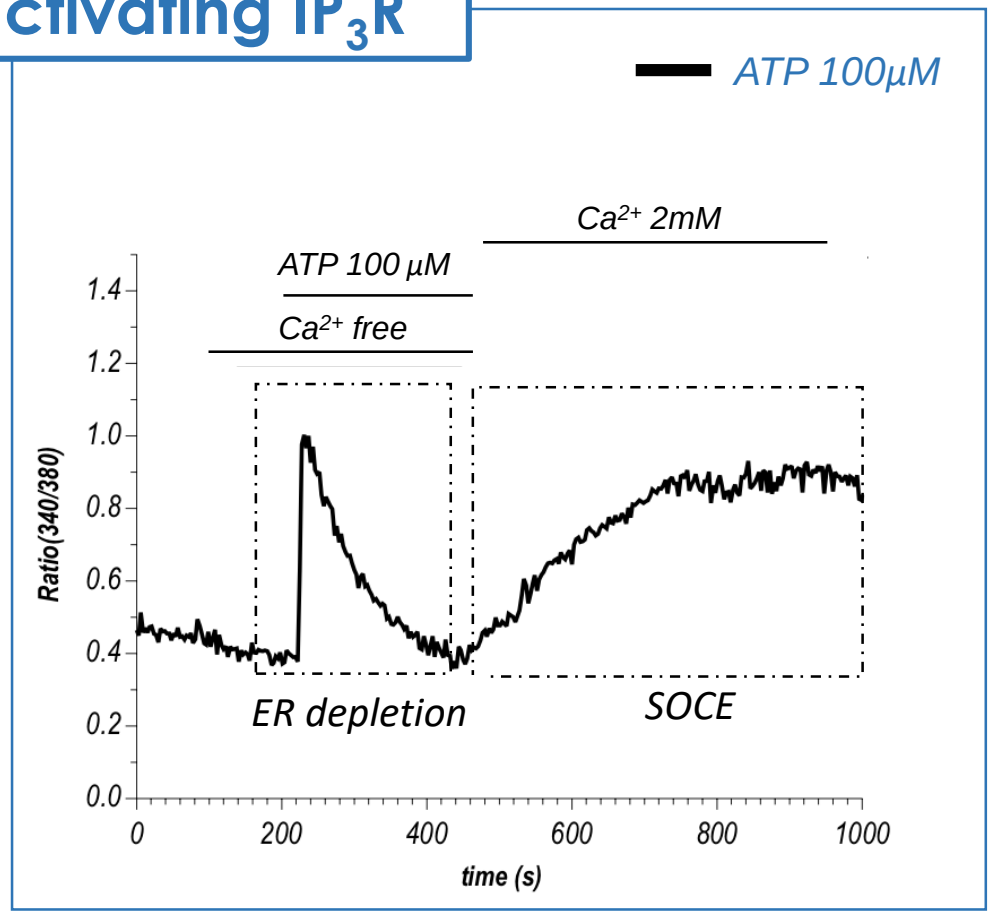
Indicator	Catalog Number		Zero Calcium			High Calcium			K_d (Ca^{2+}) (μM)
	Salt	AM Ester	λ_A † (nm)	ϵ_{max} ‡ ($cm^{-1}M^{-1}$)	λ_F § (nm)	λ_A † (nm)	ϵ_{max} ‡ ($cm^{-1}M^{-1}$)	λ_F § (nm)	
fura-2	F1200, F6799	F1201, F1221, F1225, F14185 *	363	28,000	512 **	335	34,000	505 ††	0.14

Intracellular Ca^{2+} indicators:
Fura-2 probe

blocking SERCA



activating IP_3R



examples of SOCE signals measured with Fura-2 probe

1. Introduction to Ca^{2+} signaling

see theoretical lesson

2. Experimental procedure

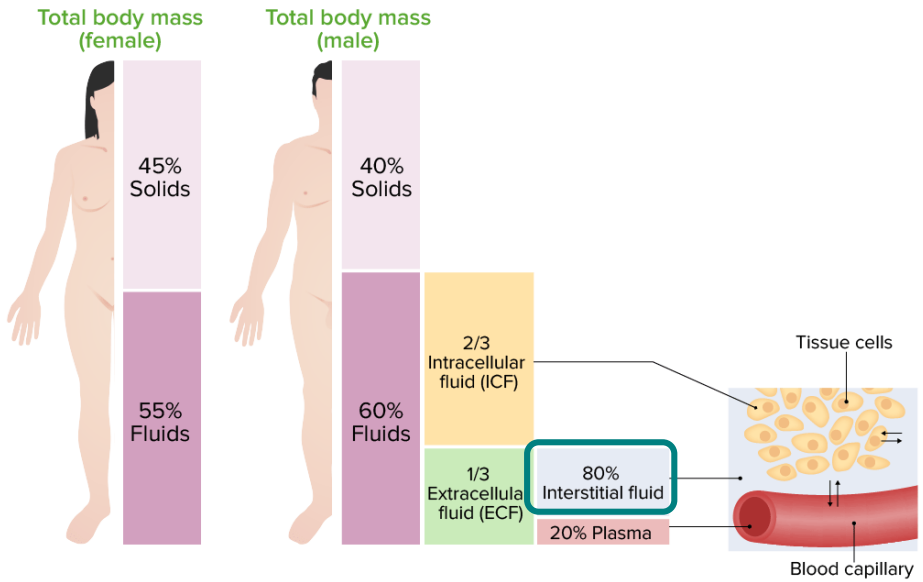
EXPERIMENTAL PROCEDURE

Scope:

Study ATP-induced Ca^{2+} signals in endothelial cells

EXPERIMENTAL PROCEDURE

1. Preparation of physiological extracellular solutions



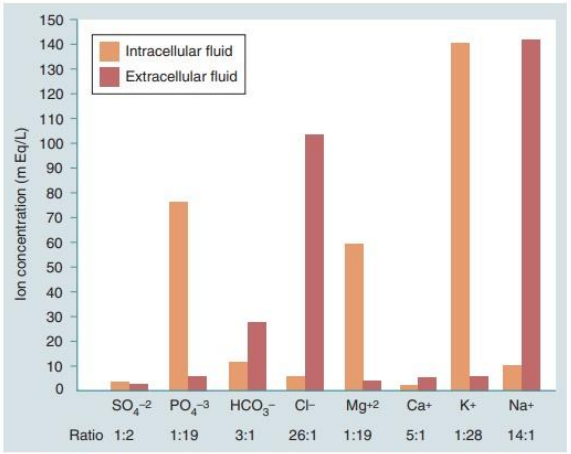


FIGURE 23-2 Concentration of various ions in extracellular and intracellular fluid.

Biochemical lab (1° floor)

Tyrode Standard	
	mM
NaCl	154
$CaCl_2$	2
KCl	4
$MgCl_2$	1
HEPES	5
Glucose	5.5

pH = 7.4

Tyrode Ca^{2+}-free	
	mM
NaCl	154
$CaCl_2$	-
KCl	4
$MgCl_2$	1
HEPES	5
Glucose	5.5

pH = 7.4

- Solution that reproduces as closely as possible the composition of physiological extracellular solutions
- A Ca^{2+}-free solution is needed to investigate SOCE signals

EXPERIMENTAL PROCEDURE

1. Preparation of physiological extracellular solutions

Biochemical lab (1° floor)

1. Calculate the amount of each reagent needed to prepare **250** or **100 mL** of a **10X solution**

Tyrode Standard

	Conc. Finale (mM)	MW	g/l	ml/l	10X g/l	10X ml/l	10X g/250ml	10X µL/250ml	10X g/100ml	10X µL/100ml
NaCl	154	58.44								
$CaCl_2$	2	147.02								
KCl	4	74.56								
$MgCl_2$	1	STOCK 2 M	/		/		/		/	
HEPES	5	238								
Glucose	5.5	180.2								

Tyrode Ca^{2+}-free

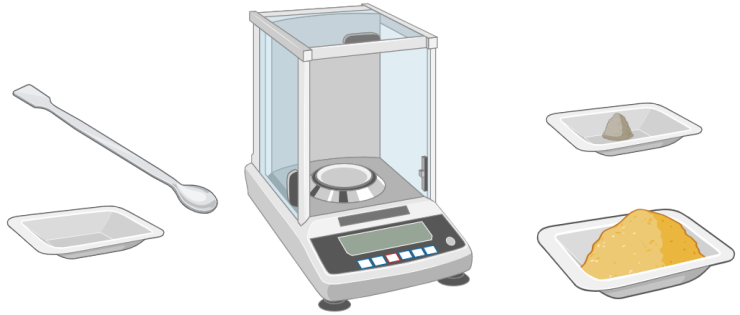
	Conc. Finale (mM)	MW	g/l	ml/l	10X g/l	10X ml/l	10X g/250ml	10X µL/250ml	10X g/100ml	10X µL/100ml
NaCl	154	58.44								
KCl	4	74.56								
$MgCl_2$	1	STOCK 2 M	/		/		/		/	
HEPES	5	238								
Glucose	5.5	180.2								

→ *You can find solutions recipes on Moodle*

EXPERIMENTAL PROCEDURE

1. Preparation of physiological extracellular solutions

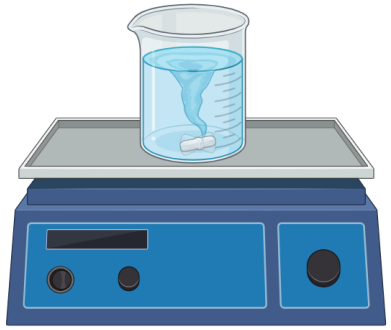
2. Weight all reagents



Analytic balance

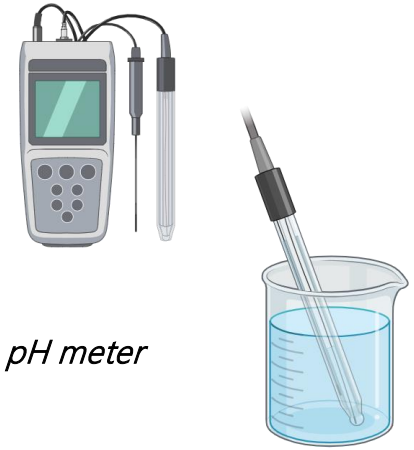
3. add milliQ water

4. dissolve the solution under stirring



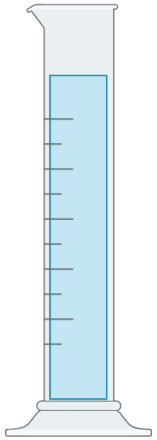
Magnetic stirrer

5. adjust the pH to 7.4



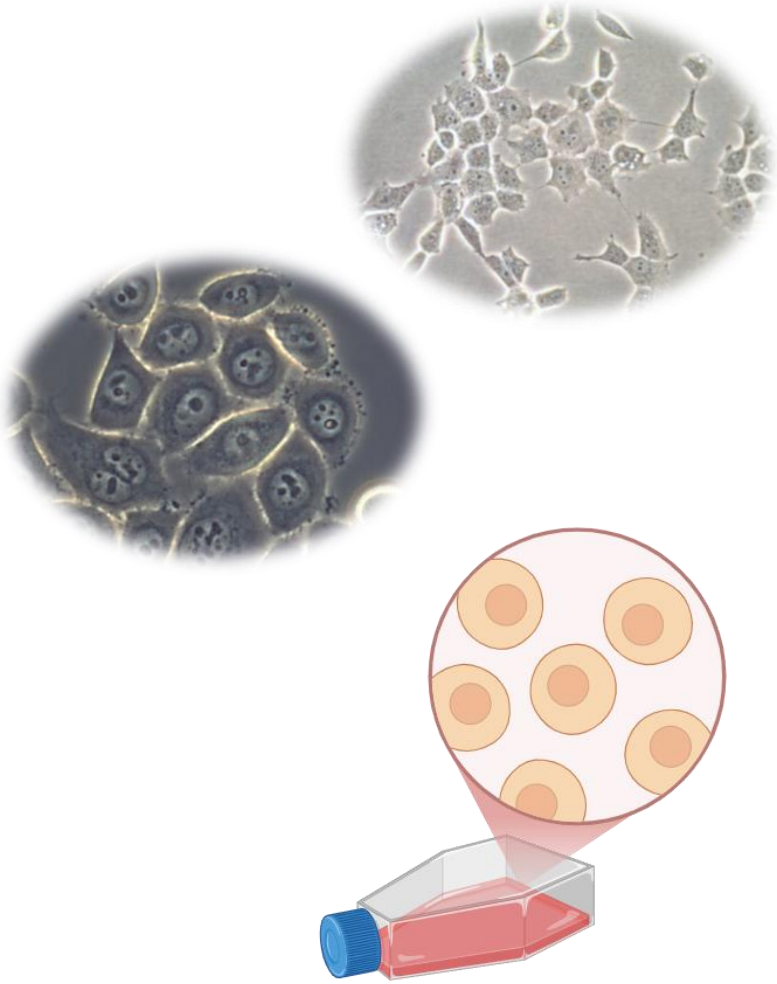
pH meter

6. bring the solution to final volume



10X solution

2. Cell culture

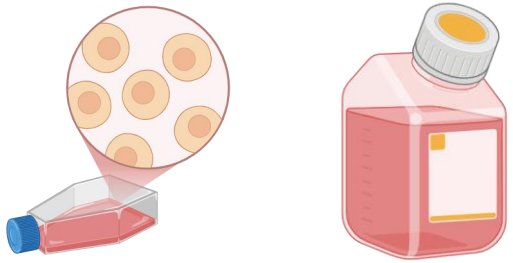


Vantaggi	Svantaggi
Sistemi semplificati ed altamente riproducibili	Sistemi semplificati rispetto ad un organismo integrato
Consentono l'analisi dei meccanismi cellulari e molecolari alla base del fenomeno in esame	Condizioni di esposizione alle sostanze diverse da quelle *in vivo*
Consentono un controllo molto fine delle condizioni ambientali	Concentrazioni *in vitro* difficilmente correlabili con quelle *in vivo*
Economicità e rapidità di risposta	Le sostanze inoculate possono interagire con il terreno di coltura
Ampia varietà di tipi cellulari disponibili	
No problemi etici e legali	

2. Cell culture:
Equipment

Flasks, dishes and plates for cell culture

Cell growth medium



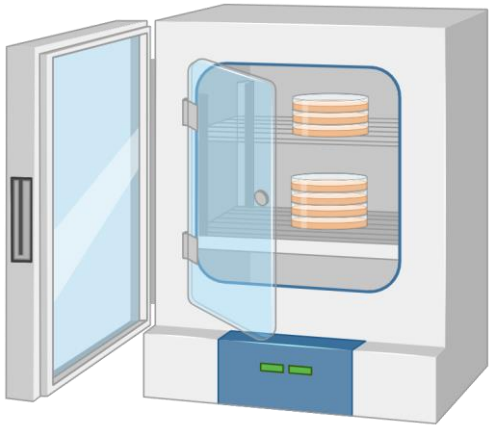
- Basal nutrients (glucose, aminoacids, mineral salts, etc.)
- Buffer system

supplemented (just before use) with:

- Glutamine (very labile essential amino acid)
- Serum (in varying amount)
- Antibiotics
- other specific supplements (growth factors, nutrients, etc.)

2. Cell culture:
Equipment

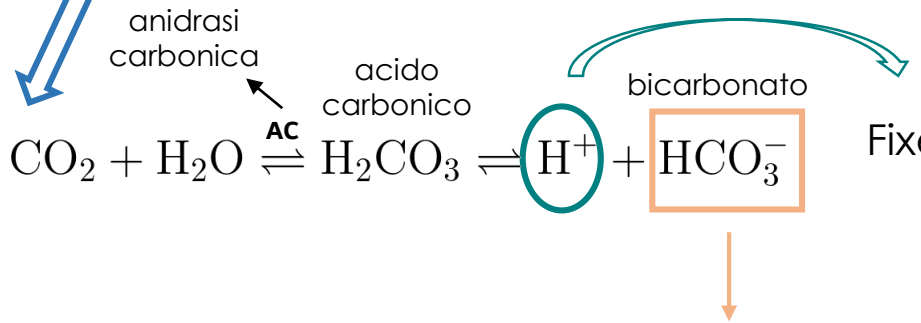
Incubator



ensures **controlled** cell growth **conditions**

Temperature: **37°C**

Gaseous phases: **5% CO_2** and 95% O_2



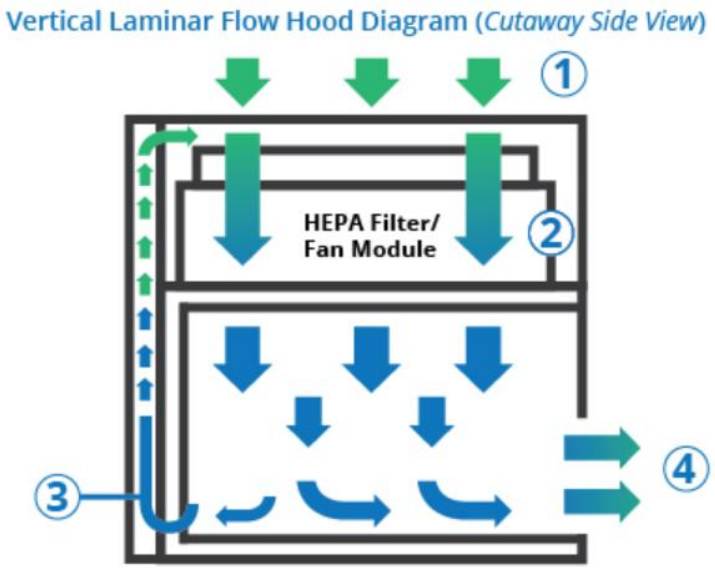
Fixed pH: **7.4**

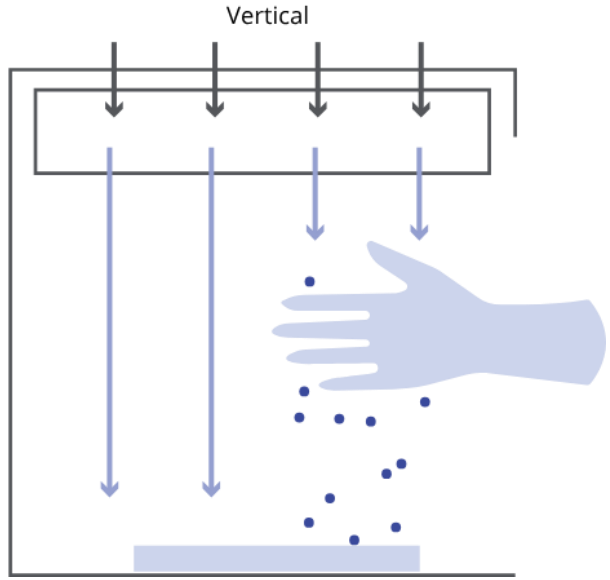
Buffer system present in the culture medium

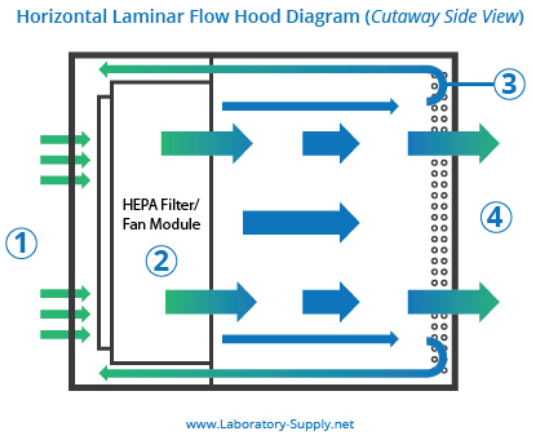
2. Cell culture:
Equipment

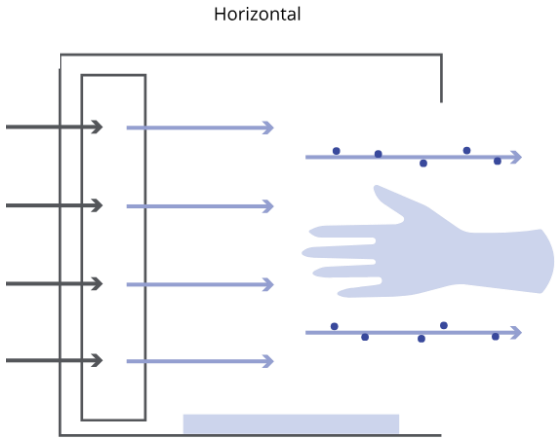
Laminar flow hood

guarantees to work in **sterile conditions**









2. Cell culture:

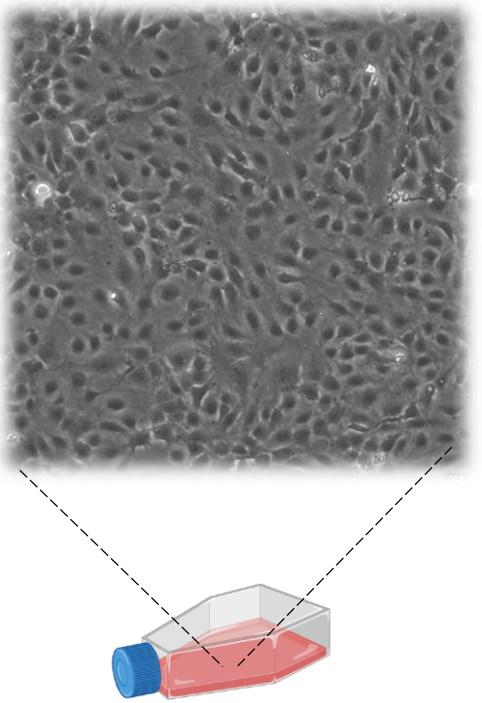
Cellular model

Cell culture lab (1° floor)

HMEC-1

immortalized
Human
dermal
Microvascular
Endothelial
Cells

ATCC®
CRL-3243™



MCDB131 medium

supplemented with:

+ 10% FBS (Fetal Bovine Serum)
+ 10 ng/mL EGF (Epithelial Growth Factor) → just for cell growth
+ 1 ug/mL Hydrocortisone
+ 10 mM Glutamine
+ 1% Penicillin-Streptomycin

EXPERIMENTAL PROCEDURE

2. Cell culture

Cell culture lab (1° floor)

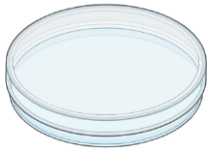
1. Detach the cells from the culture support

p20 dish

2. Count the cells

Burker Chamber

3. Plate a specific number of cells in appropriate dishes to perform the Ca^{2+} imaging experiment ($7*10^4$ the day before the experiment or $5*10^4$ two days before)



p10 dish

→ Ca^{2+} imaging experiment

→ *You can find cell passage protocol on Moodle*

→ *You can find a movie of the procedure on Moodle*

EXPERIMENTAL PROCEDURE

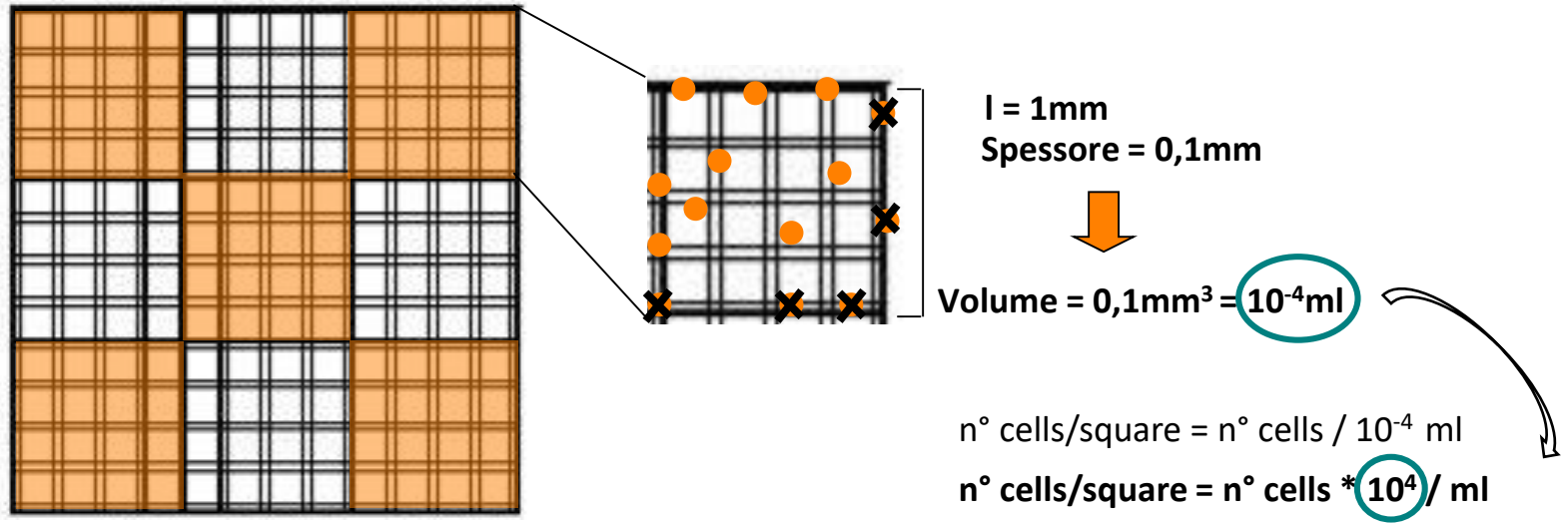
2. Cell culture:

Cell counting

Cell culture lab (1° floor)

Burker Chamber

Each Burker Chamber contains two small chambers like this:



- count the cells within the 5 squares highlighted (in both chambers of the Burker)
- calculate the average number of cells per square
- considering the volume of each square, calculate the number of cells per ml
- set up the equation needed to calculate how many µl of the cell suspension correspond to the number of cells you want to plate

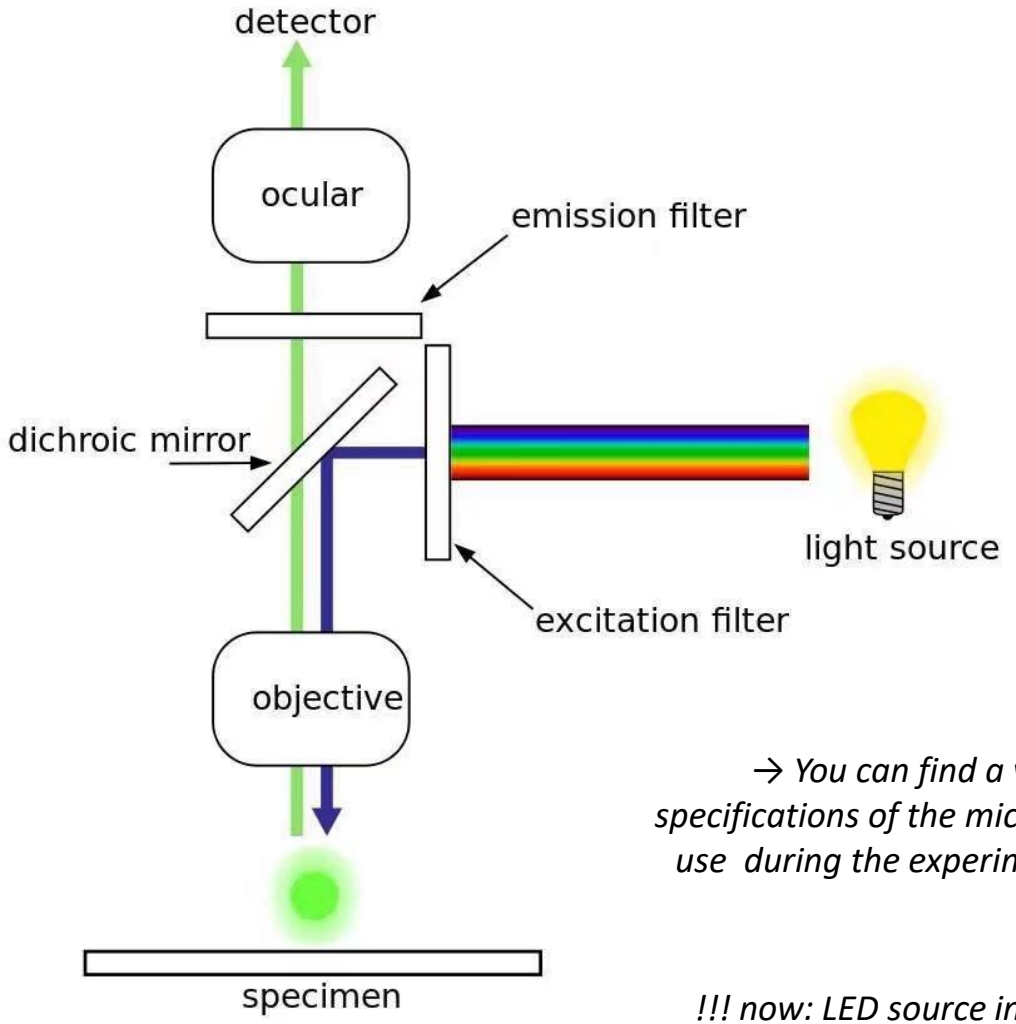
→ *You can find a movie on cell counting on Moodle*

3. Ca^{2+}-imaging experiments

Fluorescence microscope

Turin Cell Physiology Lab (1° floor)

Eclipse TE2000S Inverted Microscope



→ You can find a video about the specifications of the microscope we will use during the experiment on Moodle

⇩

!!! now: LED source instead of Xenon lamp and no monochromator

EXPERIMENTAL PROCEDURE

3. Ca^{2+}-imaging experiments

Turin Cell Physiology Lab (1° floor)

1. Load the cells with **2 µM Fura-2 probe** (stock: 1 mM) and incubate at 37°C for 30/45 minutes

2. Remove the cell medium and wash the probe excess using tyrode standard solution

3. Mount the slide on which the cells have been plated into the microscope holder

4. Perform the experiment following the desired protocol

5. Save the excel file for later analysis of the recorded signals

→ You can find Ca^{2+} imaging protocols on Moodle

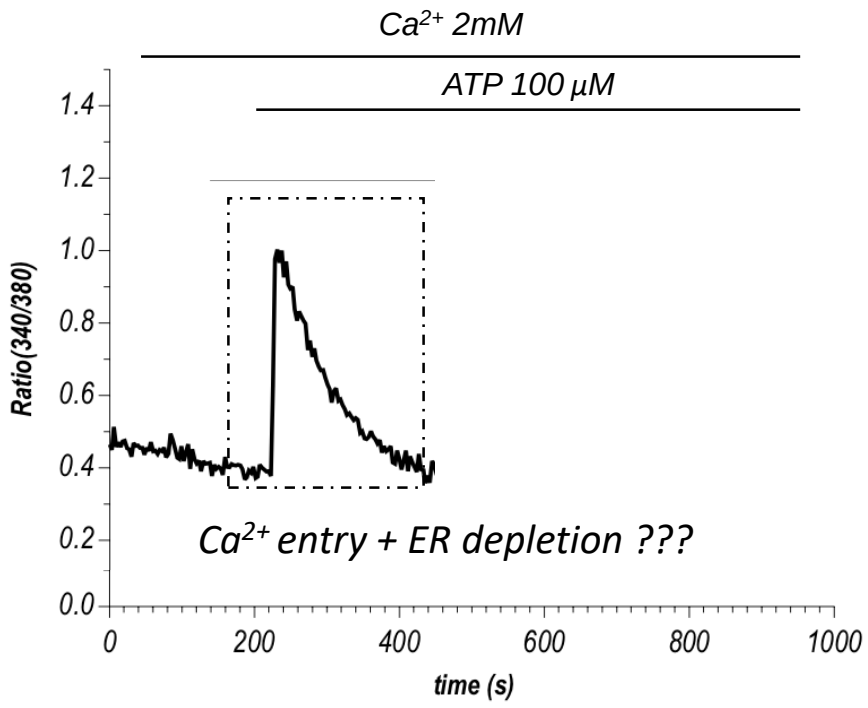
→ You can find a movie of the experiment on Moodle

EXPERIMENTAL PROCEDURE

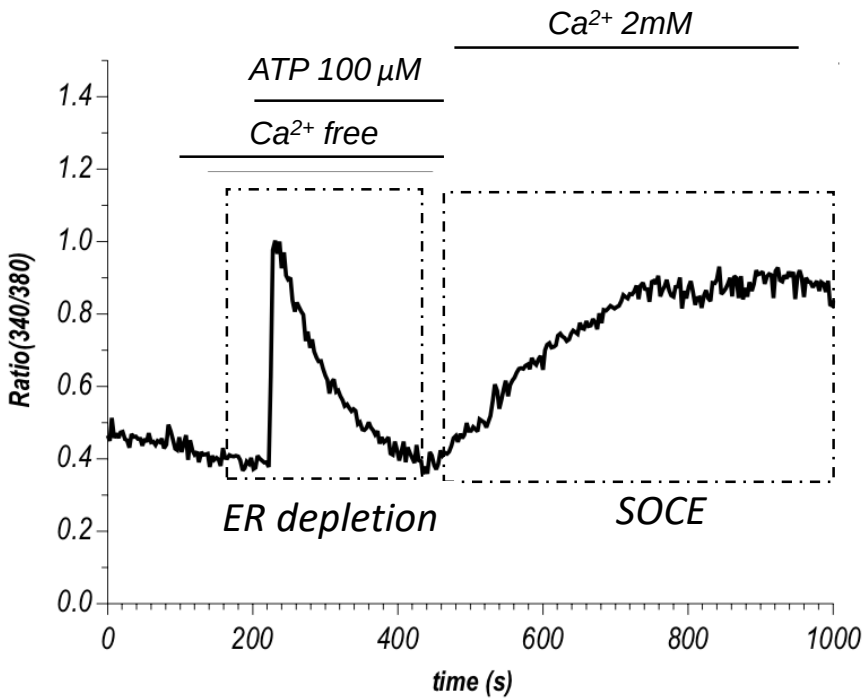
3. Ca^{2+}-imaging experiments

Turin Cell Physiology Lab (1° floor)

Protocol 1



Protocol 2



→ *You can find Ca²⁺-imaging protocols on Moodle*

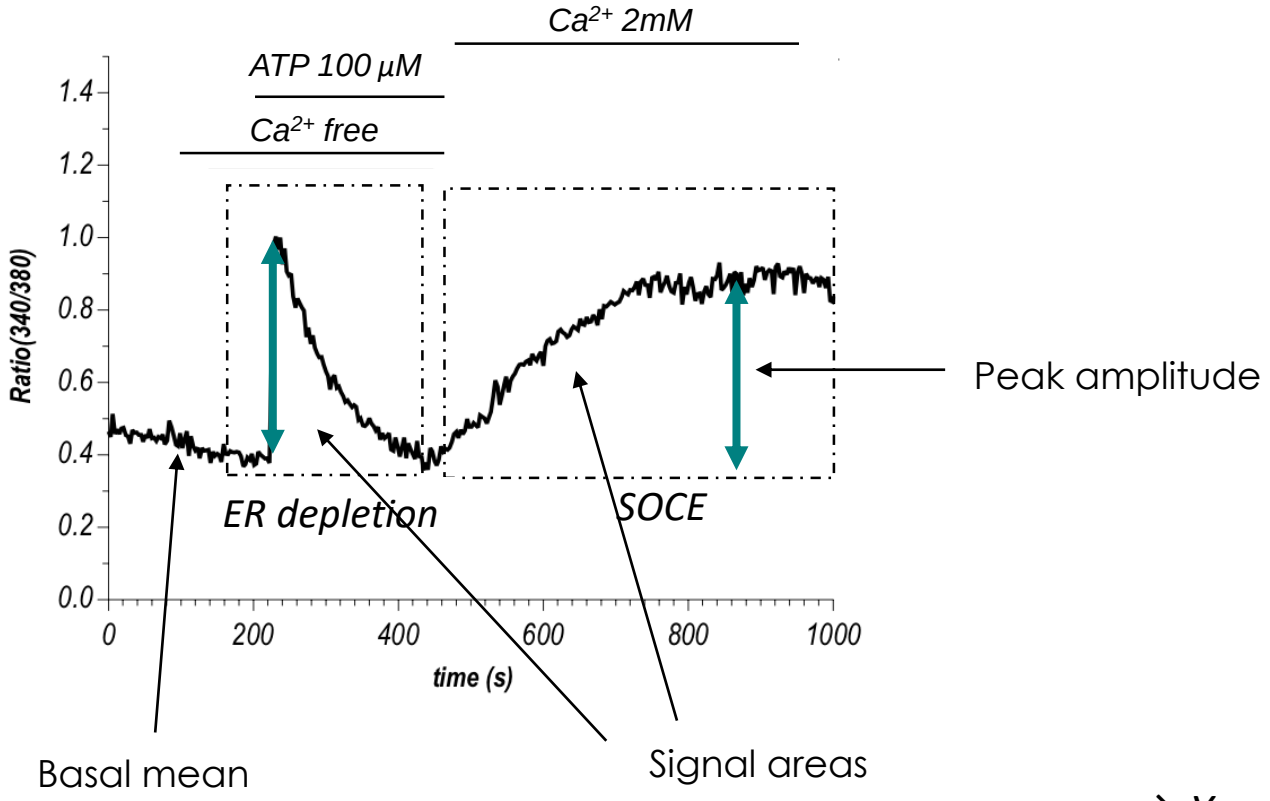
EXPERIMENTAL PROCEDURE

4. Analysis of Ca^{2+} signals

Computer room (ground floor)

1. Calculate the % of cellular responses

2. Calculate the peak amplitudes of all signals obtained during Ca^{2+} imaging experiments



→ *You can find a guide to analysis on Moodle*

Final reports

At the end of the practical experience, each group must write a report on the activities carried out, describing what was done and the results obtained.

The final report must be organized according to the following scheme:

- Introduction
- Methods
- Results and discussion

The final reports must be sent by e-mail to prof. Tullio Genova.