Antibiotic Accumulation and **M**embrane-**A**ssociated **M**echanisms of Resistance : The Translocation Challenge

Jean-Marie Pagès
UMR_MD1, AMU, Marseille, France

ANTIBIOTIC UPS AND DOWNS

As new antibiotics come on the market (upward arrows), resistance develops (downward arrows), but the … continue to be used. The growing collection of … over time is offset by the increasing resistan…

▲ Introduced ▼ Resistance

Deadline for effect

Penicillin introduced — *Staphylococcus* resistance — Pneumonia resistance

Erythromycin introduced — *Staphylococcus* resistance

Tetracycline introduced — *Shigella* resistance

Methicillin introduced — *Staphylococcus* resistance

Gentamicin introduced — *Enterococcus* resistance

Vancomycin introduced — *Enterococcus* resistance

Imipenem introduced — Enterobacteriaceae resistance

Ceftazidime introduced — Enterobacteriaceae resistance

Levofloxacin introduced — *Pneumococcus* resistance

Linezolid introduced — *Staphylococcus* resistance

Ceftaroline introduced — *Staphylococcus* resistance

PENICILLIN

Resistance in *Staphylococcus* developed before penicillin was approved, and resistance in pneumonia developed 22 years after the drug went on the market.

VANCOMYCIN

Typically used as a last resort, which could explain why it took 16 years for resistance to develop.

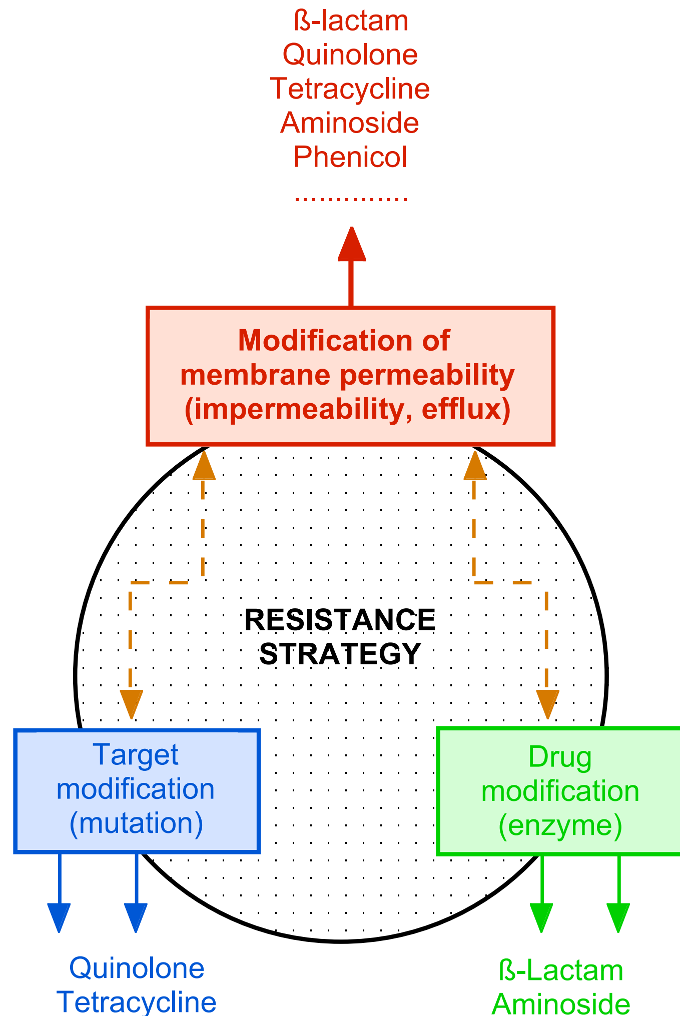
ACCELERATING RESISTANCE

Some bacteria quickly resist some of the recent antibiotics, such as levofloxacin and linezolid.

450,000 new cases of multidrug-resistant tuberculosis worldwide in 2012.

Drug transport and bacterial strategy

Membrane permeability and membrane-associated mechanisms of resistance



Various bacterial strategies face to antibiotic attack:

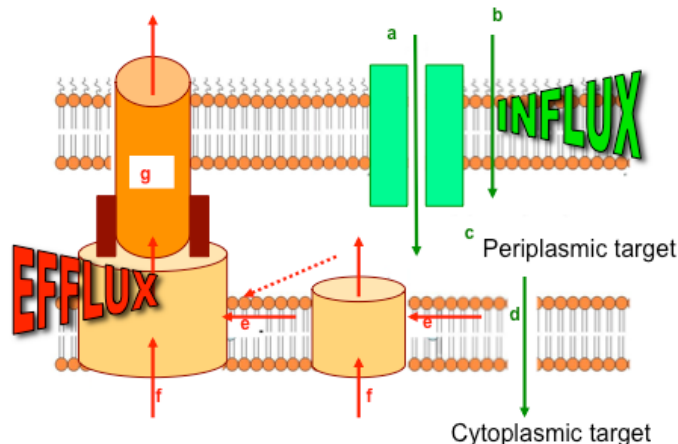
(i) Specific responses :
target mutations (mutation or import of genetic mobile elements), diverse drug modification

OR

(ii) General responses :
alteration of permeability (porines), expression of efflux pumps, poly-selective transporters

Intra-bacterial concentration of active molecule

“ new concept ”



Intracellular concentration is related to

- the compound structure
- the bacterial membrane, *e.g.* porins, LPS, lipids
- the diffusion pathway, porins are involved in a large majority (>2/3) of used antibiotics (*e.g*. ß-lactams, fluroquinolones)
- the expression and activity of efflux pumps, *e.g.* AcrAB-TolC
- the cooperativity between transporters

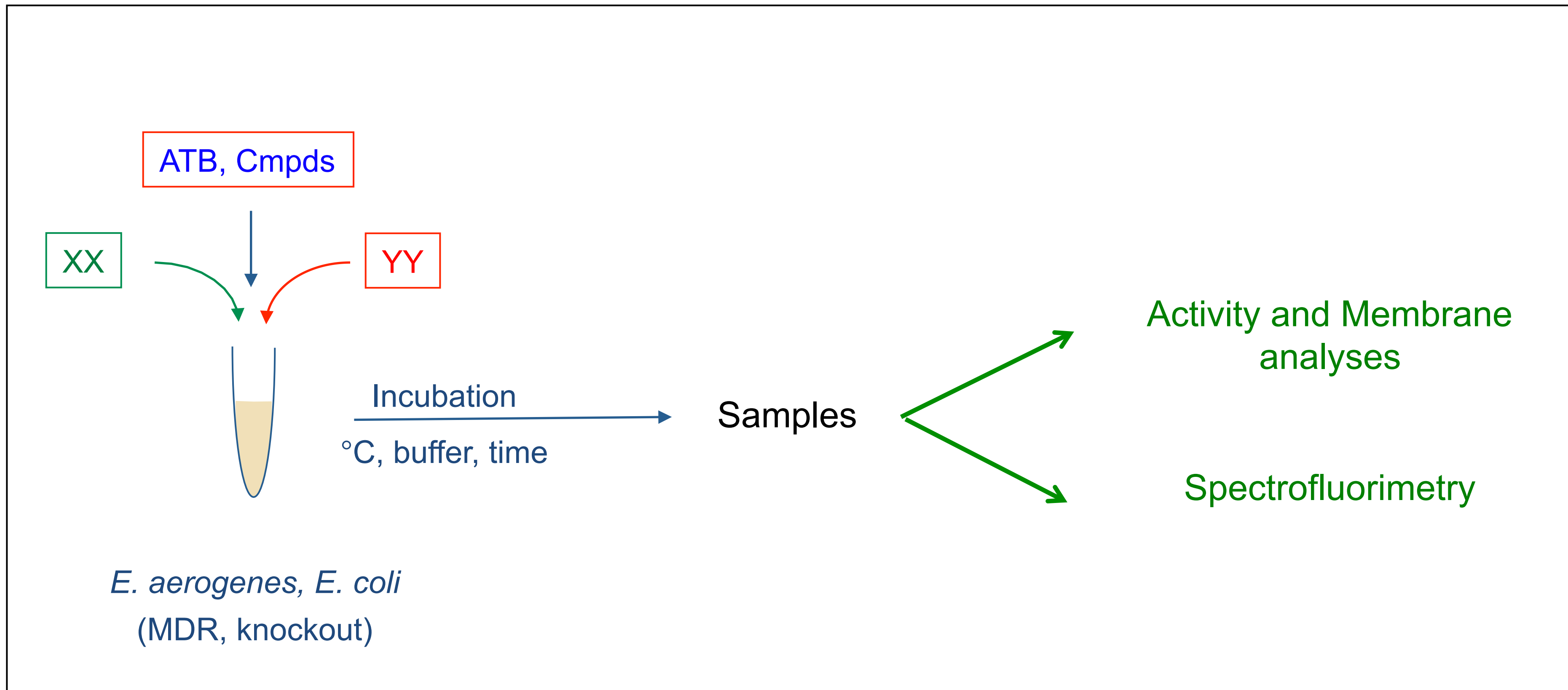
>> **global membrane permeability and transports**

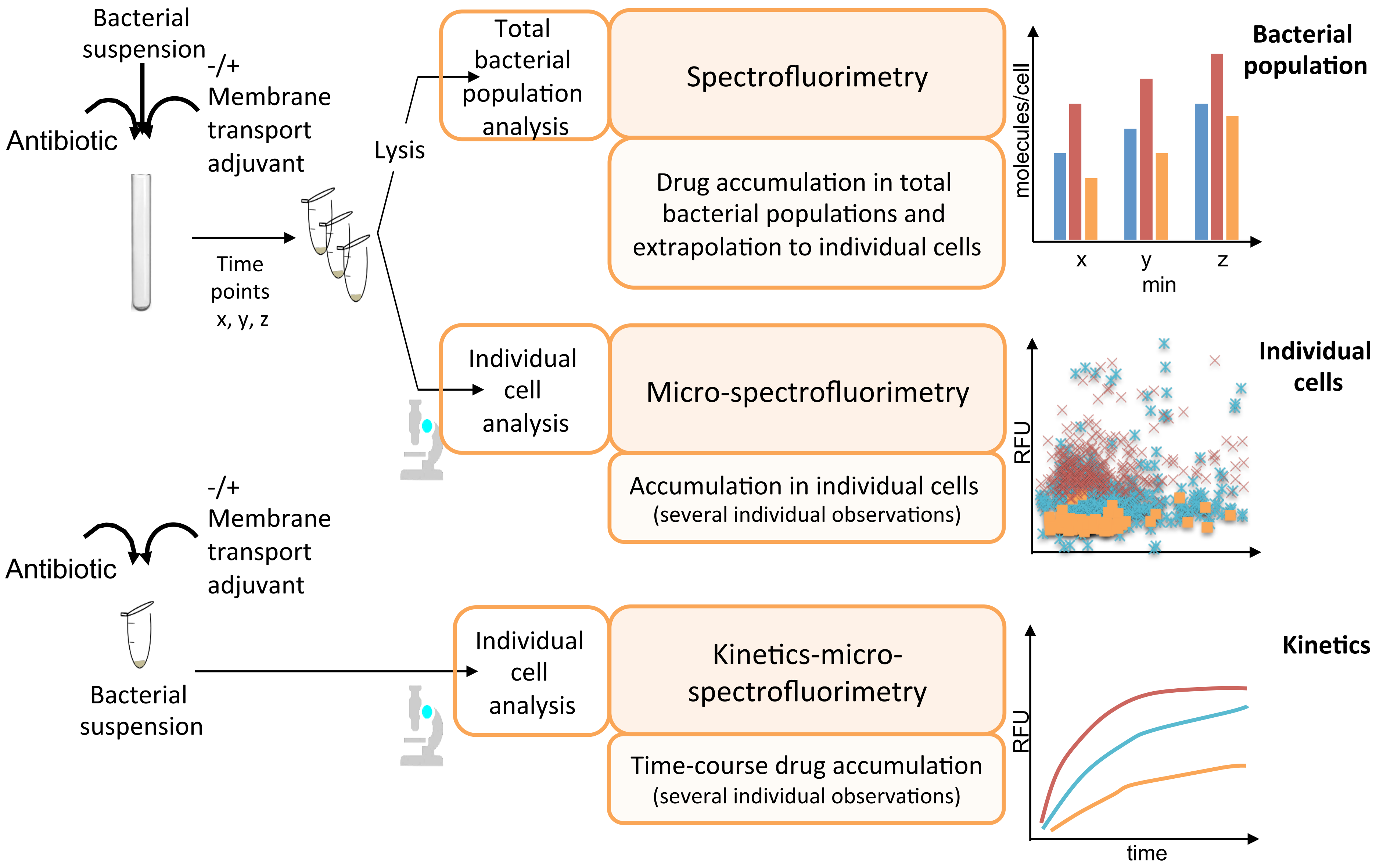
Pagès *et al.,* Nat. Rev. Microbiol. 2008.
Bialek-Davenet *et al.*, Sci. Rep. 2017.
Masi *et al.*, Nat. Microbiol., 2017

Influx & Efflux

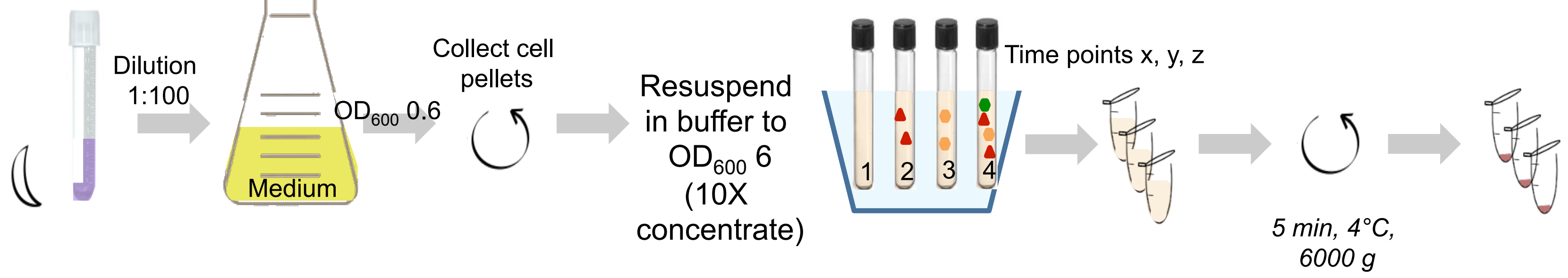
control antibiotic accumulation & activity

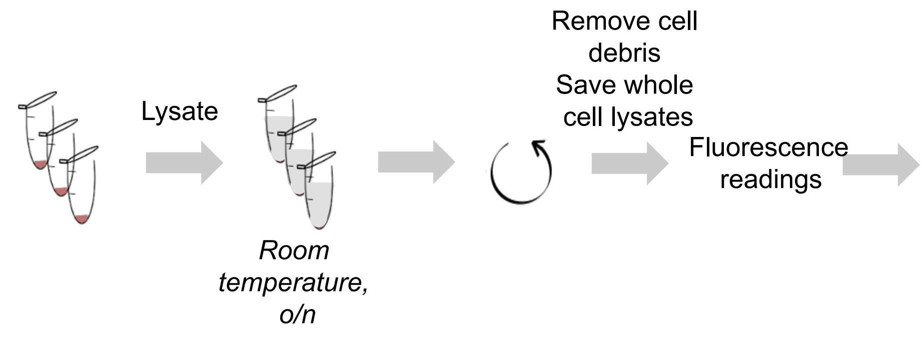
Determination of intra-bacterial concentration
From MIC to *in situ* concentration : RTC2T

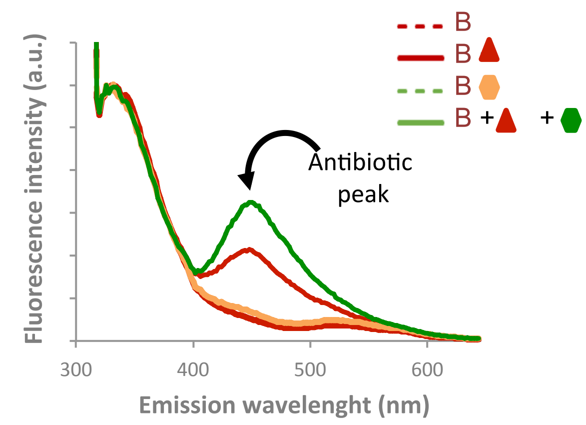


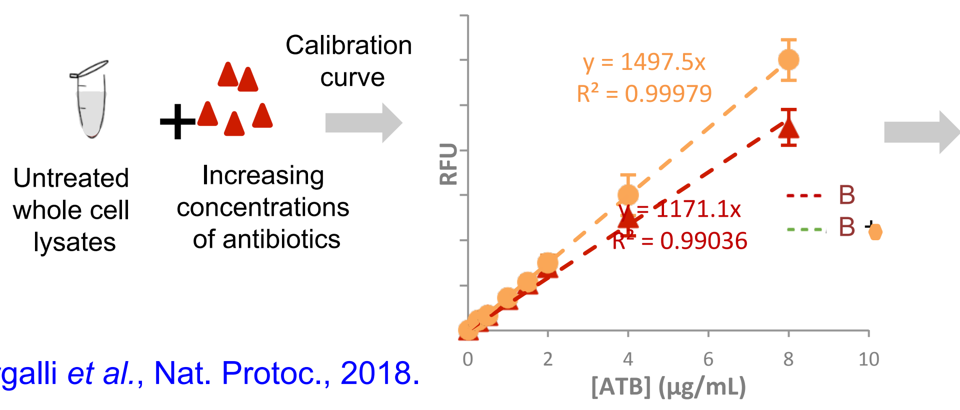


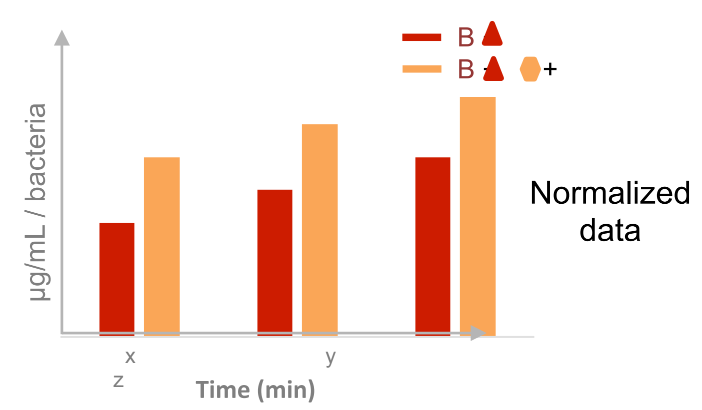
Masi *et al.*, Res. Mic., 2017.
Vergalli *et al.*, Nat. Protoc., 2018.





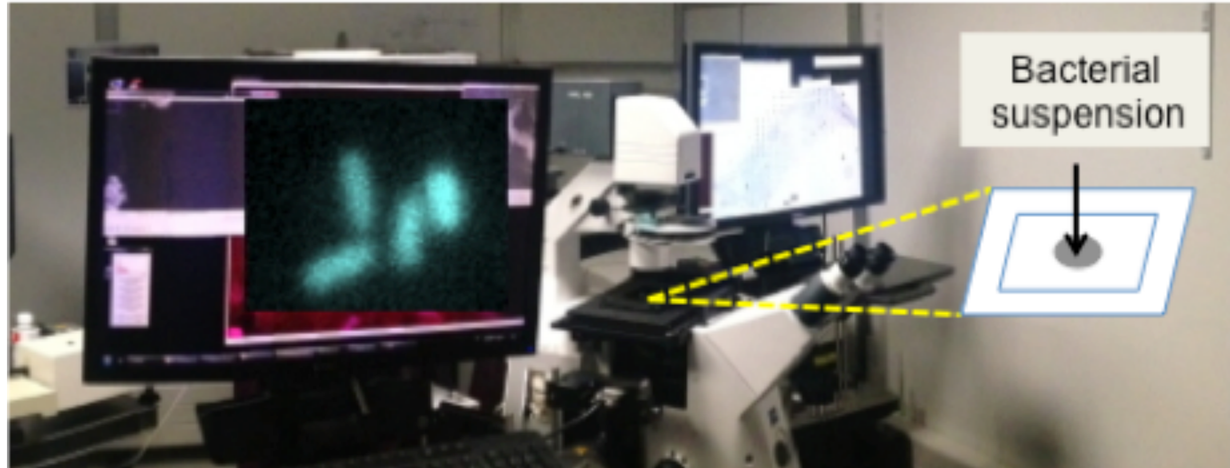




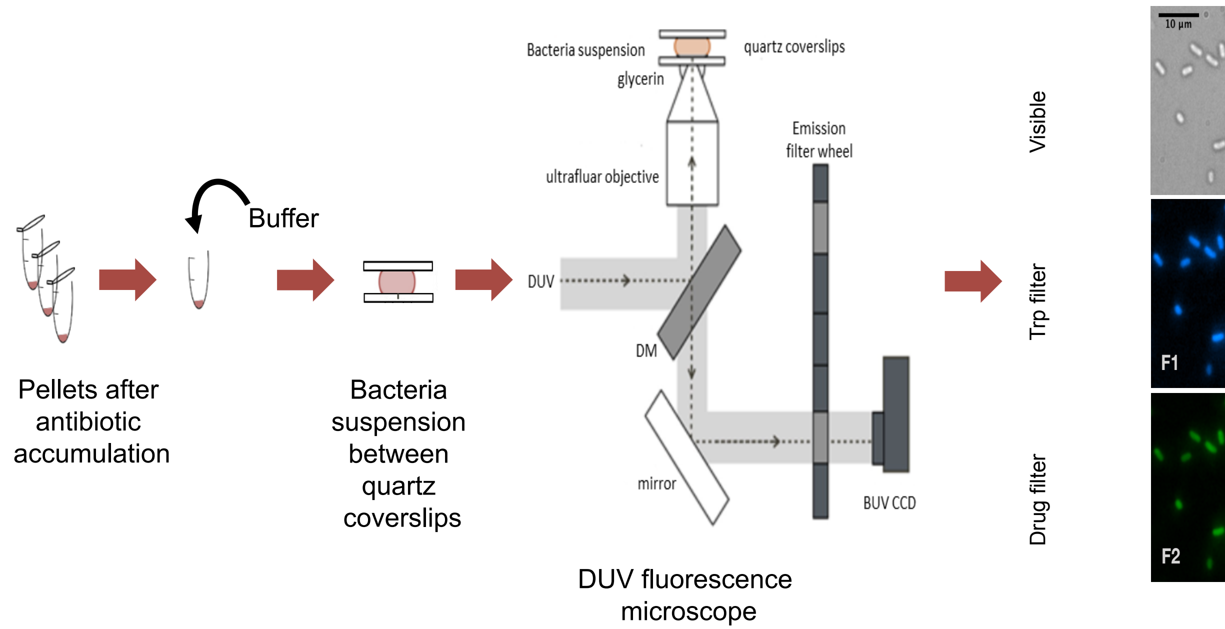


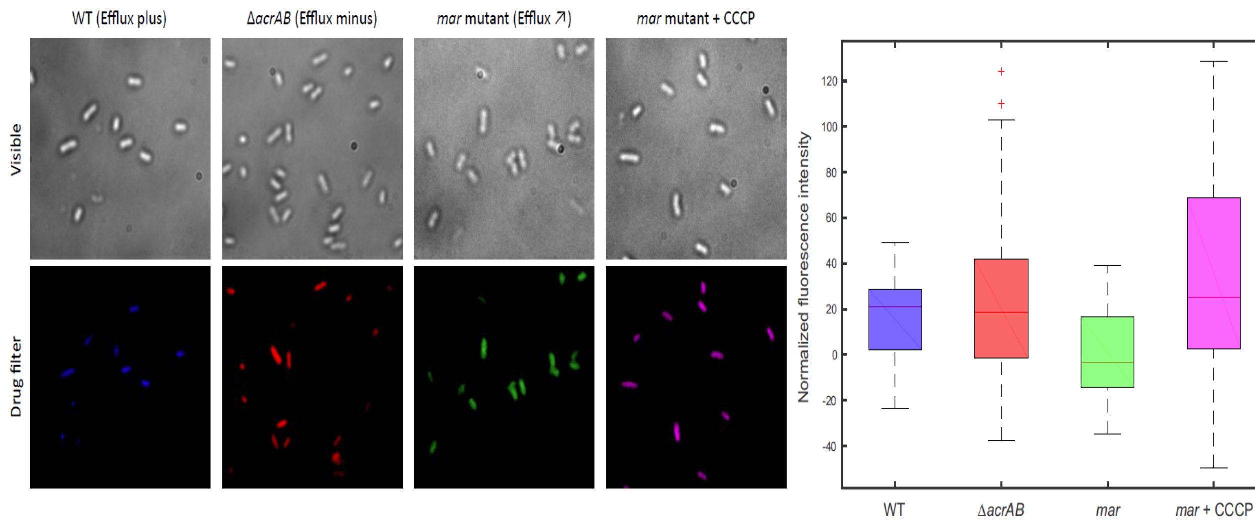
Vergalli *et al.*, Nat. Protoc., 2018.

Accumulation in individual bacterial cell



To detect the time-course accumulation in single bacteria, pellets corresponding to bacterial suspensions were re-suspended in NaPi-MgCl2 buffer and mixed without or with CCCP/ inhibitors/etc.
Aliquots of resuspended pellets were immediately deposited between two quartz coverslips and analysed by DUV fluorescence imaging.


Buffer
Pellets after antibiotic accumulation
Bacteria suspension between quartz coverslips
Bacteria suspension
glycerin
quartz coverslips
ultrafluar objective
Emission filter wheel
DUV
DM
mirror
BUV CCD
DUV fluorescence microscope
Visible
Trp filter
Drug filter
10 µm
F1
F2

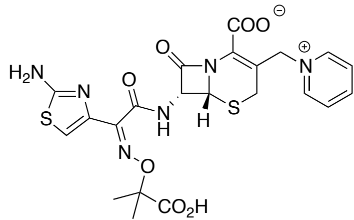

WT (Efflux plus)
ΔacrAB (Efflux minus)
mar mutant (Efflux ↗)
mar mutant + CCCP
Visible
Drug filter
Normalized fluorescence intensity
120
100
80
60
40
20
0
-20
-40
WT
ΔacrAB
mar
mar + CCCP

Influx and ß-lactams

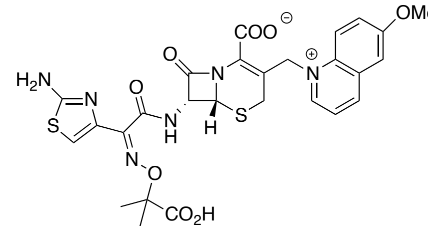
control antibiotic accumulation & activity

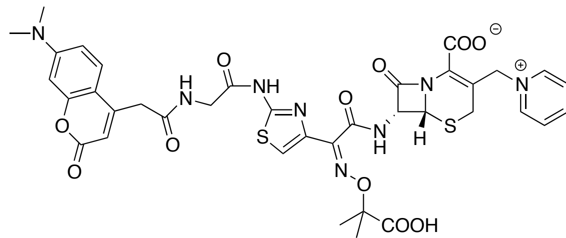
Accumulation and Location: use of CAZ*

CAZ



CAZ-2





CAZ-1

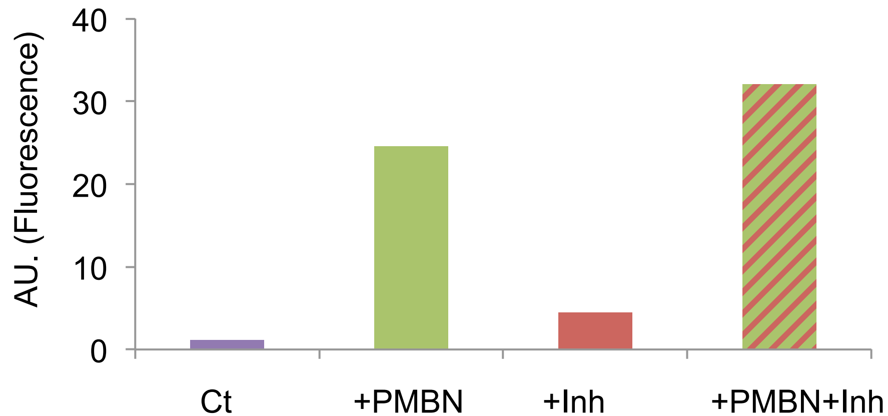
Allam *et al*., Sci. Reports, 2017.

Bacterial susceptibility to Ceftazidime and modified-CAZs

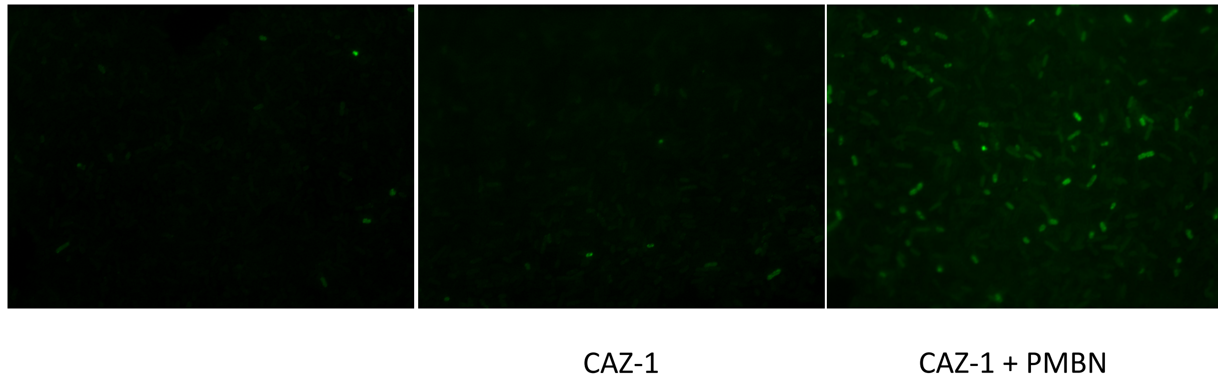
	AG100 (WT)	AG100A (AcrAB-)	ARS108 (porin-)
CAZ	0.5	0.25	64
+ PMBN	0.06	0.06	0.5
+ Inh	0.25	0.25	2
+ Inh + PMBN	0.06	0.06	0.03
CAZ-1	64	16	>128
+ PMBN	8	4	64
+ Inh	64	16	>128
+ Inh + PMBN	4	4	4
CAZ-2	2	1	64
+ PMBN	1	<0.125	4
+ Inh	1	0.5	4
+ Inh + PMBN	<0.125	<0.125	0.125

CAZ, ceftazidime; CAZ-1, fluo-ceftazidime1; CAZ-2, fluo-ceftazidime2;
PMBN polymyxine B nonapeptide
Inh: tazobactame + clavulanate

CAZ-1 accumulation

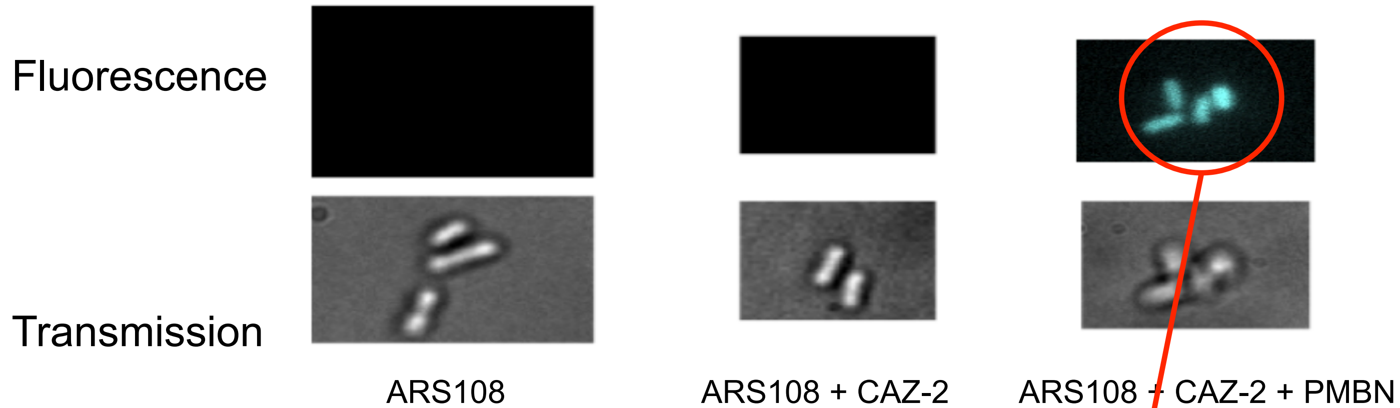


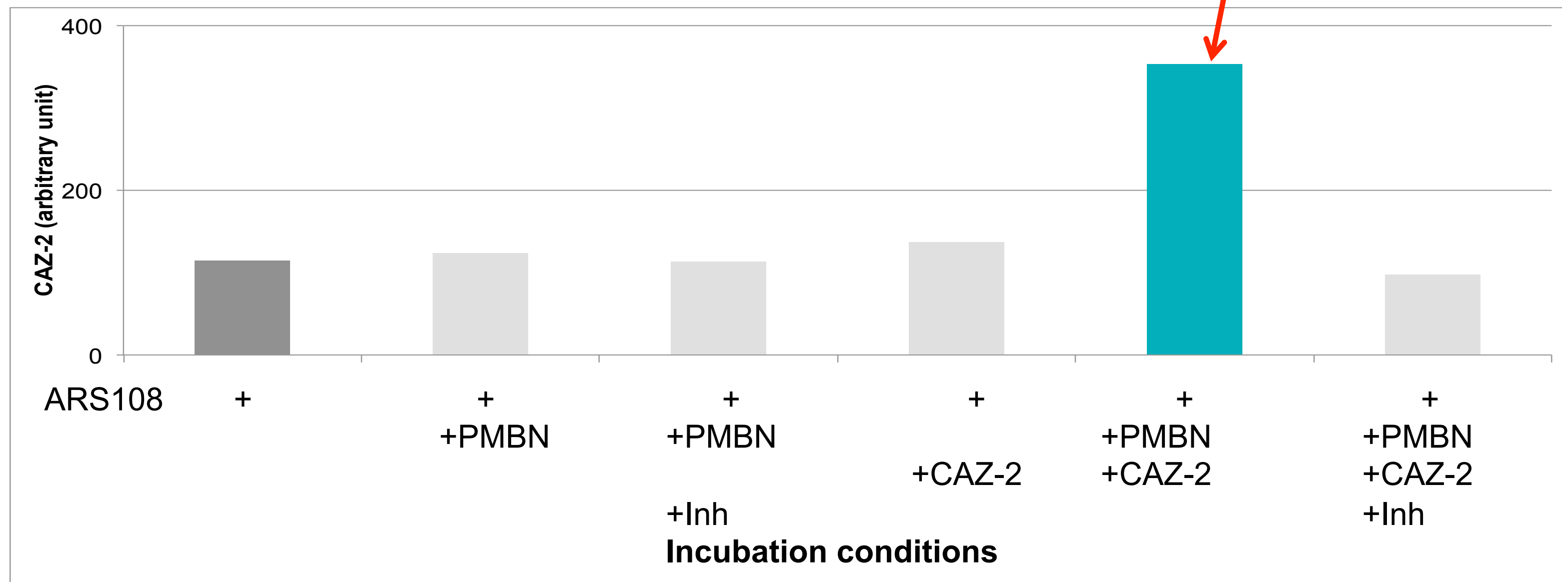
CAZ-1 accumulation in *E. coli* cells.
ARS108 cells were incubated for 30min in different conditions.
Microspectrofluorometry analyses were performed in individual bacteria (Ex372 nm, Em475-485 nm) after incubation (30min). Signals were corrected using the tryptophan peak and negative controls (without CAZ-1).



In porin- background, PMBN is able to restore the level of intracellular accumulation and support CAZ activity

CAZ-2 localization in individual bacterial cell





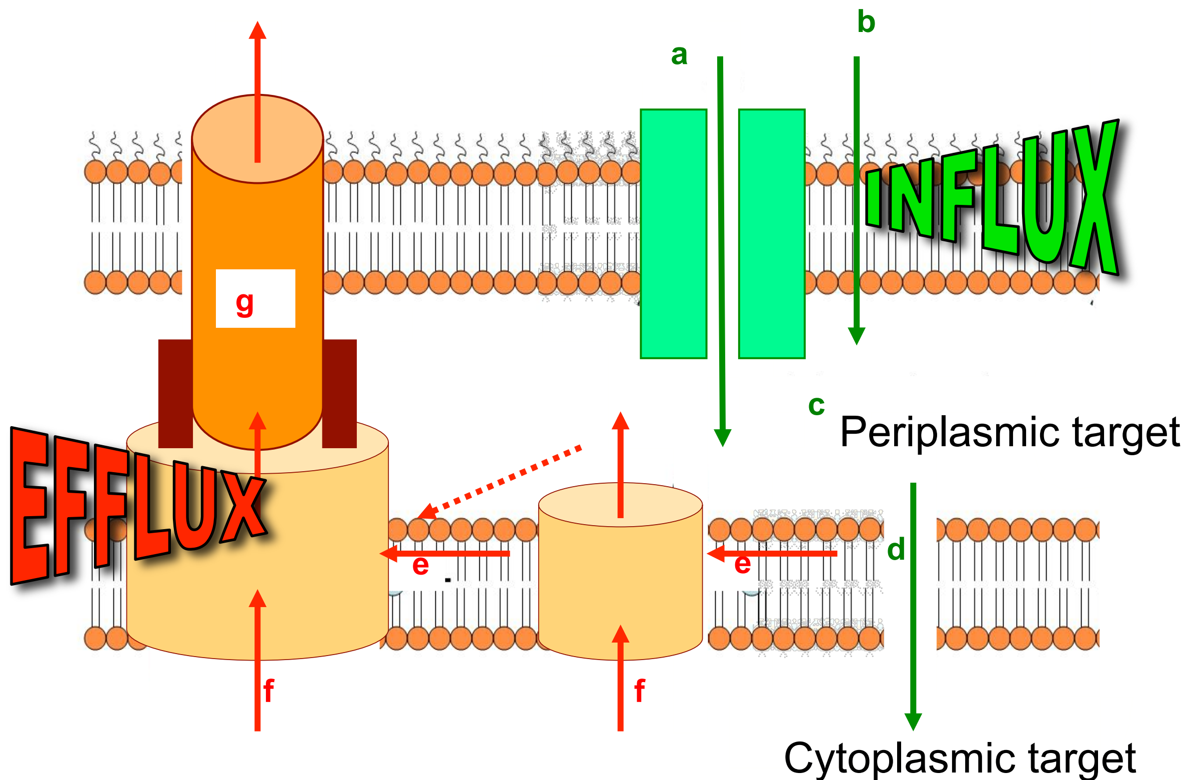
CAZ-2 accumulation in bacterial cell. Filtered fluorescence signals were standardized using the tryptophan signal to standardize with the treated bacterial amount .

The fluorescence signal is associated with the permeation of the CAZ-2 inside periplasmic space and the ß-lactamase activity that releases the fluo-product.

Efflux and FQs

control antibiotic accumulation & activity

Efflux, the Out

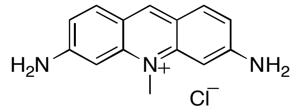


"OUT", Efflux, pump out

- Intrinsic bacterial driving force is required (transport against the gradient concentration), membrane potential (with antiport H^+),
- Poly-selectivity (large panel of substrates), pump flexibility
- Pump cooperativity

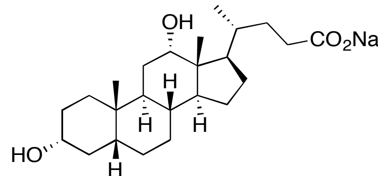
Efflux pumps and substrate poly-selectivity

DYES



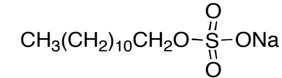
Acriflavine

BILE SALTS



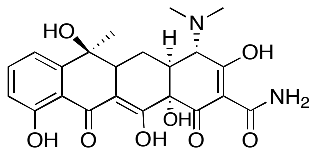
Sodium deoxycholate

DETERGENTS

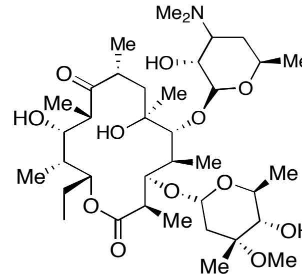


SDS

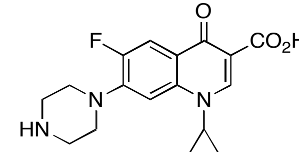
ANTIBIOTICS



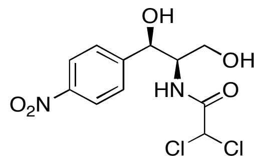
Tetracycline



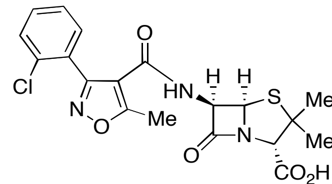
Erythromycin



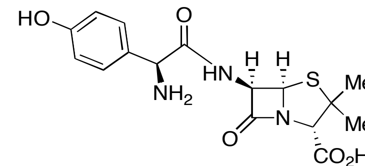
Ciprofloxacin



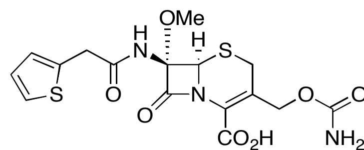
Chloramphenicol



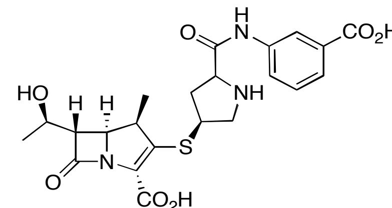
Cloxacillin



Amoxycillin



Cefoxitin

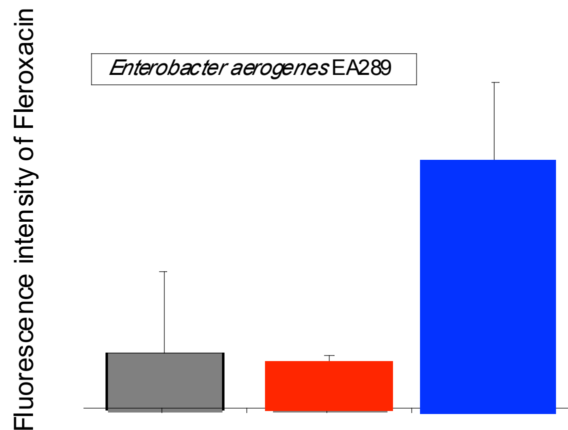


Ertapenem

Intra-bacterial concentration

DUV microspectrofluorimetry

	MIC (mg/L)		
E. aerogenes	NFL	CIP	FLE
EA289	256	64	128
EA298 (*tolC*)	32	4	16



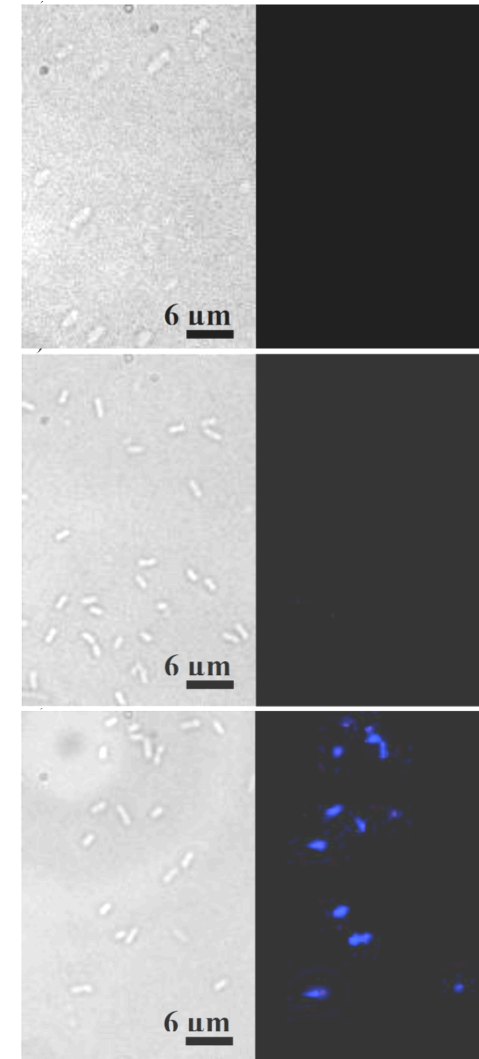
Fluorescence intensity of Fle measured by UV-VIS microspectrofluorimetry from individual EA289 bacteria:
(A) Fle (c = 64 µg/ml);
(B) Fle (c = 64 µg/ml) + Glu (0.4 %);
(C) Fle (c = 64 µg/ml) + CCCP (c = 25 µM).

Synchrotron DUV fluorescence imaging

Ct

Fleroxacine + Glucose

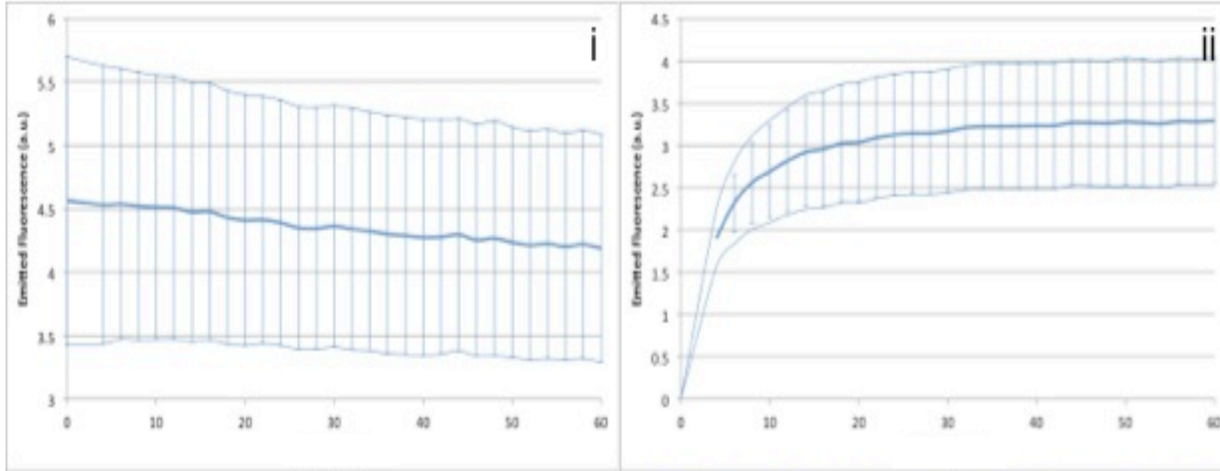
Fleroxacine + CCCP

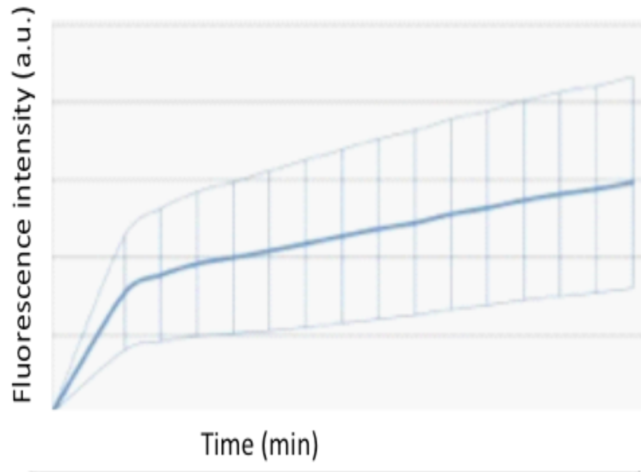


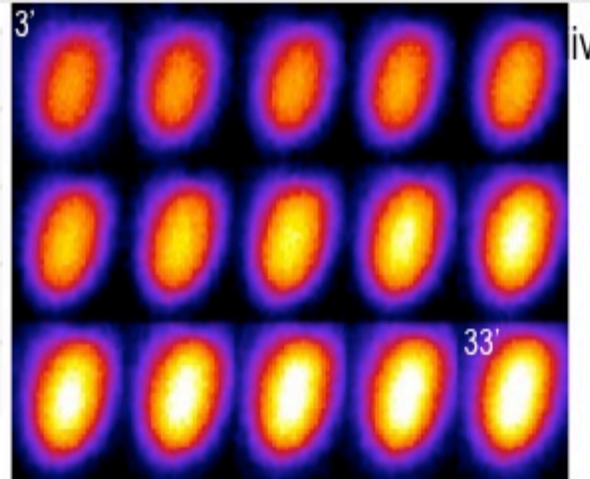
Kaščáková *et al.*, PloS ONE 2012.

Accumulation in individual bacterial cell

In situ concentration, following the kinetics of intra-bacterial accumulation







i) Kinetic accumulation of FQ without bleaching correction

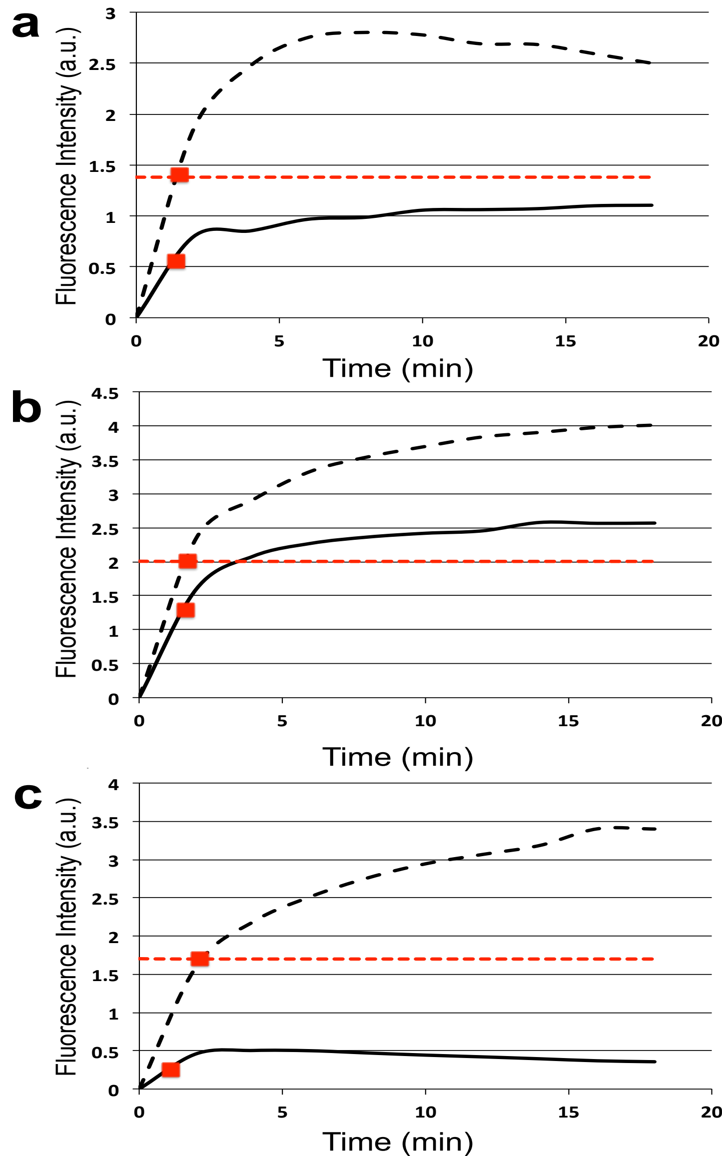
ii) with bleaching correction,

iii) Kinetic accumulation of FQ with correction of photobleaching and crosstalking effect,

iv) Accumulation of FQ in a bacterium after corrections.

>> Kinetics-micro-spectrofluorimetry in individual bacterial cells

Accumulation and molecule structure



Time course of bacteria accumulation.

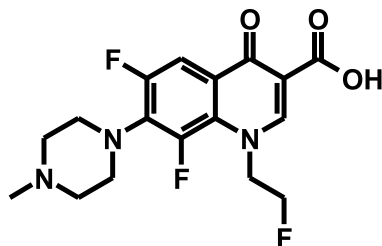
Average Fluorescence intensity of 100 individual efflux (solid line) or efflux-deleted strain (dashed line) bacteria incubated with “**a**”, “**b**” or “**c**” compounds respectively.

We consider that the maximum (steady state) of accumulation is reached at 15-20 min in efflux-deleted strains. 50 % of accumulation is presented as the horizontal dashed red line. The red dots presents 50% of accumulation when the plateau is reached for each strain.

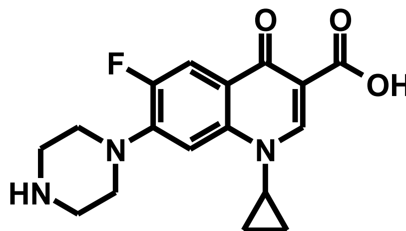
Cinquin *et al.*, Sci. Rep. 2015.

Activity and molecule structure

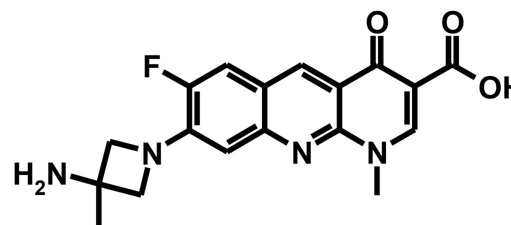
FLE



CIP



SANO1



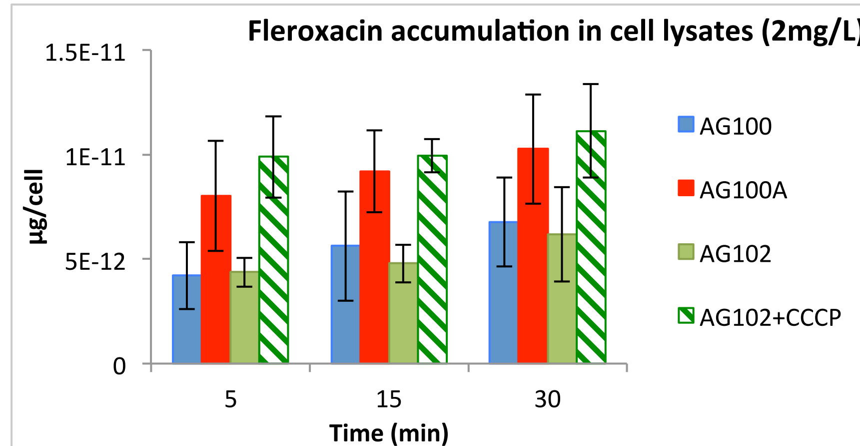
		MIC (mg/L)		
		FLE	CIP	SANO1
	Molecular weight	369	331	356
	LogP	-0.35	-0.5	0.45
E. coli strains	AcrAB			
AG100*	+	0.125 (0.062)	0.016 (0.008)	0.5 (0.125)
AG100A*	-	0.031 (0.031)	0.004 (0.004)	0.125 (0.125)
AG102*	++	0.5-1 (0.125)	0.0625 (0.016)	2-4 (0.25)

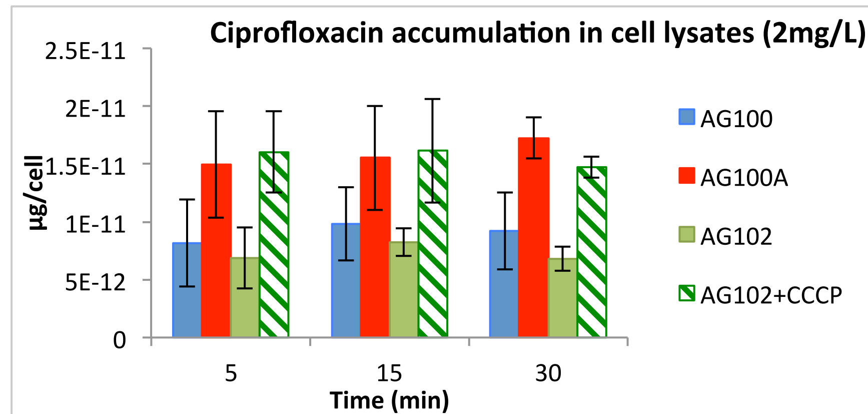
* *E. coli* isogenic strains have been previously described.

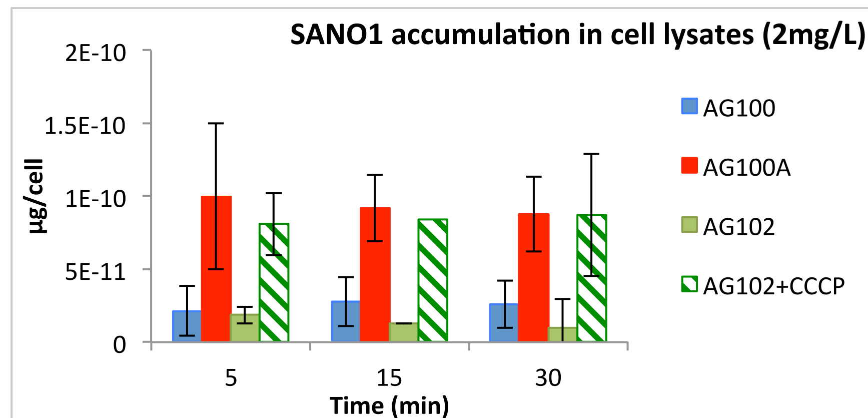
In parenthesis, susceptibility assays performed in the presence of PAßN (20 mg/L)
Assays were carried out in triplicate and the resulting medians were presented.
LogP was calculated with ACDLabs 2016 (Build 2911).
FLE, fleroxacin; CIP, ciprofloxacin, SANO1, SANOFI compound.

Vergalli *et al.*, Sci Rep. 2017.

SICAR: intra-bacterial Influx and Efflux







SICAR ^{IN} and SICAR ^{EF}

Compound	Accumulated drug, 5min (10^{-11} µg/bacterial cell)			$SICAR^{IN}$
	AG100A*	AG100*	AG102*	
FLE	**0.80**	0.42	0.44	**1**
CIP	**1.50**	0.82	0.69	**1.8**
SANO1	**9.26**	2.5	1.86	**11**

Compound	Accumulated drug, 15 min (10^{-11} µg/bacterial cell)			$SICAR^{EF}$ R	$SICAR^{EF}$ R'
	AG100A*	AG100*	AG102*	**AG100A/AG100**	**AG100A/AG102**
FLE	0.92	0.56	0.48	**1.6**	**1.9**
CIP	1.55	0.98	0.83	**1.6**	**1.9**
SANO1	9.12	2.98	1.27	**3.1**	**7.2**

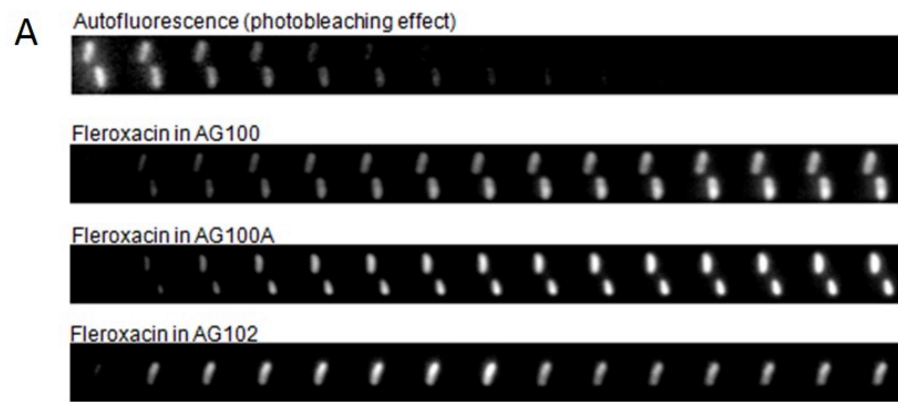
Means of three independent assays performed in triplicates

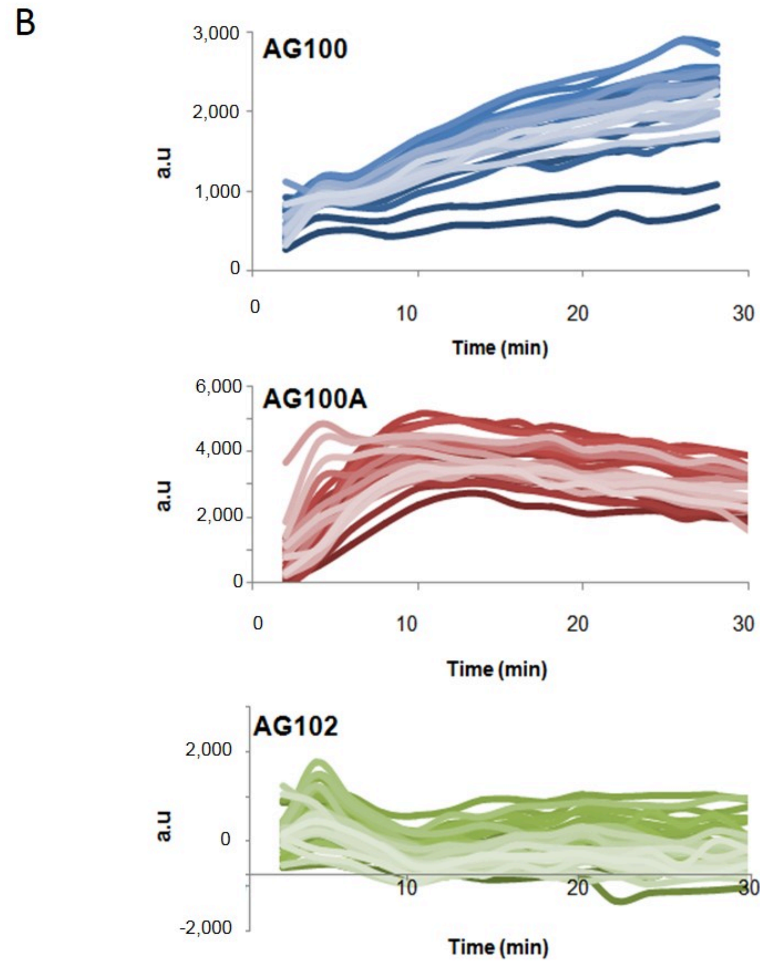
SICAR (Structure Intracellular Concentration Activity Relationship) relies the physico-chemical properties of the antibiotic to the translocation efficacy across the bacterial membrane (Masi et al, Nat. Microbiol, 2017).

So, we can conclude that:

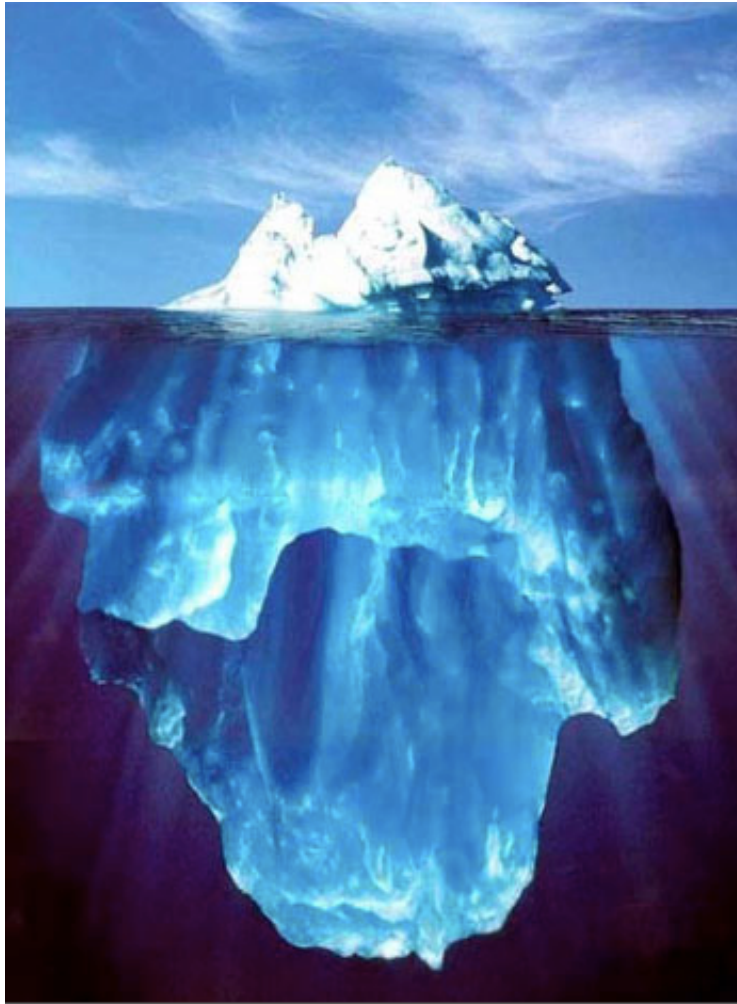
- ✓ SANO1 exhibits a high penetration rate, **$SICAR^{IN}$**, compare to CIP and FLE.
- ✓ In contrast, CIP et FLE have a relative low SICAR efflux index, **$SICAR^{EF}$**, when SANO1 displays a high index reflecting a high sensitivity to efflux pump activity.

Vergalli *et al*., Sci Rep. 2017.


A
Autofluorescence (photobleaching effect)
Fleroxacin in AG100
Fleroxacin in AG100A
Fleroxacin in AG102


B
AG100
3,000
2,000
1,000
0
a.u
0
10
20
30
Time (min)
AG100A
6,000
4,000
2,000
0
a.u
0
10
20
30
Time (min)
AG102
2,000
0
-2,000
a.u
10
20
30
Time (min)

CONCLUSION



Membrane associated mechanisms
and translocation,
the “masked side” of
susceptibility/resistance

“ Mechanical/Membrane
barrier ”

SICAR , RTC2T

new concepts/tools for dissecting
"drug translocation"

Masi *et al.*, Nat. Microbiol, 2017.
Masi *et al.*, Res. Microbiol, 2017.

“ RTC2T ”

Resident time concentration close to target

$$\text{Accumulation}_{t0} = \text{Influx}_{t-1} - [\text{Efflux} + \text{degradation} + X]_t$$

The intracellular concentration of antibiotics (C_{in}), as well as the critical intracellular concentration of antibiotics needed to inhibit bacterial growth (C_{inh}), are key points.
The concept **"Resident Time Concentration Close to Target"** (RTC2T) and the precise determination of both C_{in}, and C_{inh} for a given antibacterial molecule for a given bacterial strain: it represents a marker of the correct addressing of the drug taking into account the membrane-associated mechanisms used by bacterial cells.

The intra-bacterial concentration of antibacterial molecules must be associated with the killing rate to approach the « real time activity » of a drug.

“ SICAR ”

The concept of **"Structure Intracellular Concentration Activity Relationship"** (SICAR) can be defined as follow: based on the SAR studies for biologically active molecules, it will correlate the chemical structure to the efficacy of translocation across bacterial membrane and the resulting intracellular accumulation.

It is of interest to identify structural pharmacophoric groups that favor the intracellular accumulation (increasing Influx, decreasing Efflux): The favorable chemotype.

Antibiotic accumulation: MassSpect // Fluorimetry

Mass Spect

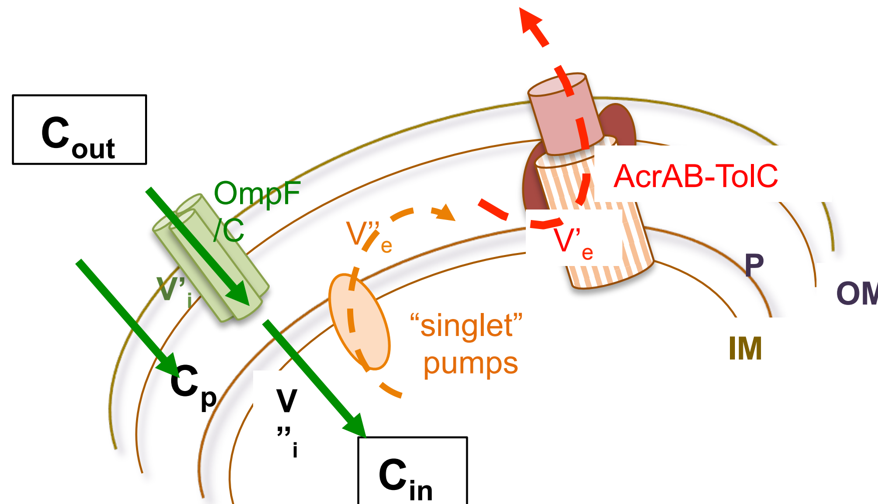
- rapid and precise
- reproducible and feasible in microplates
- very sensitive (compared to fluorimetry assays for low concentrations)
- detection of variations in accumulation rate
- recent good correlation with data obtained by Fluo analyses

In progress, but

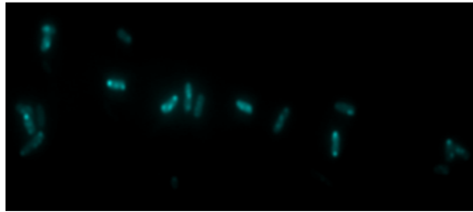
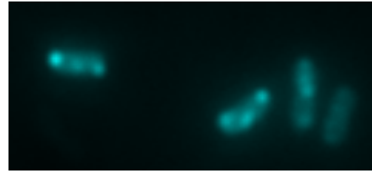
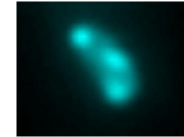
Require internal standard to normalize the data, between strains, assays, etc...

“RTC2T - SICAR ”

Accumulation assays can be used for modeling translocation of a small molecule (*i. e.* influx) with Fick’s law of diffusion together with the contribution of porins and efflux by Michaelis-Menten kinetics, while taking into account fluorescent properties of the studied molecule, the evolution of its intracellular concentration can be written as a differential equation integrating the following steps:



Masi *et al.*, Nat. Microbiol, 2017.
Vergalli *et al.*, in preparation, 2018.

DISCO beamline Synchrotron Soleil (M. Réfrégiers)

Univ. Paris Descartes (I. Artaud)

GSK (R. Stavenger)

SANOFI (M. Mourez)

Jacobs Univ. Bremen (M. Winterhalter)

IMI-Translocation partners

etc..

and UMR_MD1 members involved in this study

Estelle Dumont　　Laure Maigre*　　Muriel Masi

Elizabeth Pinet*　　Julia Vergalli　　Anne-Marie Tran

Anne Davin

ND4BB
TRANSLOCATION

Innovative Medicines Initiatives 115525

Initial Training Networks (ITN) - Marie Curie Actions
Translocation

Membrane and Therapeutic Targets
UMR_MD1, U-1261, Marseille, France