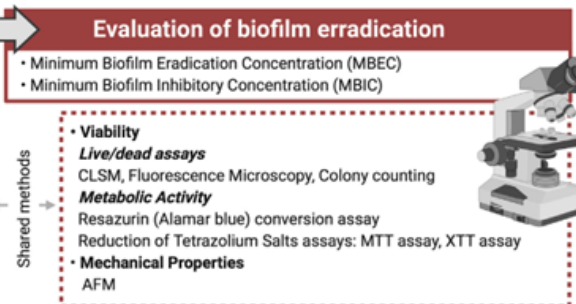
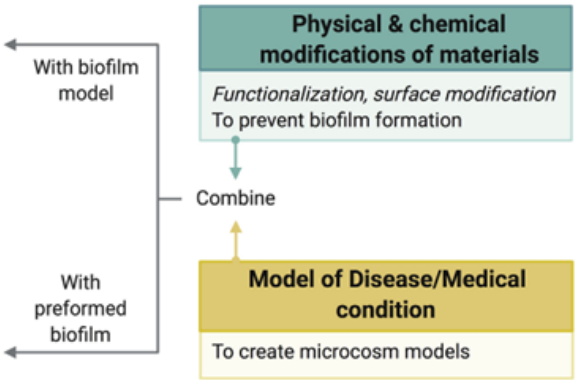
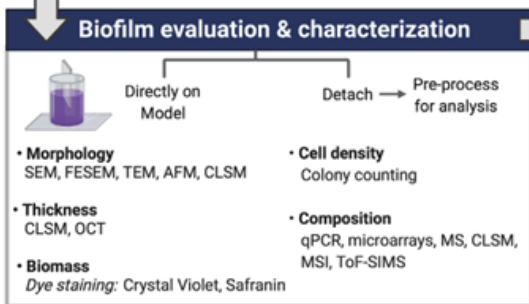
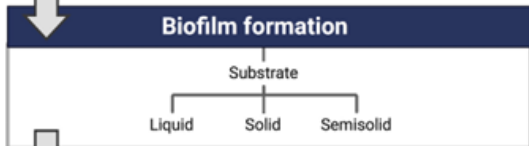
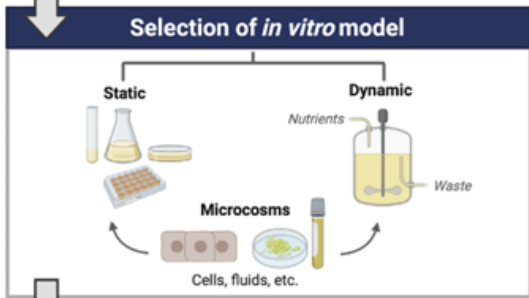
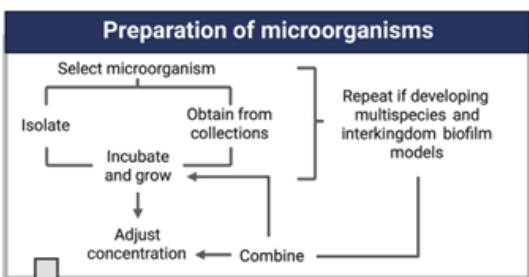




Models for the Study of Microbial Biofilms



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Depto. de Microbiología, IIBCE



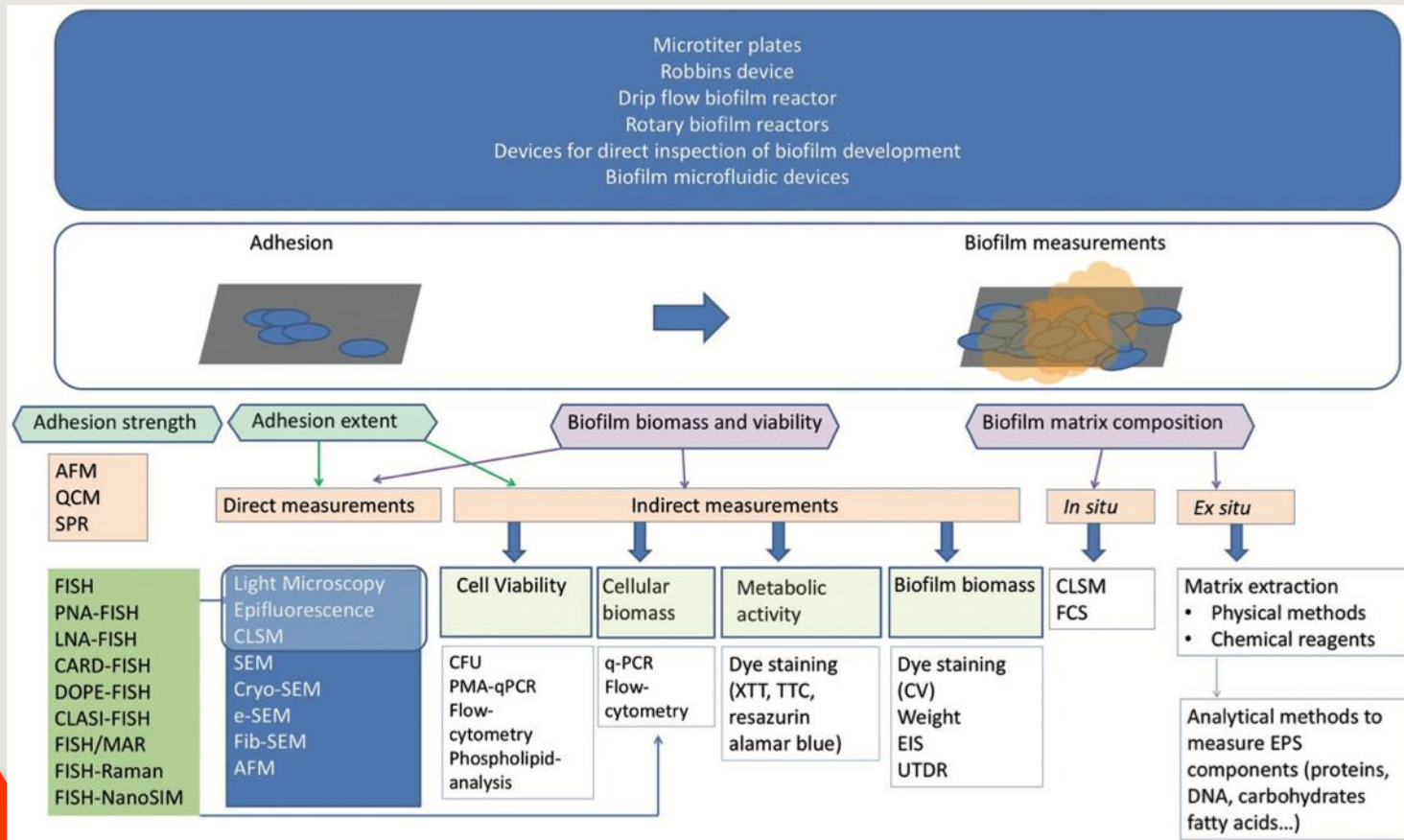
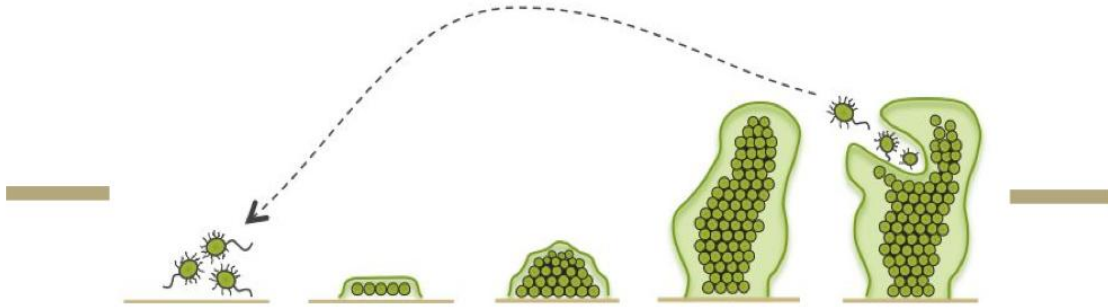


Figure 1. Overview of methods to grow and characterize biofilms, which includes different biofilm devices, methods to assess adhesion extent and strength, and techniques to measure biofilm biomass, viability and matrix composition. © Joana Azeredo.

BIOFILMS IN STATIC SYSTEMS



Commercial models

Format/technique	Experimental features	Applications and examples	References
Static biofilms	<ul style="list-style-type: none"> Low or no shear No replacement of medium No cell washout 		
Microtiter plate	<ul style="list-style-type: none"> High throughput Limited biomass 	<ul style="list-style-type: none"> Phenotypic screening of mutant libraries Attachment and early biofilm development studies Biomass quantification with staining 	26, 81
Calgary device (MBEC)	<ul style="list-style-type: none"> High throughput Peg material may be modified Biomass may be recovered from pegs 	<ul style="list-style-type: none"> Phenotypic screening of mutant libraries Antibiotic susceptibility studies Microscopy with fluorescent probes Biomass quantification with staining 	82
Colony biofilm	<ul style="list-style-type: none"> Limited amount of biomass Large biomass in short amount of time Inexpensive laboratory materials Low throughput 	<ul style="list-style-type: none"> Antibiotic susceptibility and penetration studies Chemical gradient measurements using microelectrodes Heterogeneity studies using microscopy and fluorescent probes Cryosectioning studies for gene expression heterogeneity 	38, 40, 61, 62, 85, 87

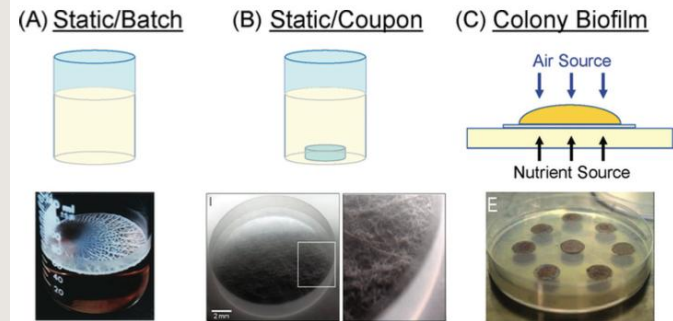


FIGURE 1 Examples of methods for biofilm cultivation under static conditions. (A) Biofilm cultured at the air-water interface, forming a pellicle. Published with permission from reference 83. (B) Biofilm cultured on a glass coupon under static conditions. Published with permission from reference 84. (C) Example of biofilm growth as a colony biofilm. Published with permission from reference 84. doi:10.1128/microbiolspec.MB-0016-2014.f1

Classic model of screening

Pre-inoculum of
each isolate

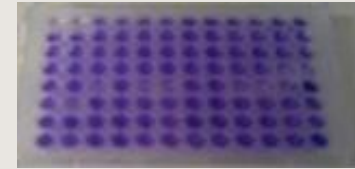


Culture of
strains in LB
medium

Incubation for 48
h at 37°C

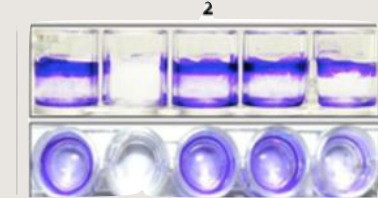


Removal of
planktonic
bacteria



Absorbance
measurement at
590 nm

Staining with cristal
violet of the
attached biofilm



<https://youtube.com/playlist?list=PLG8B8Uyfh7-D4oBxTzx0JI4-MhBnET3nq>



Classification according to Villegas *et al.* 2013

Valores de la absorbancia de acuerdo a la clasificación para la formación de biofilm.

Clasificación	$DO_c = 0,21$	Valores
No formador	$DO \leq DO_c$	$\leq 0,21$
Débil formador	$DO_c < DO \leq 2 \times DO_c$	$0,21 < x \leq 0,42$
Moderado	$(2 \times DO_c) < DO \leq (4 \times DO_c)$	$0,42 < x \leq 0,84$
Gran	$(4 \times DO_c) < DO$	$> 0,84$



Antimicrobials evaluation

Pre-inoculum of
each isolate



Culture of
strains in LB
medium

Incubation for 48
h at 37 °C



Removal of
planktonic
bacteria



Staining with
cristal violet of
the attached
biofilm

Absorbance
measurement at
590 nm

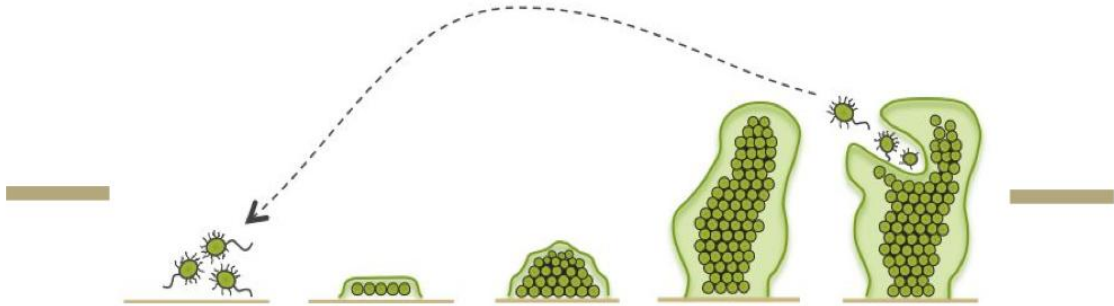


To keep in mind

- The optimal conditions need to be adjusted and are specie-dependent.
- The conditions to be optimized are the culture medium, temperature, time, and the surface support.



BIOFILMS IN DYNAMIC SYSTEMS



Continuous flow biofilms

- | | | | |
|--------------------|---|--|----------------|
| | <ul style="list-style-type: none">• Continuous supply of fresh medium• Adjustable shear force• Low to medium throughput | | |
| CDC reactor | <ul style="list-style-type: none">• Special surface materials may be used• Multiple biofilms are formed simultaneously• Suitable for time-course study• May be used for anaerobic cultures | <ul style="list-style-type: none">• Antibiotic susceptibility/viability studies• Microscopy studies with fluorescent probes• Applicable for omics studies | 88 |
| Drip flow reactor | <ul style="list-style-type: none">• Special surface materials may be used• High gas transfer• Heterogeneous biofilm• Large biomass in short time | <ul style="list-style-type: none">• Antibiotic susceptibility/viability studies• Chemical gradient measurements using microelectrodes• Heterogeneity studies using microscopy fluorescent probes• Cryosection and laser capture microdissection followed by transcriptomic analysis• Biofilm-immune cell interaction• Real-time imaging• Monitoring attachment, development, and detachment phases• Microscopy with fluorescent tags• Attenuated total reflection Fourier transform infrared spectrometry• Hydrodynamics in biofilm by nuclear magnetic resonance | 38, 61, 89 |
| Imaging flow cells | <ul style="list-style-type: none">• Real-time detection• Surfaces can be modified• Appropriate for short-time experiments | | 40, 79, 80, 91 |

Commercial models

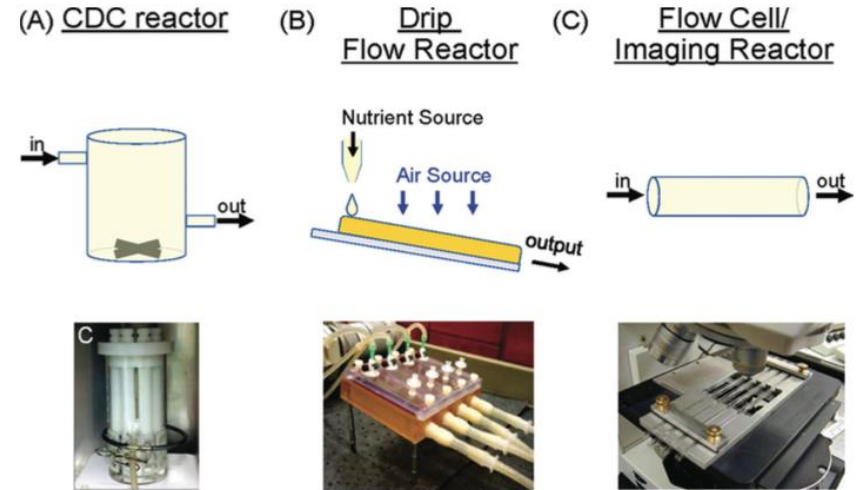


FIGURE 2 Examples of continuous-flow reactors for biofilm cultivation. (A) CDC reactor with medium inlet and outlet ports. Biofilms form on coupons arranged on removable Teflon rods. Published with permission from reference 88. (B) Drip-flow reactor with medium inlet and outlet ports and air exchange ports. Biofilms form on removable slides. Published with permission from reference 89. (C) Capillary flow cell for imaging biofilms. Published with permission from http://centerforgénomicsciences.org/research/biofilm_flow.html. doi:10.1128/microbiolspec.MB-0016-2014.f2

CHARACTERISTICS OF THE MODEL



Three-dimensional structure and spatial organization



Monitoring over time



Mimicking physiological characteristics

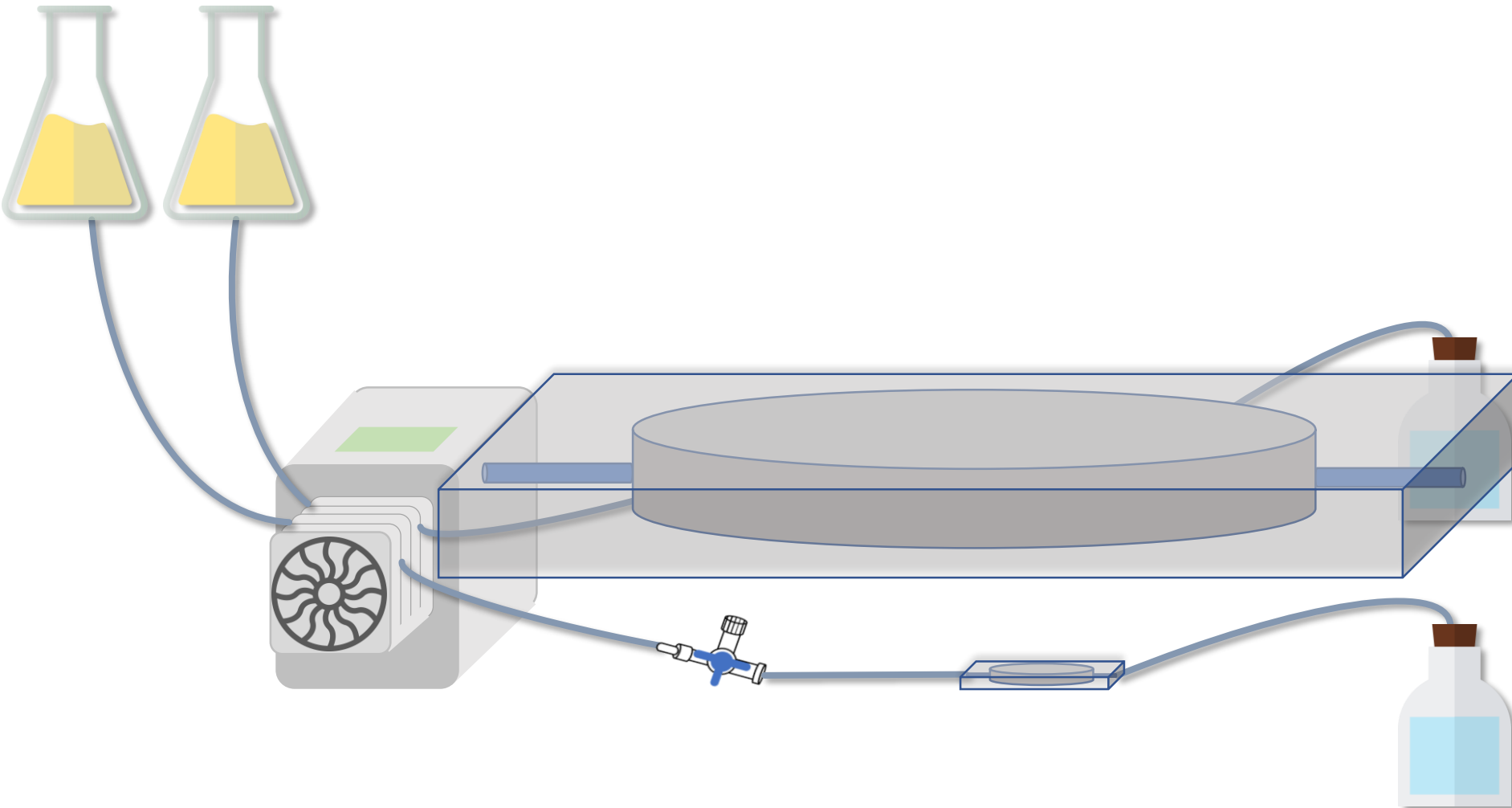


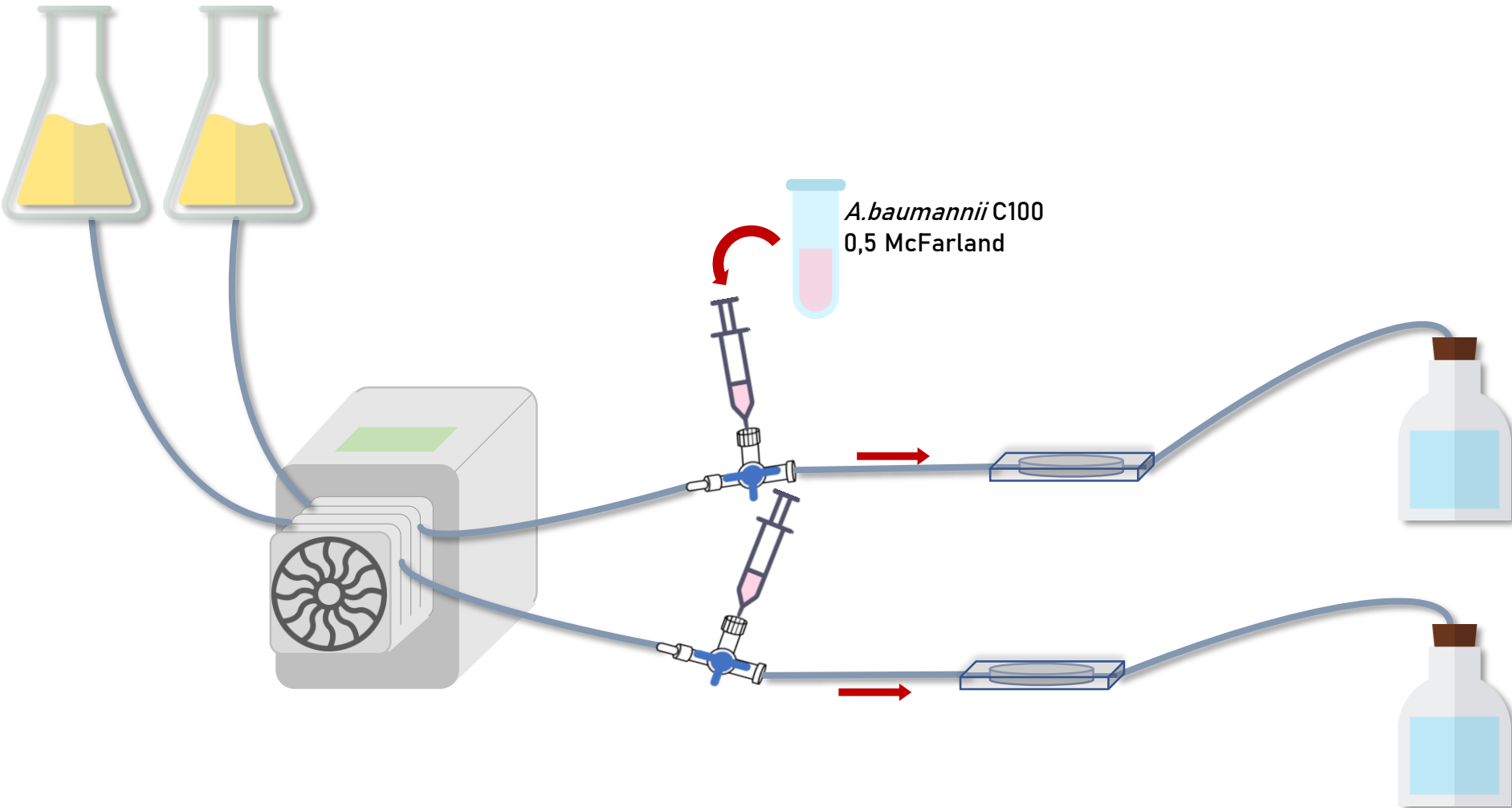
Microorganisms exposed to flow and turbulence



Non-invasive

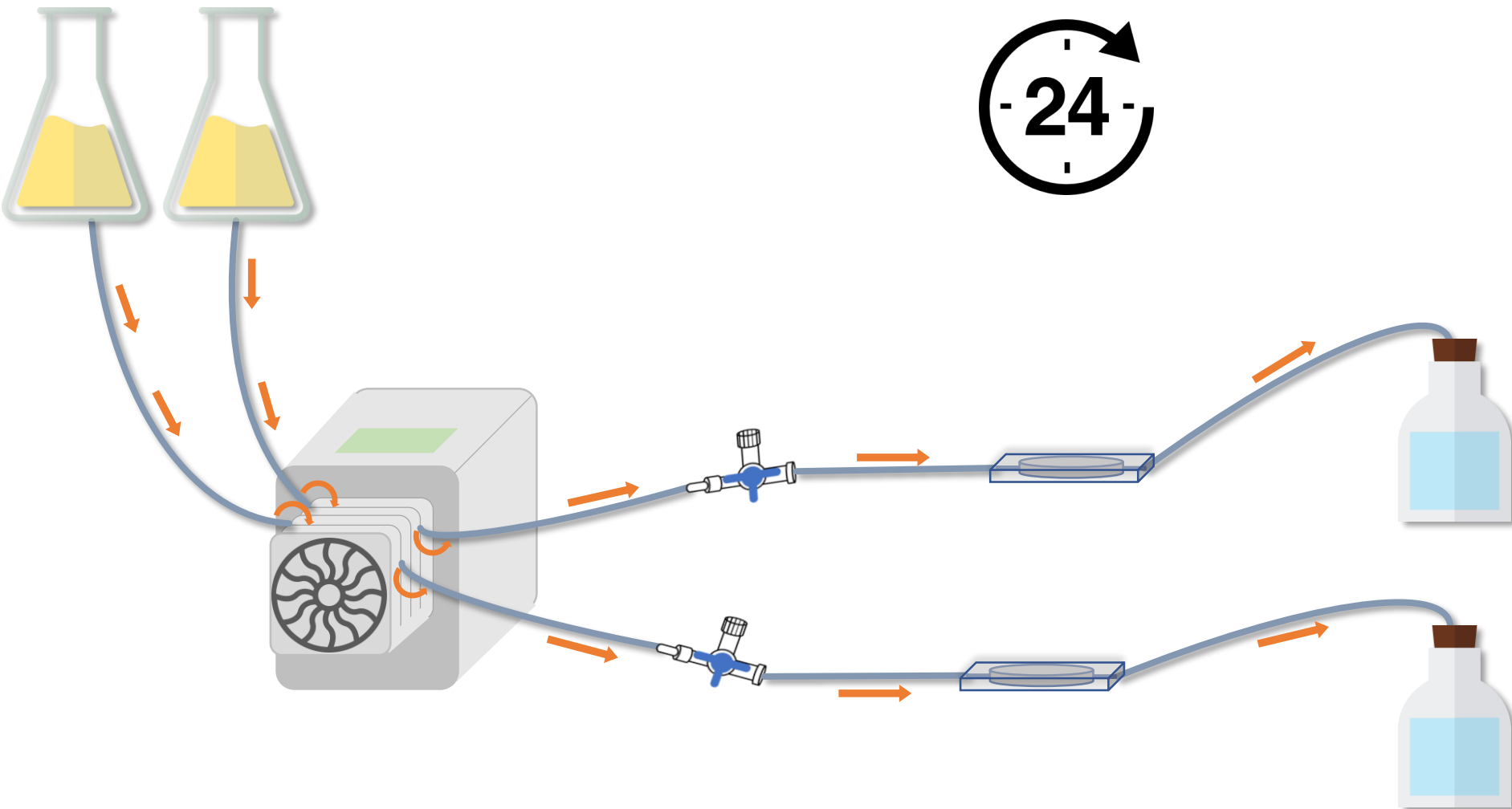


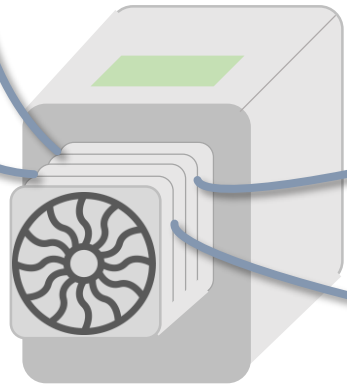




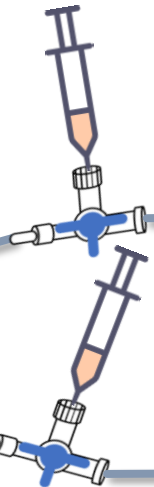
A.baumannii C100
0,5 McFarland

24





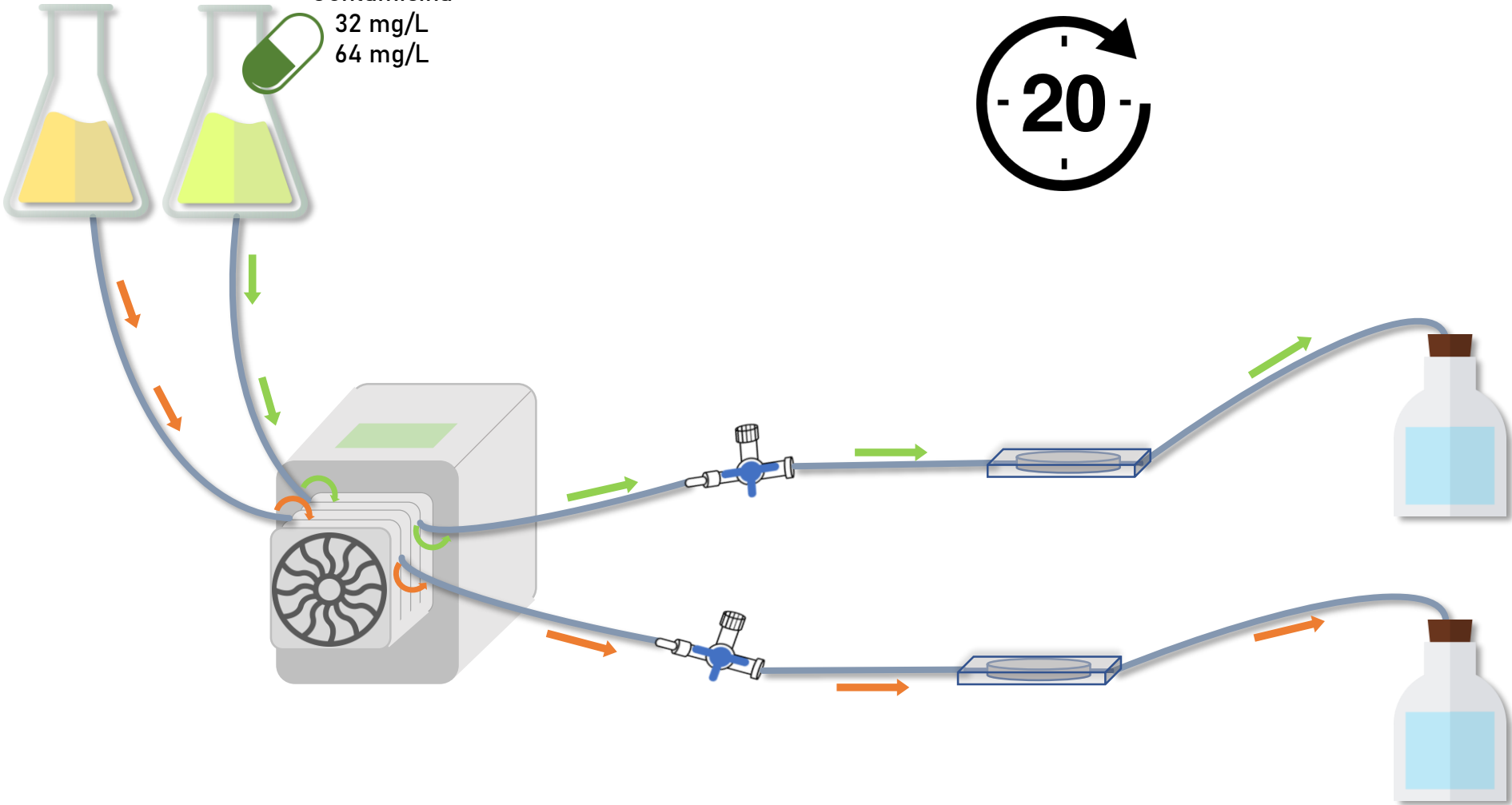
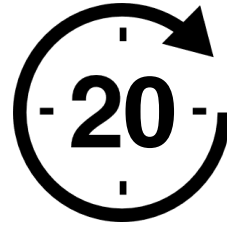
Naranja de acridina
0,01 mg/ml

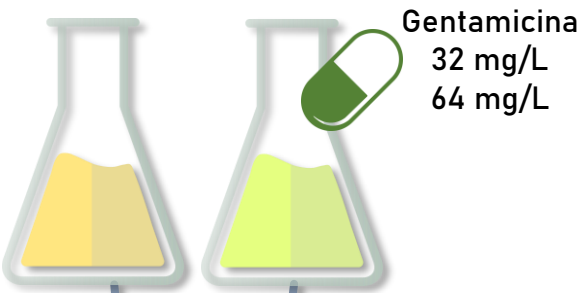


Microscopía
láser confocal



Gentamicina
32 mg/L
64 mg/L





Naranja de acridina
0,01 mg/ml

Microscopía
láser confocal

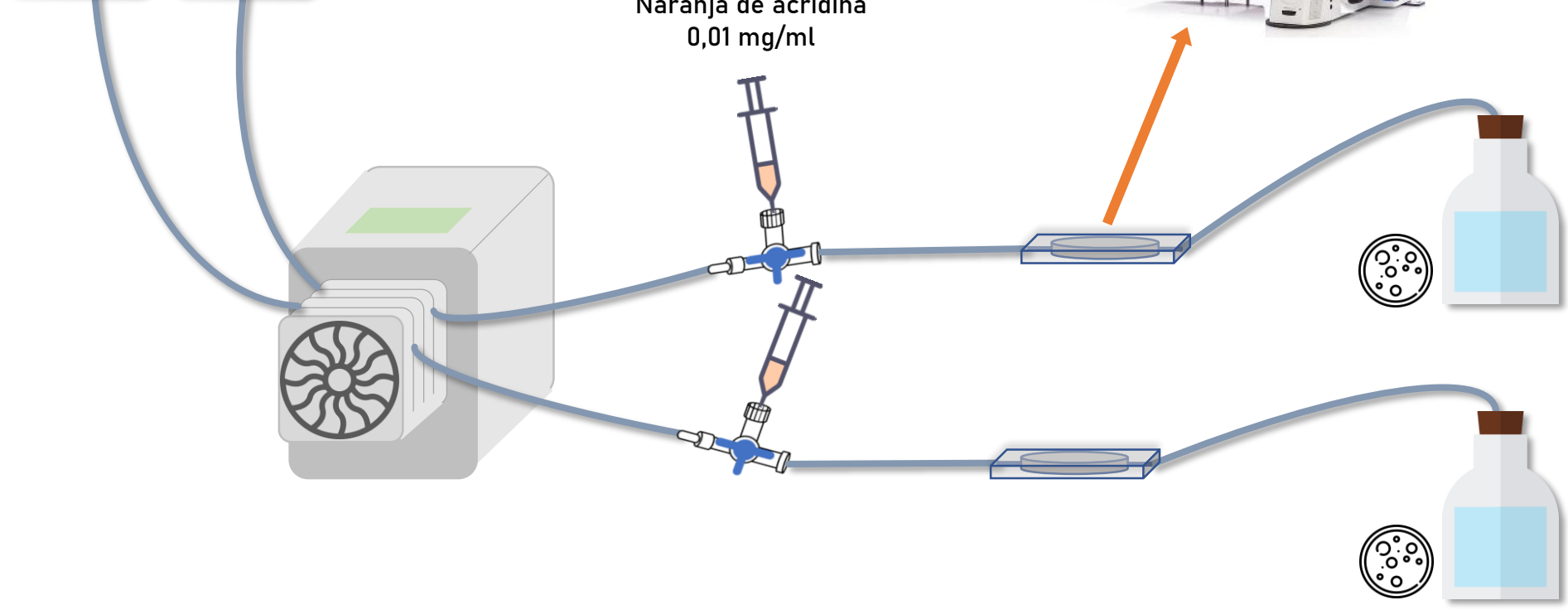
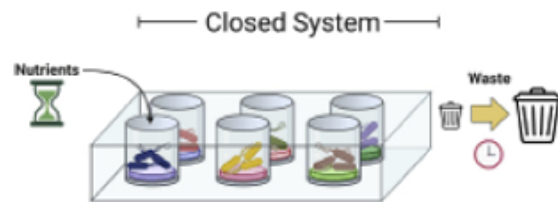
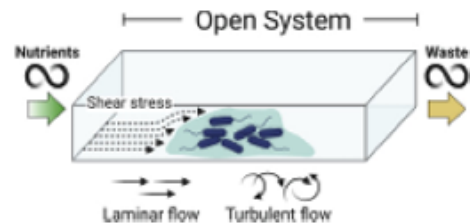


Table 1. General characteristics of *in vitro* static and dynamic biofilm models

Static



Dynamic



Nutrient supply	Finite (may be replenished periodically)	Continuous
Flow	No	Yes, over the biofilm surface
Shear stress	Null to mild (if shaken)	Adjustable: Different hydrodynamic conditions can be adapted
Waste and planktonic cells	Accumulated	Removed as nutrients are replenished
Length of experiments	Short (days)	Long (from days to weeks)
Throughput screening	High: Different species/strains and conditions can be simultaneously evaluated	Low to medium
Technical complexity	Low	Medium to high
Complexity of setup procedures	None	Considerable, time consuming
Specialized equipment	Little (requires mostly common laboratory equipment)	Required (e.g., reactors or fermenting systems)
Cost per replicate	Low	Medium to high
Particularly useful for	Early stages of biofilm development Genetic screening	Study of mature biofilms

Stainings



TABLE 2 Assays applied for biofilm quantification and viability determination^f

Assay or reagent	Quantification ability	Assay combination	Advantage(s)	Disadvantage(s)	Reference(s)
Fluorescent dyes					
CV	Biofilm matrix biomass		Easy Inexpensive Wide applicability	Dependent on absorption of the dye into the biomass Nonspecific to multispecies biofilms No dimensional information Sample destruction Poor reproducibility	110, 124, 162
Congo red	Biofilm matrix biomass		Easy	Low accuracy for biofilm visual analysis pH-dependent binding ability	149, 442
DMMB Live/Dead BacLight (Syto 9 and PI)	Biofilm matrix biomass Semiquantitative	Resazurin, XTT, BTA, FDA CLSM	Inexpensive Strain specific (<i>S. aureus</i>) Cell viability assessment	Reagent instability Expensive	154, 162 443, 444
AO	Apoptotic quantification	Ethidium bromide, epifluorescence microscopy	Time efficient	Intermediate "unknown" population Underestimation of living cells Large no. of samples required Lab safety requirements due to high mutagenicity	157, 445
DAPI	Live-cell biomass	CTC	DNA and RNA labeling Detects apoptotic phenomena Feasible combination with other probes Nuclear integrity	Used only for fixed cells High concn is required for live-cell staining	200, 446
XTT	Counts metabolically active cells		Cell viability assessment Reproducible Nondestructive	Requires highly respirative bacteria Variations due to biofilm heterogeneity	162, 437
AB/resazurin	Counts metabolically active cells		Cell viability assessment Reproducible	Time-consuming Large no. of samples required Heat and light sensitive	447, 448
CTC	Counts metabolically active cells	DAPI, epifluorescence microscopy	Cell viability assessment Bright red fluorescence Discrimination between active cells and abiotic parts Cell viability assessment	Detects only highly metabolically active cells Toxicity Solute-associated inhibition	166, 449–451

TABLE 2 (Continued)

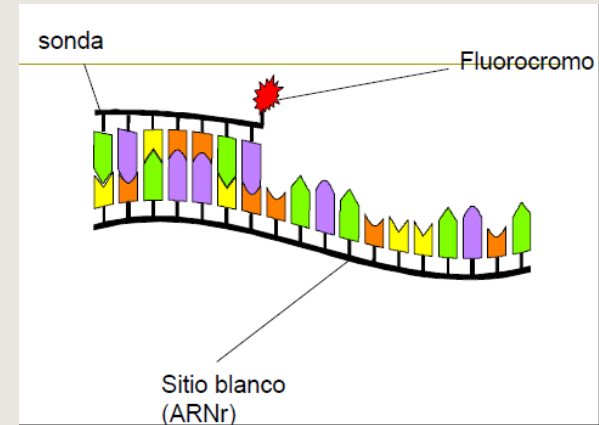
Assay or reagent	Quantification ability	Assay combination	Advantage(s)	Disadvantage(s)	Reference(s)
SYBR Green I	Multispecies biofilm cell quantification Can synthesize DNA in real time	Real-time PCR	Detects bacteria with low metabolic activity Cell viability assessment Reliable and reproducible No specific probes required Cell viability assessment	Risk of sample contamination	454, 455
Genetic/molecular approaches RT-PCR	Multispecies biofilm cell quantification	Gel electrophoresis (DGGE)	Detects uncultivable or challenging-to-culture species, live and dead cells, matrix components DGGE detects predominant species, gives early clinical diagnosis	Risk of sample contamination Expensive and complex procedure	456
Real-time PCR	Can synthesize DNA in real time Counts cells in multispecies biofilms	SYBR green I	Easy, rapid, reliable, and reproducible High sensitivity Cell viability assessment High sensitivity	Risk of sample contamination	455, 457
Next-generation sequencing (NGS)	Quantification of genomic sequences	PCR, RT-PCR ^e	Entire transcriptome available in a single analysis (RNA-seq) ^e Biofilm phenotype, protein profile determinant, and resistance pattern analysis	Expensive	458
Proteomic analysis	ECM protein component	Mass spectroscopy/NMR	Independent of growth conditions	Low permeability of DNA probes	175, 200, 459, 460
Microscopy FISH	Semiquantitative	CLSM	Applicable to multispecies biofilms Detects all viable microorganisms	Low sensitivity Hybridization between complementary PNA probes	
IF	Antibody-antigen complexes	Fluorescently labeled antibodies	Visualization and spatial distribution Simple procedure	Expensive and lengthy multistep procedure Less flexible procedure	461

Costly

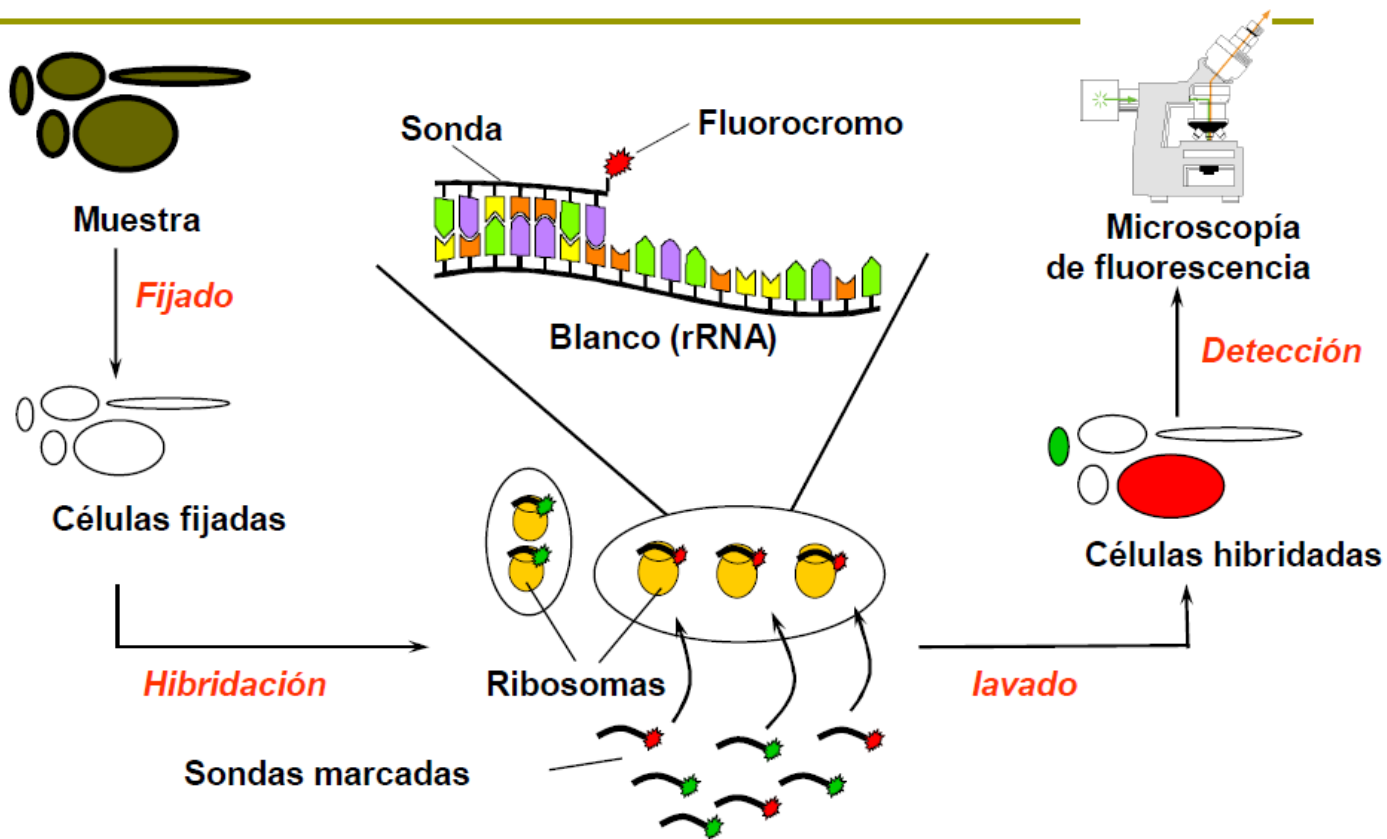
Assay or reagent	Quantification ability	Assay combination	Advantage(s)	Disadvantage(s)	Reference(s)
CLSM	Quantitative imaging	Fluorescence assay, FISH, FCS	Nondestructive	Probe efficacy dependent on biofilm EPS complexity	162, 204, 205, 462, 463
SIM	Live-cell biomass imaging	Fluorescent probes	3D imaging Cell and EPS spatial distribution Applicable to thick sample 3D imaging of living cells	Special equipment required Specimen instability during multiple-image recording	210, 211, 464
OCT	Biomass, structure, and porosity identification	Ultra-broad-bandwidth lasers	Enhanced resolution Computational amplification Imaging of thick samples Real-time 3D imaging	No cell-level resolution	216, 465–467
TEM	Total biofilm matrix biomass imaging		Speedy measurements Noninvasive Label-free High resolution	Limited penetration depth Sample prepn required	204, 468, 469
SEM ^a	Synergy with focus ion beam for inner biofilm study	EDS ^b	Surface visualization ^a	Special equipment required Risk of sample distortion due to dehydration ^a	162, 220, 223, 227, 470, 471
ESEM ^b Cryo-SEM ^c ASEM ^d			Detailed 3D visualization ^a No structural damage ^b No sample prepn ^b Imaging of EPS ^b No dehydration required ^c Nonconductive surfaces ^c Time efficient ^c Nanostructure biofilm surface visualization in liquids ^d	Low resolution ^b Artifacts due to sample prepn ^c Low resolution ^c Multiple labeling ^d	
STXM	Total biofilm biomass	X-ray fluorescence	Macromolecule distribution	Applicable to thin samples	162, 204, 224, 472, 473
	Chemical biofilm components		Visualization of biological and environmental components and spatial distribution	Special equipment required	
AFM	Chemical biofilm component imaging		Real-time 3D imaging Little/no sample prepn Performed in both air and water Elucidation of molecular interactions High resolution	Artifacts and sample damage due to incorrect tip elections Deformation of soft samples Poor image quality in water Special equipment required	227, 228, 474–476

Fluorescence in situ hybridization, FISH

- Staining technique that allows the identification of bacteria in complex samples using fluorescence and confocal microscopy.
- Use of fluorescently labeled DNA or RNA probes.



Protocolo para FISH



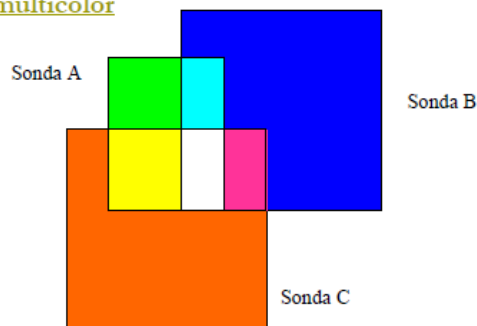
Sondas: aproximación jerárquica

sonda	blanco	Domi- nio	Clase	Sub- clase	Género
ARCH915	16S	Archaea			
EUK516	18S	Eucarya			
EUB338	16S	Bacteria			
CF319a	16S		CFB		
HGC69a	23S		Actinobacteria		
PLA30	23S		Planctomycetes		
ALF1b	16S		Proteobacteria	α	
BET42a	23S			β	
GAM42a	23S			γ	
SRB385	16S			δ	
DNMA657	16S			ϵ	<i>Desulfonema</i> sp.
ARC94	16S				<i>Arcobacter</i> sp.

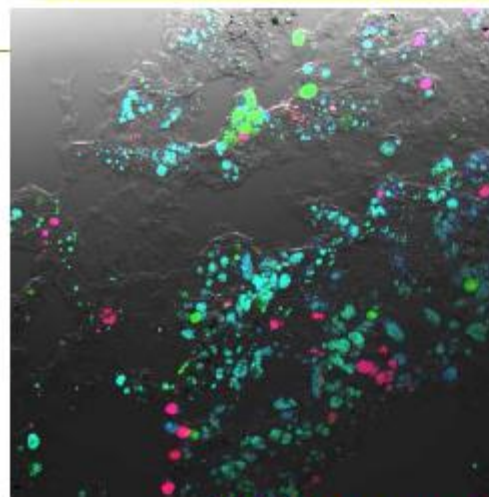
Fluorocromos para FISH

	Excitatio n	Emission
Alexa488	490 nm	520 nm (verde)
Fluos	495 nm	523 nm (verde)
Cy3	550 nm	570 nm (rojo)
Alexa546	555 nm	570 nm (rojo)

FISH multicolor



Aplicación múltiples sondas



Composition of the AOB Community



NSO1225 (β -subclass AOB)

Nc. mobilis { NEU653 (*Nitrosomonas europaea/eutropha/halophila*)
NmV (*Nitrosococcus mobilis*) } *N. europaea/eutropha*