

# Topic 10

1. Main methodologies in microbiology mainly related to the cultivation, isolation and identification of a microorganism and its genetic manipulation

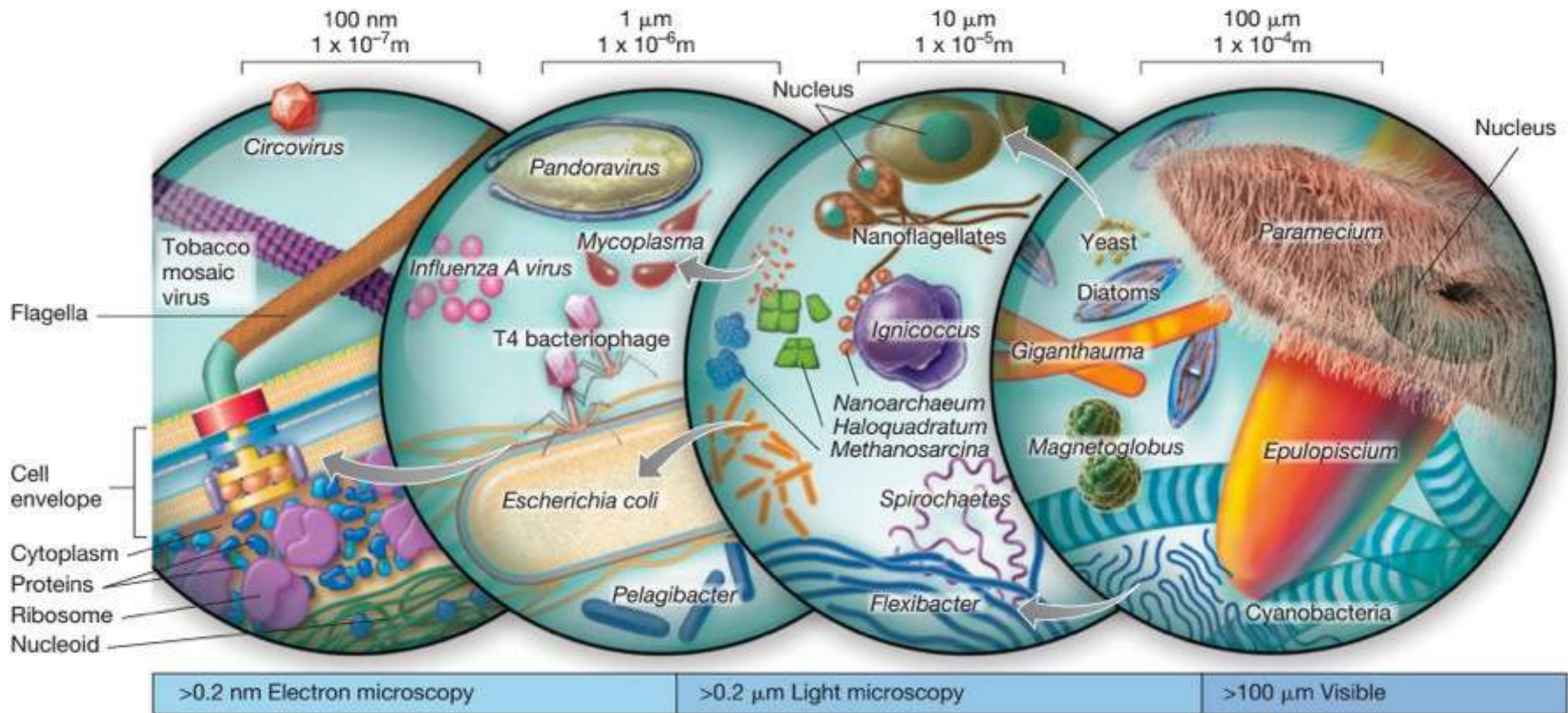
- Microscopy
- Molecular biology

2. Omics-techniques and how they are revolutionizing environmental microbiology research (genomics, transcriptomics, proteomics, metabolomics, meta-genomics, meta-transcriptomics, meta-proteomics, meta-metabolomics)

3. Integrated single cell investigations

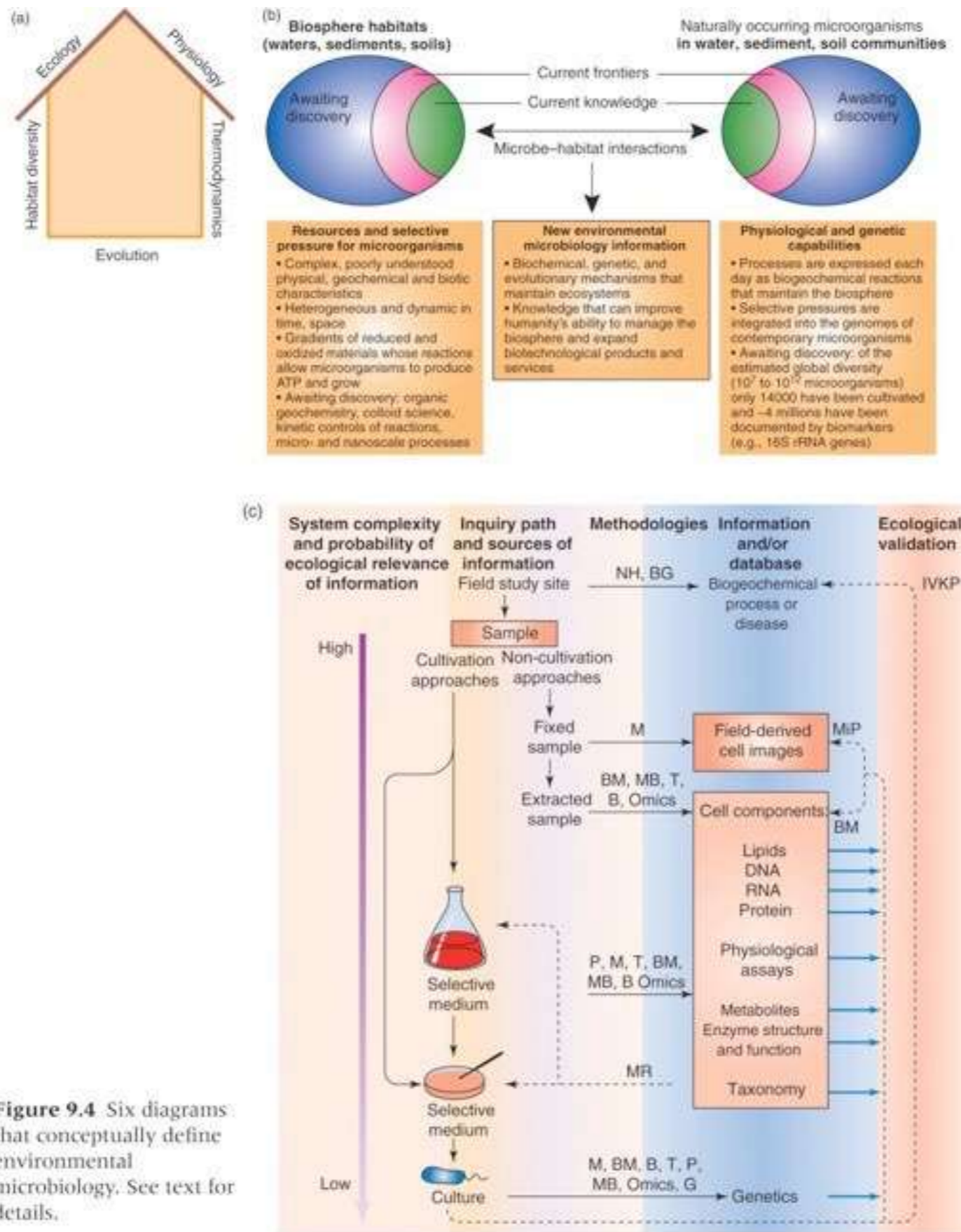
4. Novel culturing techniques

# Size matters



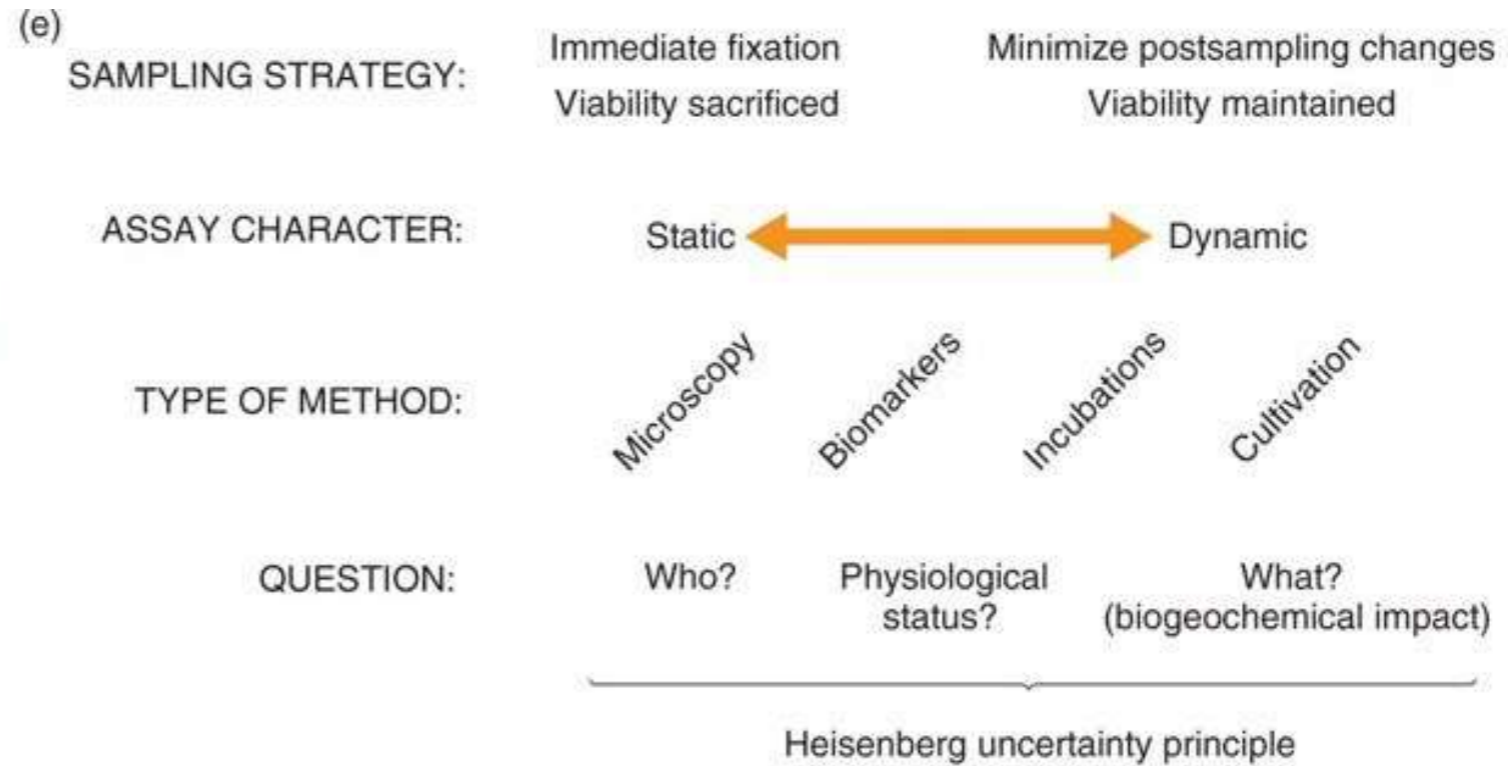
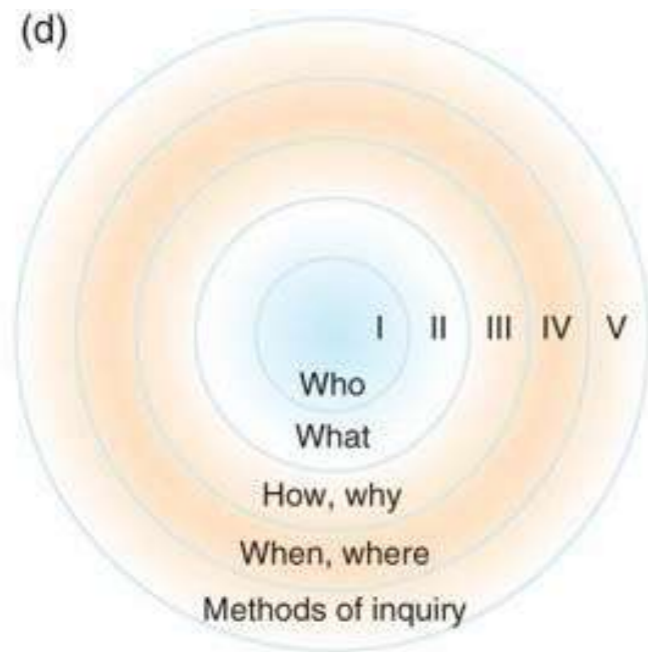
Madigan et al. 2018

- Flow cytometry
- Raman and IR spectroscopy
- Optical and Magnetic Tweezers
- Gel-based microbial diversity assessment
- Microsensor
- Radioactive compounds to assess microbial metabolisms and growth ( $^{14}\text{C}$ ,  $^3\text{H}$ ,  $^{35}\text{S}$ ,  $^{33}\text{P}$ )
- Stable isotopes and their fractionations by biological processes



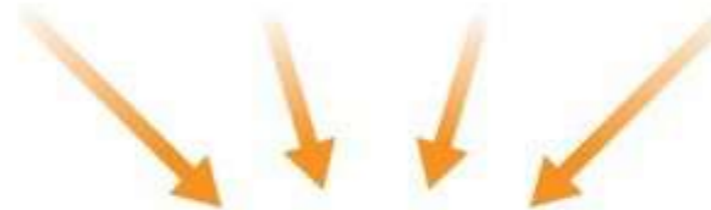
**Figure 9.4** Six diagrams that conceptually define environmental microbiology. See text for details.

WILEY Blackwell



(f) *No single approach* leads to a thorough understanding or answer to a given question. Information from all four approaches can complement and confirm one another. When this confluence occurs, the discipline of environmental microbiology is advanced.

Microscopy    Cultivation    Incubations    Biomarkers

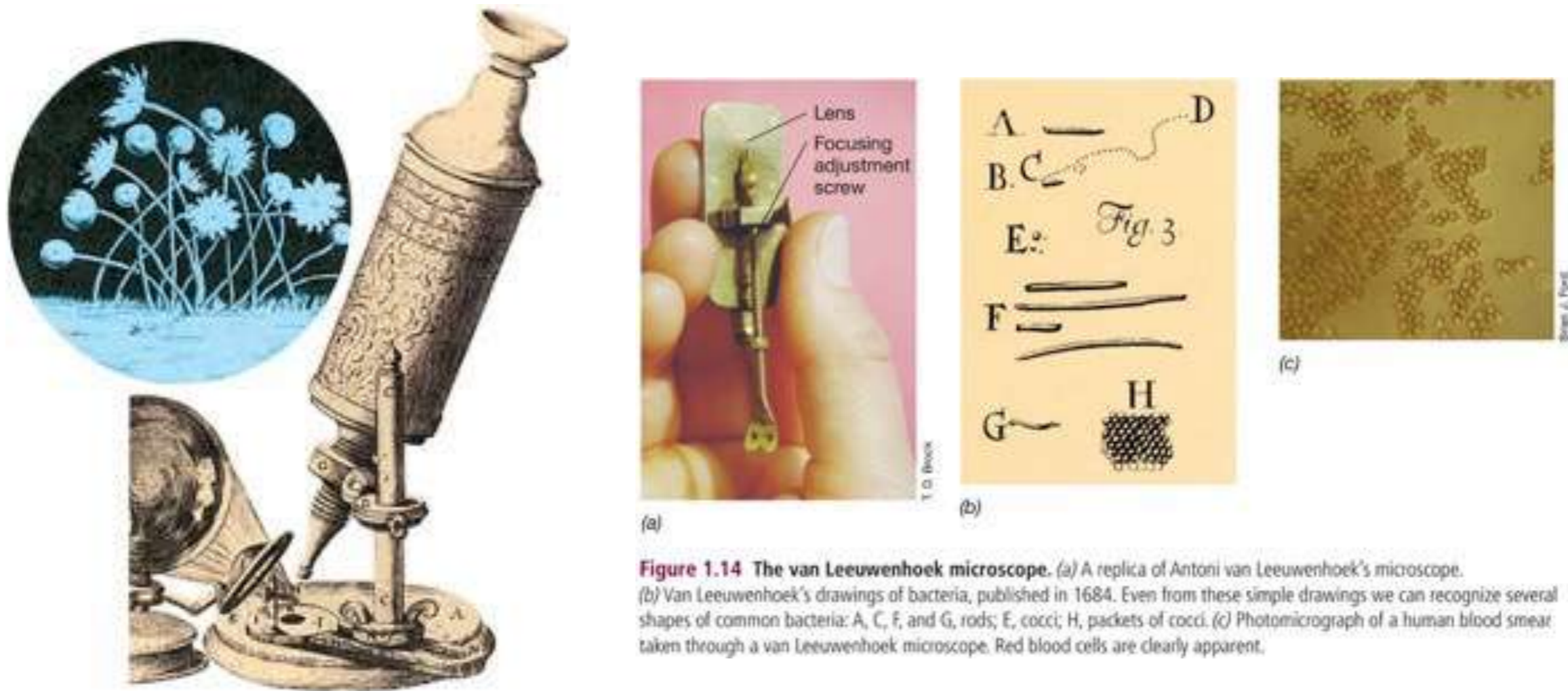


PROGRESS

**Figure 9.4** *Continued*

*Environmental Microbiology: From Genomes to Biogeochemistry*, Second Edition, Eugene L. Madsen.  
 © 2016 Eugene L. Madsen. Published 2016 by John Wiley & Sons, Inc.  
 Companion Website: [www.wiley.com/go/madsen/enviromicrobio2e](http://www.wiley.com/go/madsen/enviromicrobio2e)

# Invention drives discovery microorganisms



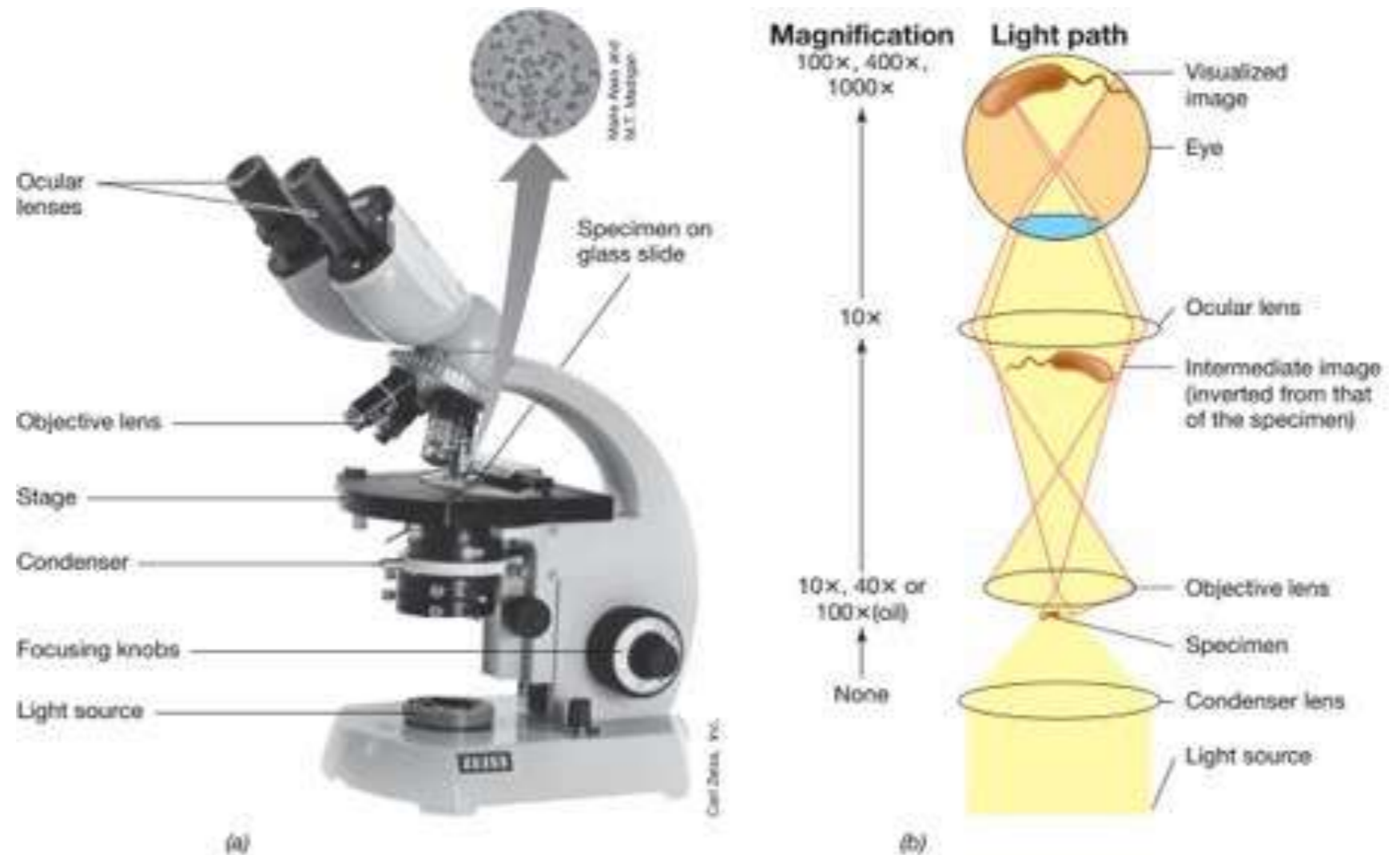
**Figure 1.14** The van Leeuwenhoek microscope. (a) A replica of Antoni van Leeuwenhoek's microscope. (b) Van Leeuwenhoek's drawings of bacteria, published in 1684. Even from these simple drawings we can recognize several shapes of common bacteria: A, C, F, and G, rods; E, cocci; H, packets of cocci. (c) Photomicrograph of a human blood smear taken through a van Leeuwenhoek microscope. Red blood cells are clearly apparent.

Light Microscopy and the Discovery of Microorganisms: English mathematician and natural historian Robert Hooke (1635–1703) was an excellent microscopist → book *Micrographia* (1665), the first book devoted to microscopic observations, Hooke illustrated many microscopic images including the fruiting structures of molds

First person to see bacteria, the smallest microbial cells, was the Dutch draper and amateur microscopist Antoni van Leeuwenhoek (1632–1723) → constructed extremely simple microscopes containing a single lens to examine various natural substances for microorganisms → discovery of bacteria in 1676 while studying pepper–water infusions: “wee animalcules”

# Microscopy

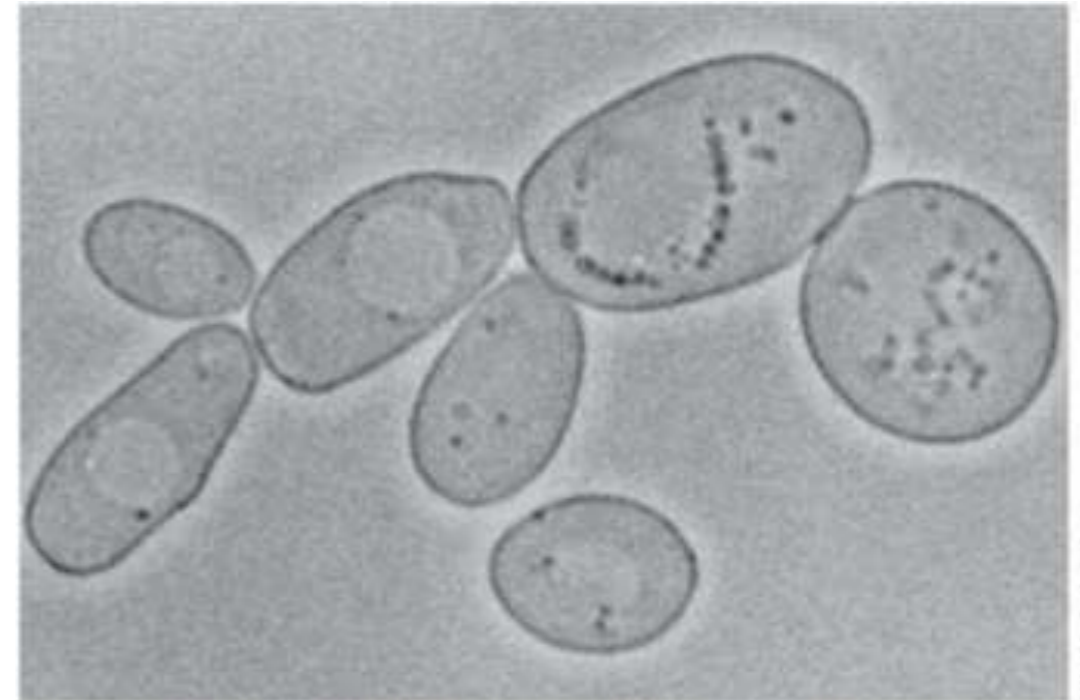
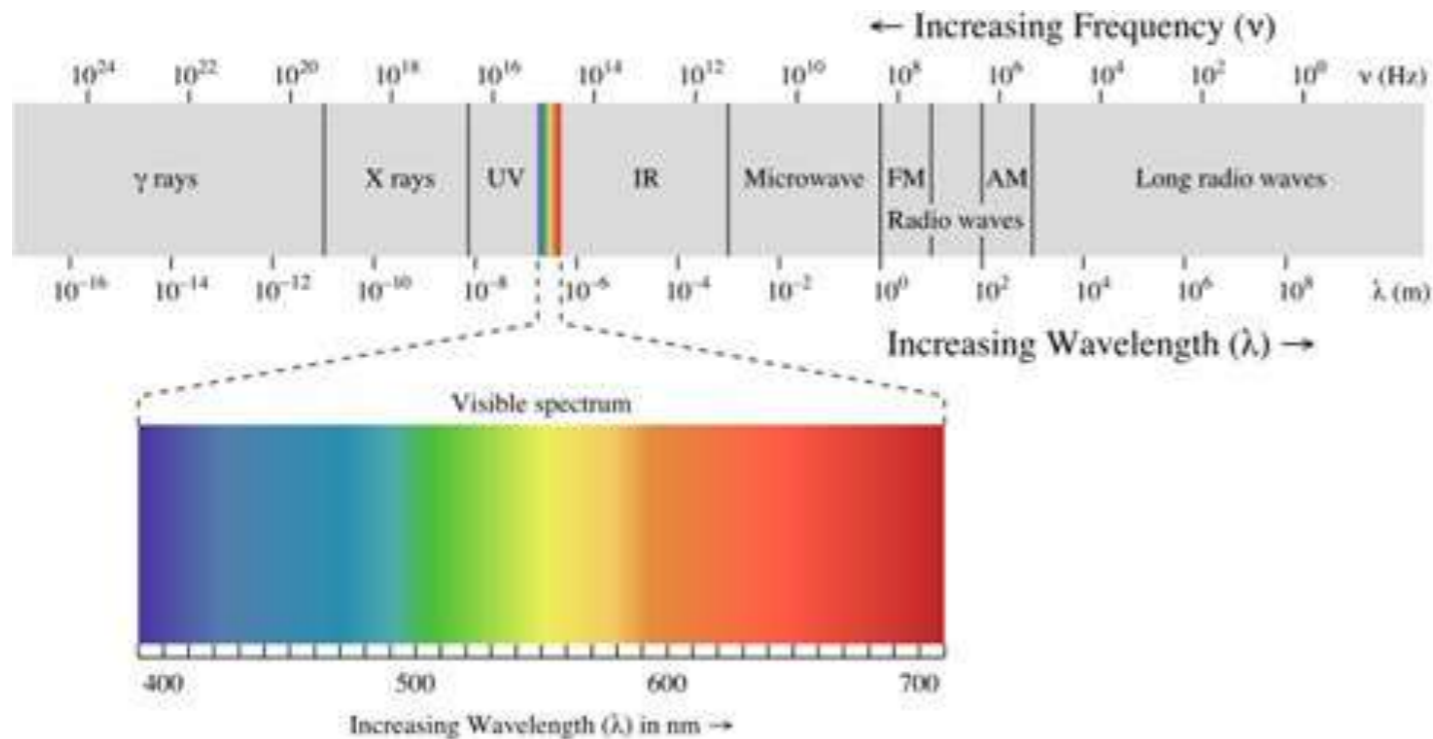
- Magnification describes the capacity of a microscope to enlarge an image → lenses
- Resolution that governs our ability to see the very small → Resolution is the ability to distinguish two adjacent objects as distinct and separate
- The limit of resolution for a light microscope is about  $0.2\ \mu\text{m}$  ( $\mu\text{m}$  is  $10^{-6}\text{m}$ ) → two objects that are closer together than  $0.2\ \mu\text{m}$  cannot be resolved as distinct and separate



Madigan et al. 2018

- Modern compound light microscope, light from a light source is focused on the specimen by the condenser, and this light passes through the sample and is collected by the lenses
- Two types of lenses, objective (10-100x) and ocular (10-30x), that function in combination to magnify the image
- Total magnification of a compound light microscope is the product of the magnification of its objective and ocular lenses → Magnification of 1000\* is required to resolve objects  $0.2\ \mu\text{m}$  in diameter.
- The limit of resolution for a light microscope is a function of the wavelength of light used and the light-gathering ability of the objective lens, a property known as its numerical aperture
- There is a correlation between the magnification of a lens and its numerical aperture; lenses with higher magnification typically have higher numerical apertures and smaller working distance and total field of view
- With objectives that have a very high numerical aperture (such as the 100\* objective), an optical grade oil is placed between the microscope slide and the objective → Immersion oil increases the light gathering ability of a lens, that is, it increases the amount of light that is collected and viewed by the lens

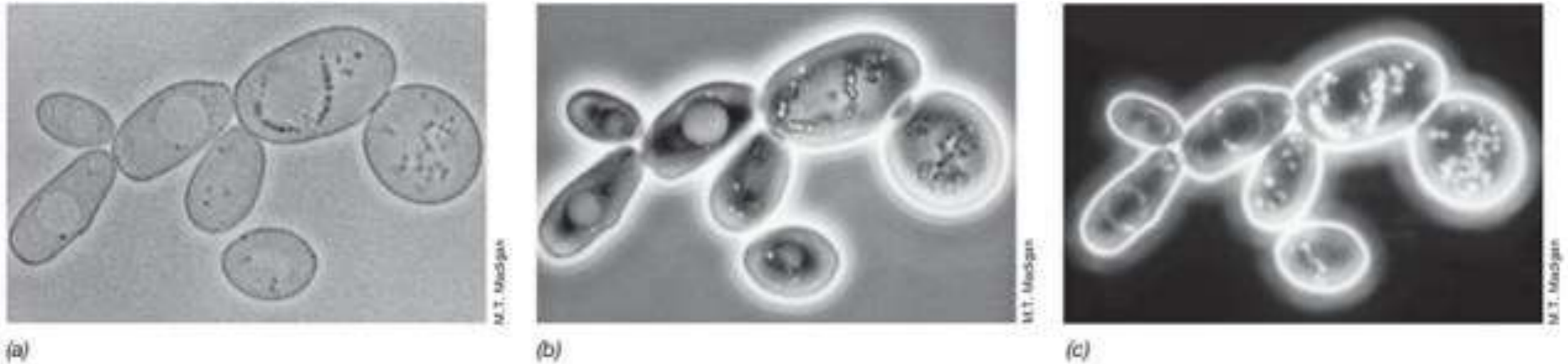
# Light Microscopy



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M.T. Madigan

- Several types of light microscopy: bright-field, phase-contrast, differential interference contrast, dark-field, and fluorescence
- In light microscopy, specimens are visualized because of differences in contrast that exist between them and their surroundings
- In bright-field microscopy, contrast results when cells absorb or scatter light differently from their surroundings
- Contrast is necessary in light microscopy to distinguish microorganisms from their surroundings  $\rightarrow$  cells can be stained to improve contrast  $\rightarrow$  staining often kills cells and can distort their features
- Many dyes used in microbiology are positively charged (basic dyes)  $\rightarrow$  bind strongly to negatively charged cell components, such as nucleic acids and acidic polysaccharides and cell surfaces



**Figure 1.19** Cells visualized by different types of light microscopy. The same field of cells of the yeast *Saccharomyces cerevisiae* visualized by (a) bright-field microscopy, (b) phase-contrast microscopy, and (c) dark-field microscopy. Cells average 8–10  $\mu\text{m}$  wide.

- **Phase-contrast** microscopy is based on the principle that **cells differ in refractive index** (that is, the ability of a material to alter the speed of light) from their surroundings  $\rightarrow$  light passing through a cell thus differs in phase from light passing through the surrounding liquid  $\rightarrow$  subtle difference is amplified by a device in the objective lens (phase ring), resulting in a dark image on a light background
- **Dark-field microscope, light does not pass through** the specimen  $\rightarrow$  light is directed from the sides of the specimen and only light that is scattered when it hits the specimen can reach the lens  $\rightarrow$  the specimen appears light on a dark background
- **Differential interference contrast (DIC)** microscopy is a form of light microscopy that employs a **polarizer in the condenser** to produce polarized light (light in a single plane)
- Polarized light then passes through a prism that generates 2 distinct beams  $\rightarrow$  pass through the specimen and enter the objective lens, where they are recombined into one  $\rightarrow$  b/c the two beams pass through substances that differ in refractive index, the combined beams are not totally in phase but instead interfere with each other  $\rightarrow$  3D perspective, which enhances subtle differences in cell structure



**Figure 1.21** Differential interference contrast microscopy. The yeast cells are about 8  $\mu\text{m}$  wide. Note the clearly visible nucleus and compare to the bright-field image of yeast cells in Figure 1.19a.



# Fluorescence Microscopy and CSLM

Figure 3 - Fluorophore Absorption and Emission Profiles

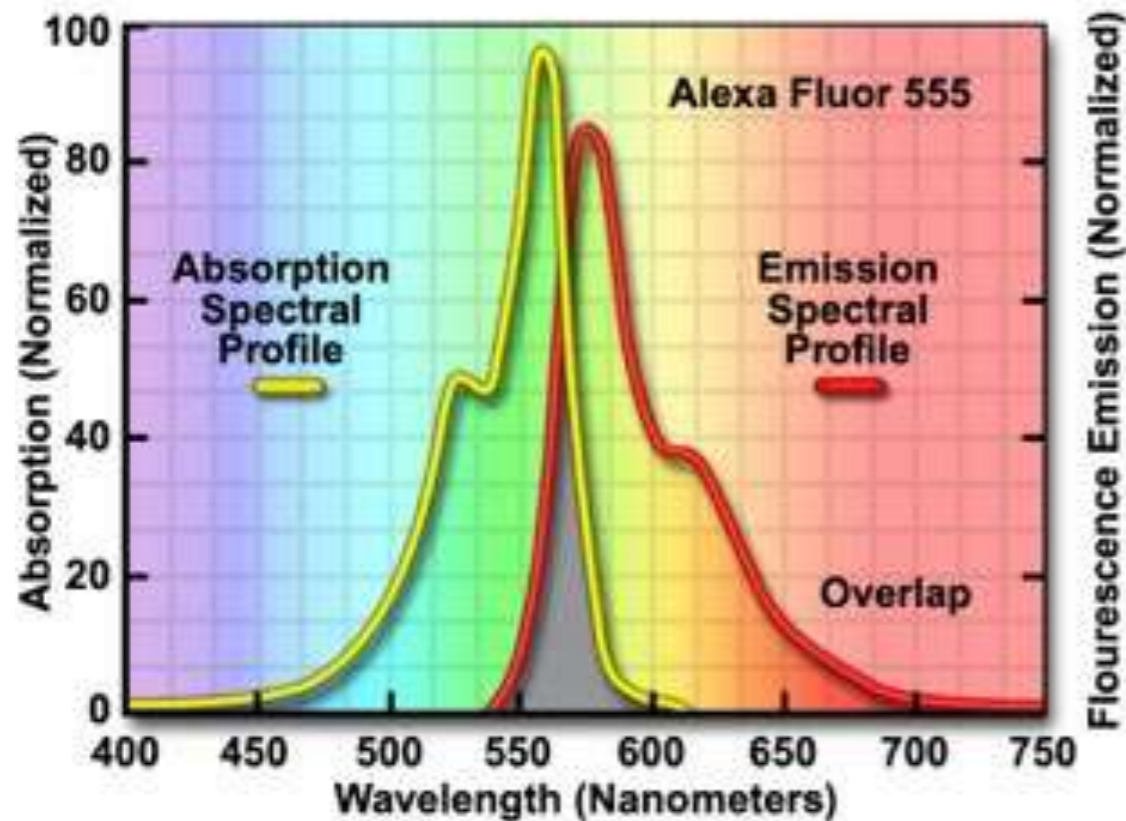
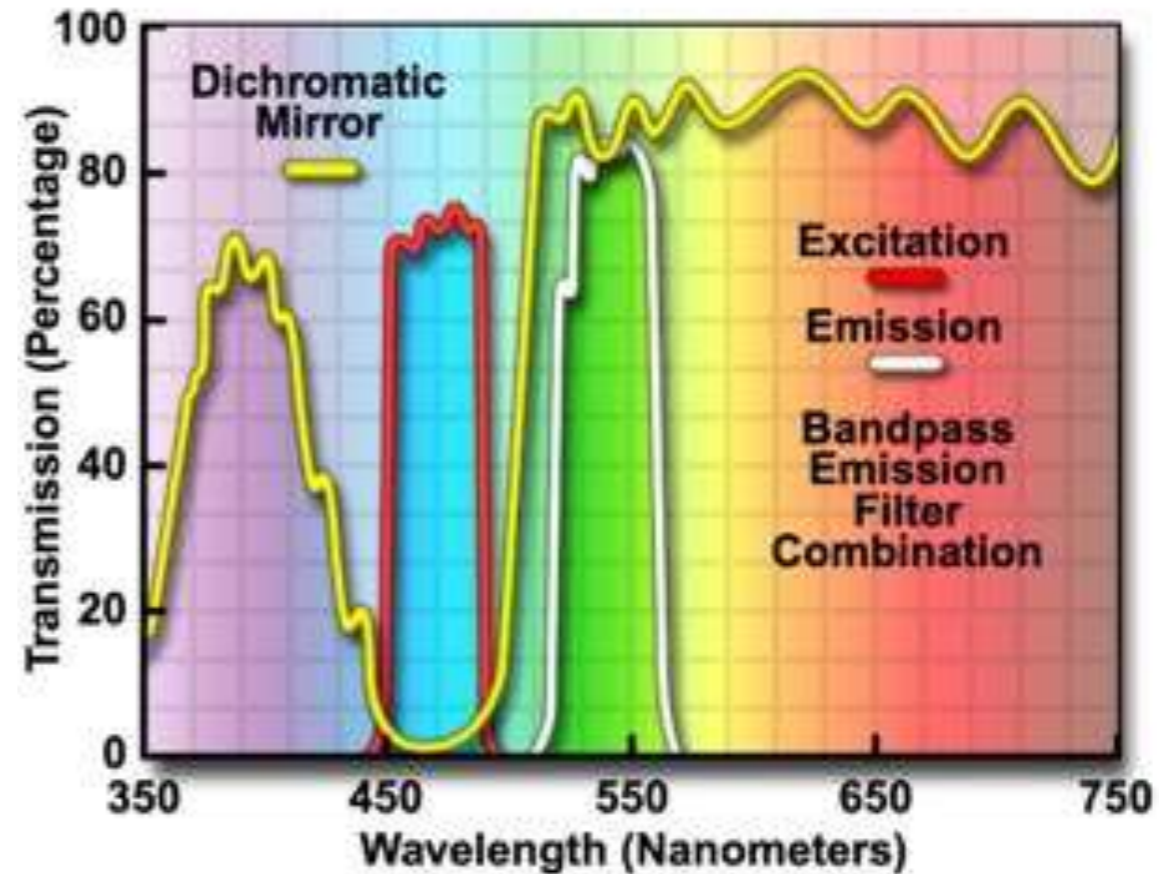


Figure 6 - Nikon B-2E (Medium Band Blue Excitation)



- **Fluorescence Microscopy:** fluorescence microscope visualizes specimens that fluoresce → cells are made to fluoresce (to emit light) by illuminating them from above (epi-fluorescence) or below (inverted scope) with light of a single color (wide light, full spectrum)
- Filters are used so that only fluorescent light is seen, and thus cells appear to glow in a black background
- **Confocal Scanning Laser Microscopy (CSLM)** (light amplification by stimulated emission of radiation, coherent and can be focused) is a computer controlled microscope that couples a laser to a fluorescence microscope → the laser generates a high-contrast, 3D image and allows the viewer to access several planes of focus in the specimen → The laser beam is precisely adjusted such that only a particular layer within a specimen is in perfect focus and illuminated
- The laser then scans up and down through the layers of the sample, generating an image for each layer → computer assembles the pictures to compose the many layers into a single high-resolution 3D image

# Anatomy of a microscope

Figure 1 - Epi-Fluorescence Microscope

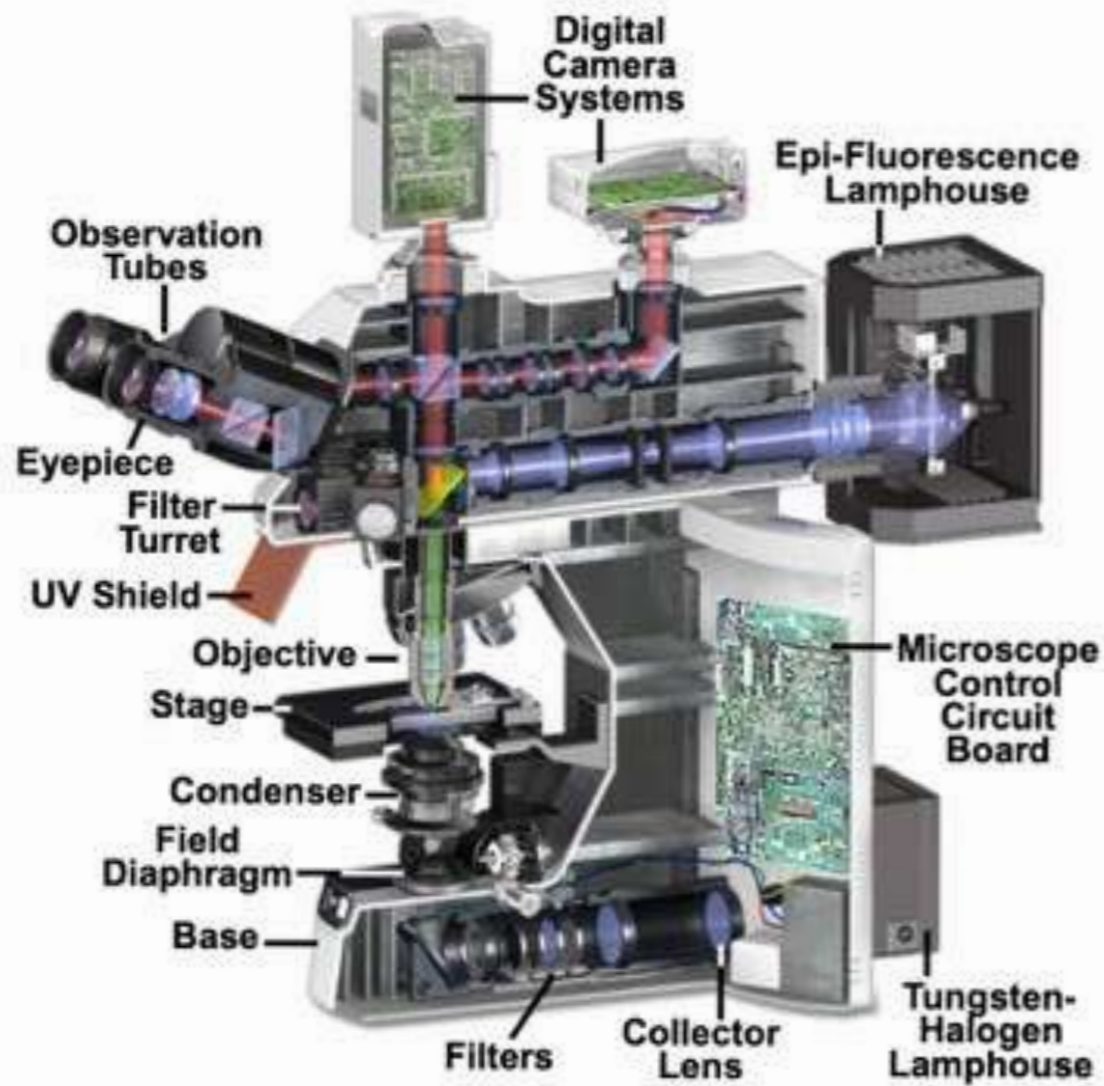
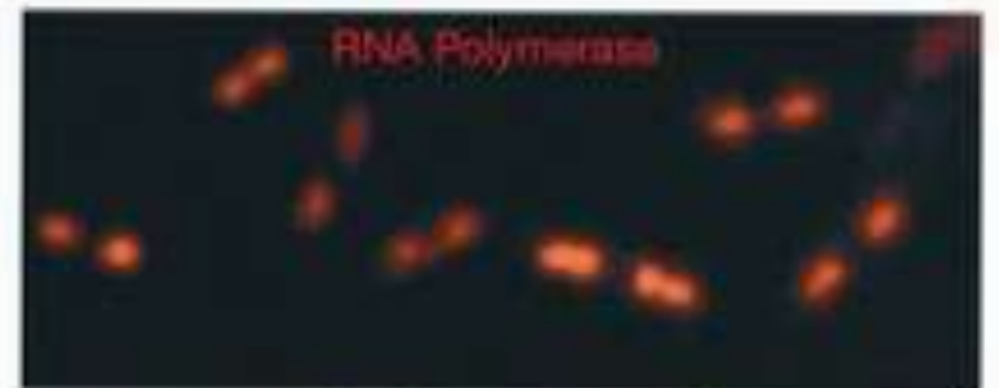
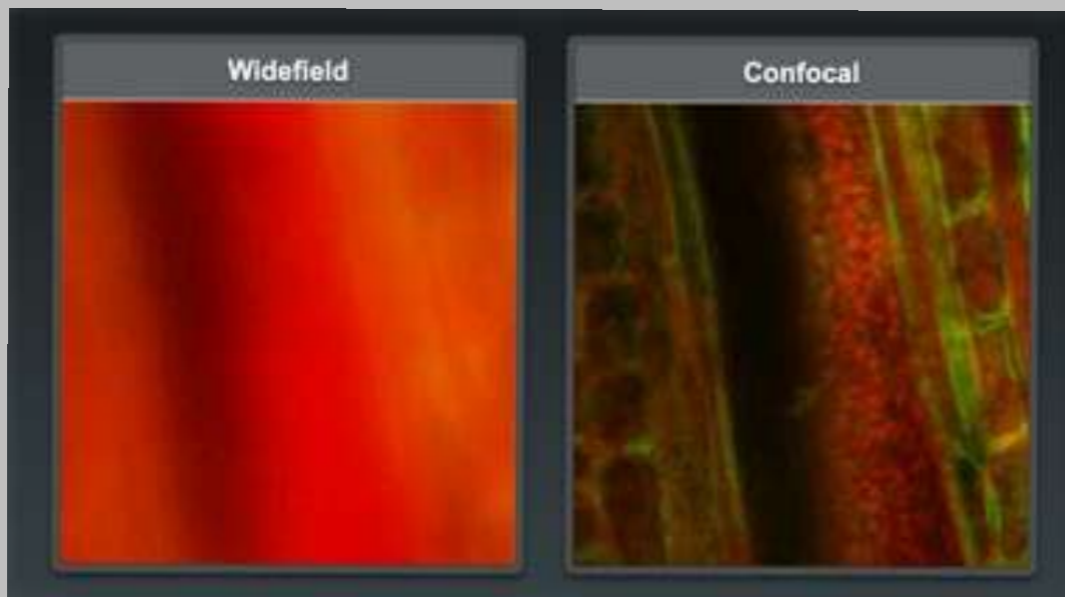
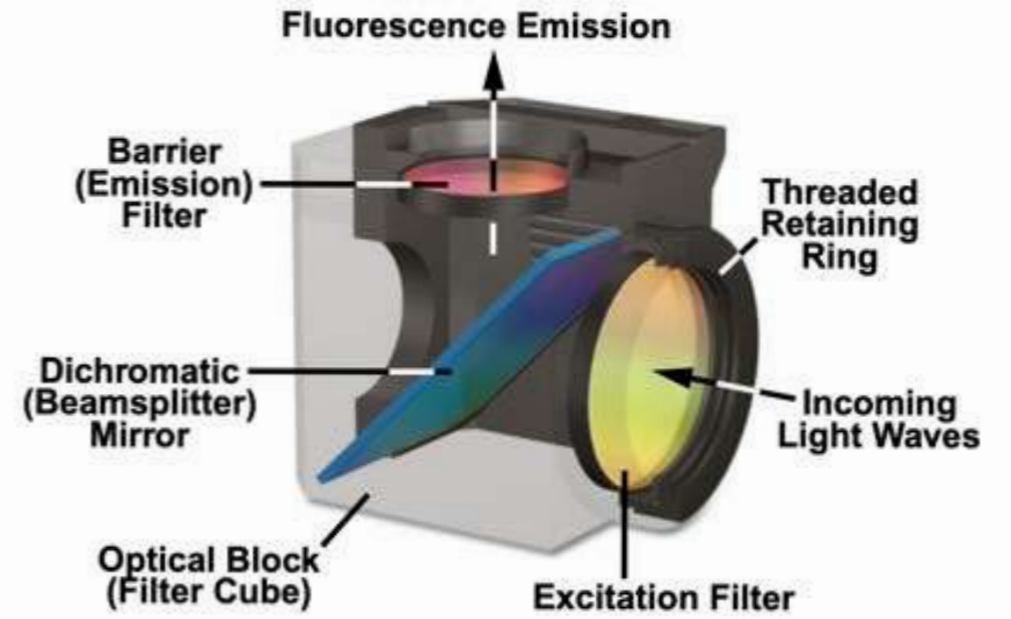
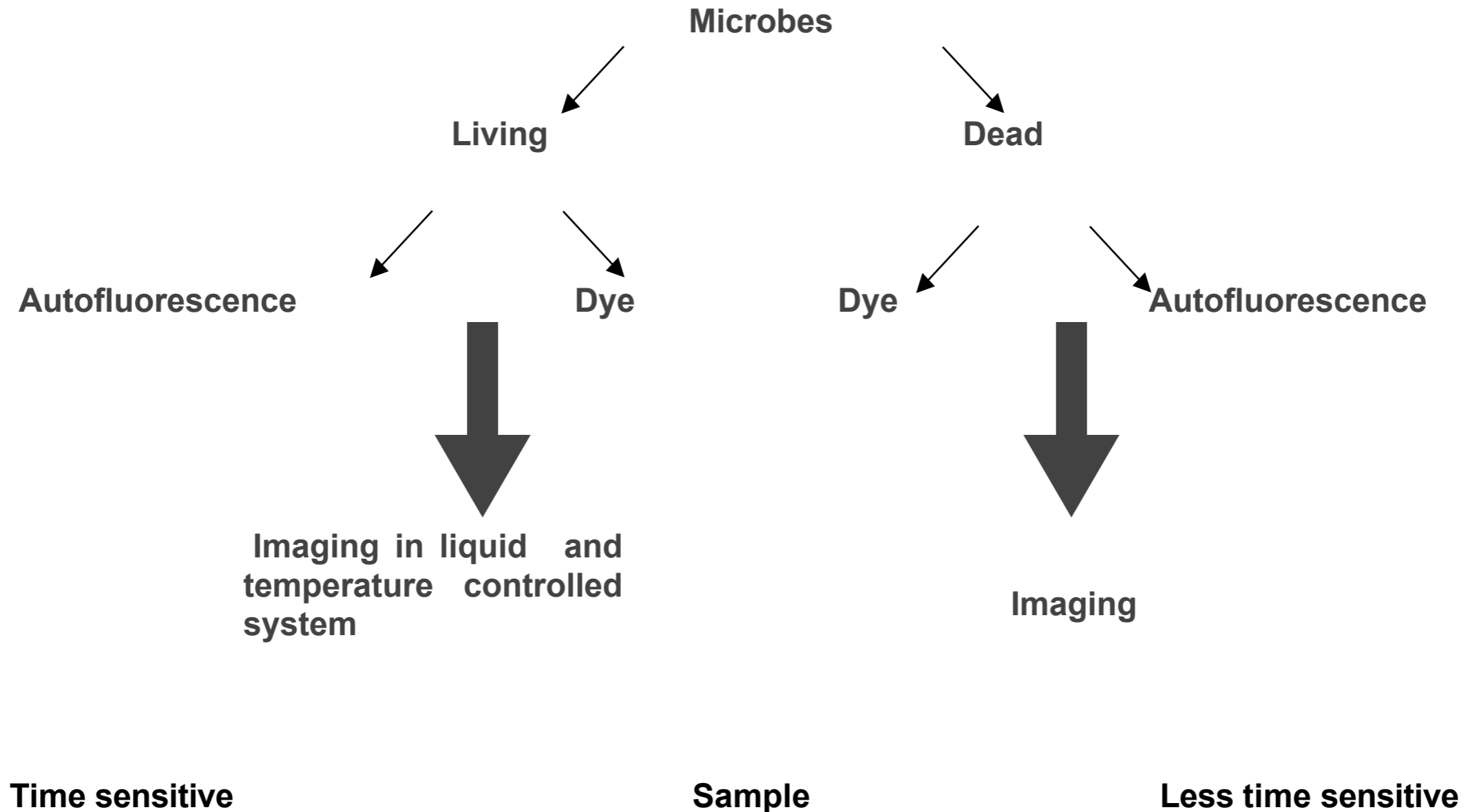


Figure 2 - Fluorescence Filters



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# Sample preparation: Fluorescence Microscopy and CSLM

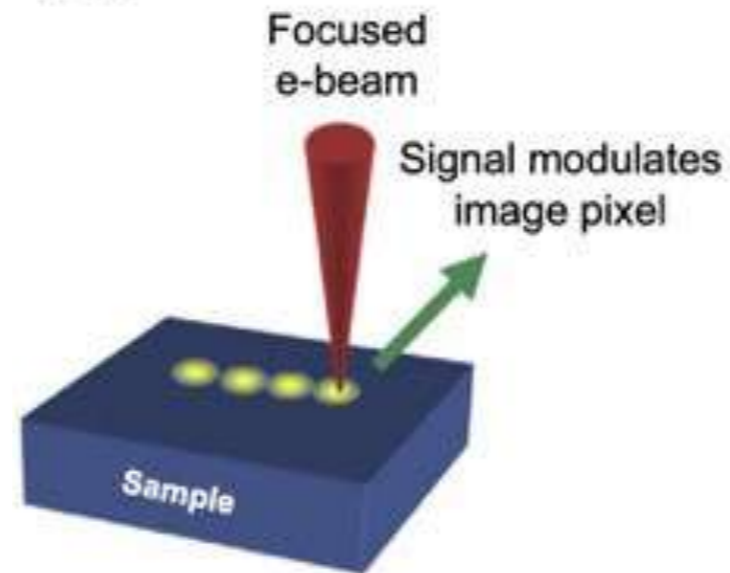


# Electron microscope

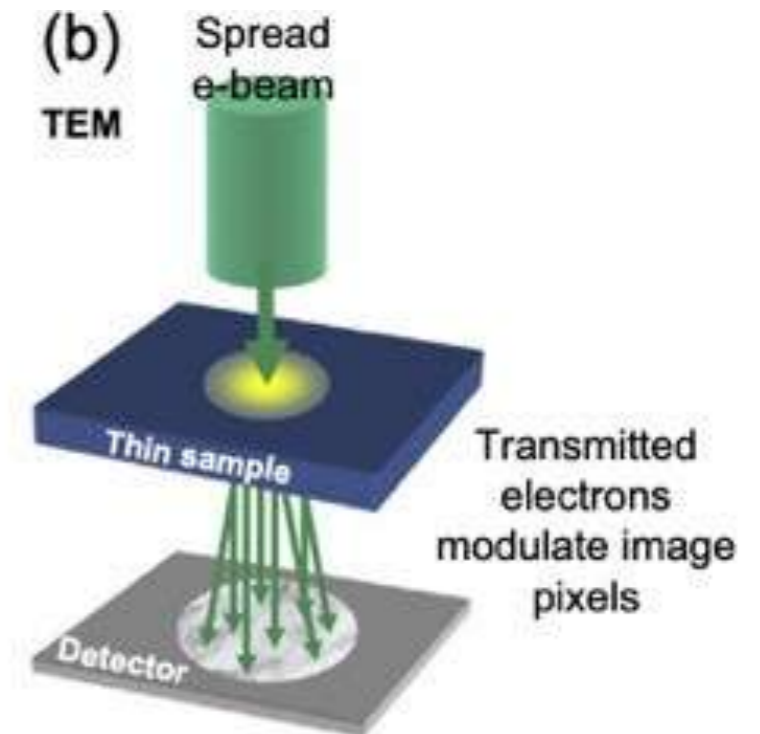


Electron source  
Evacuated chamber  
Sample port  
Viewing screen

(a)  
SEM



(b)  
TEM

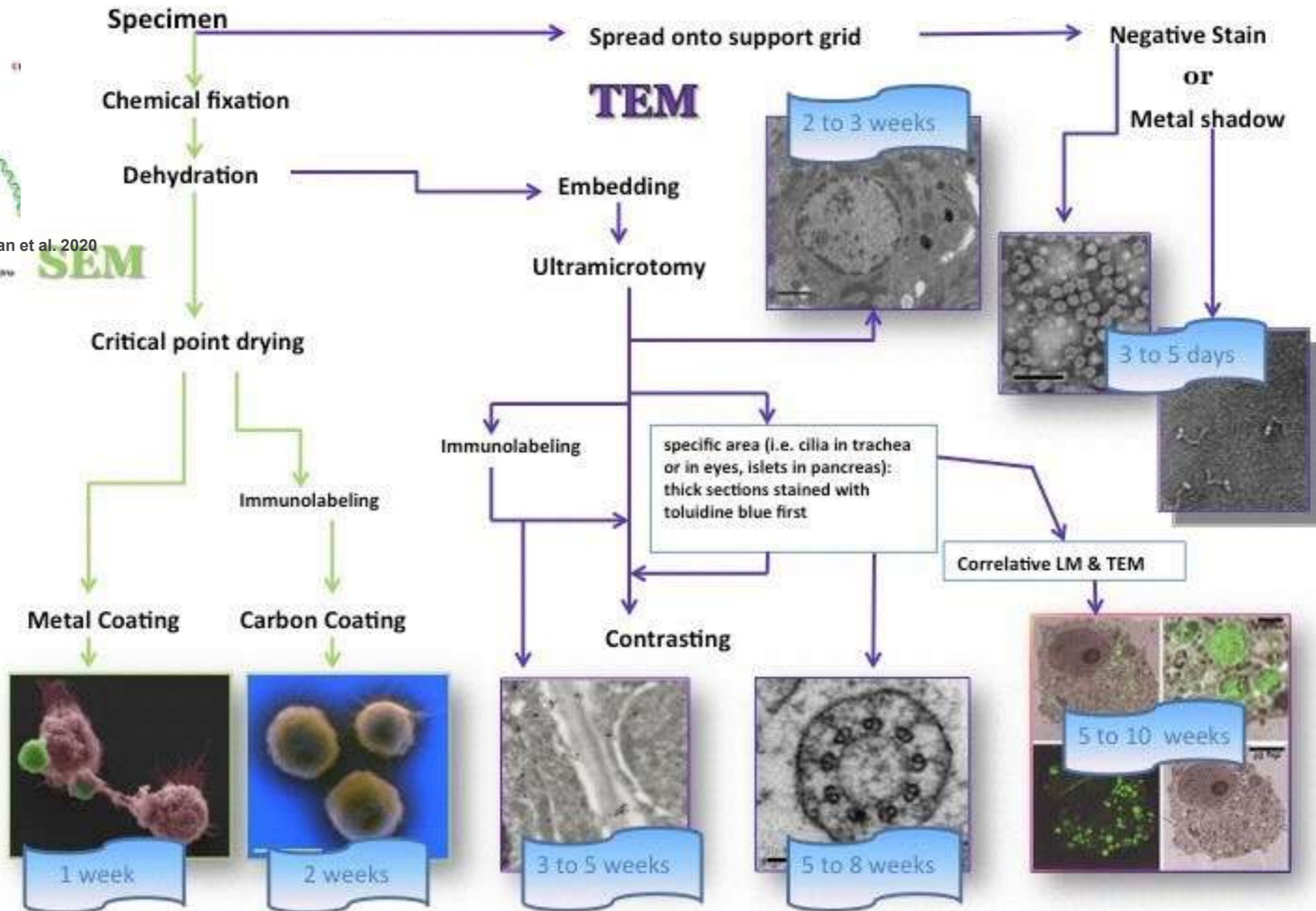
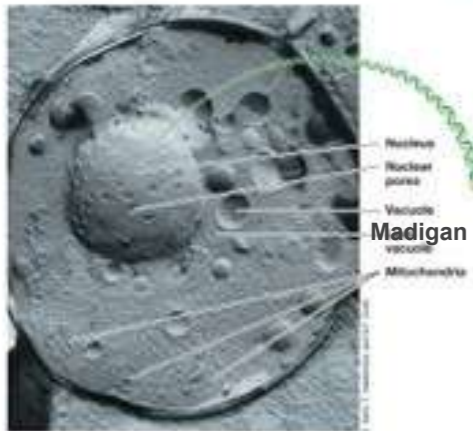
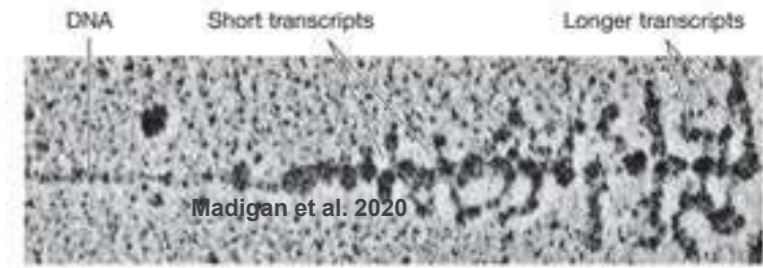


**Figure 1.23** The electron microscope. This instrument encompasses both transmission and scanning electron microscope functions.

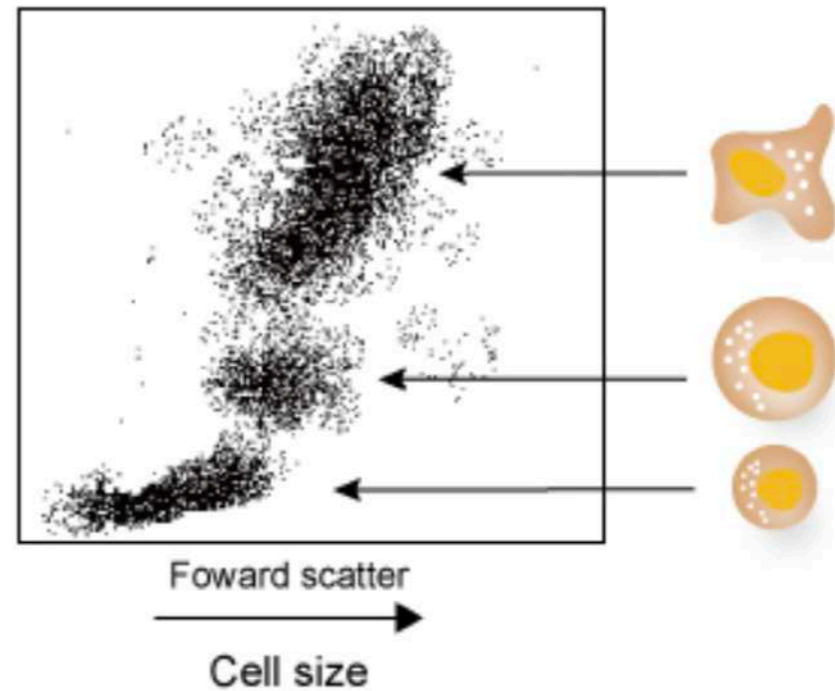
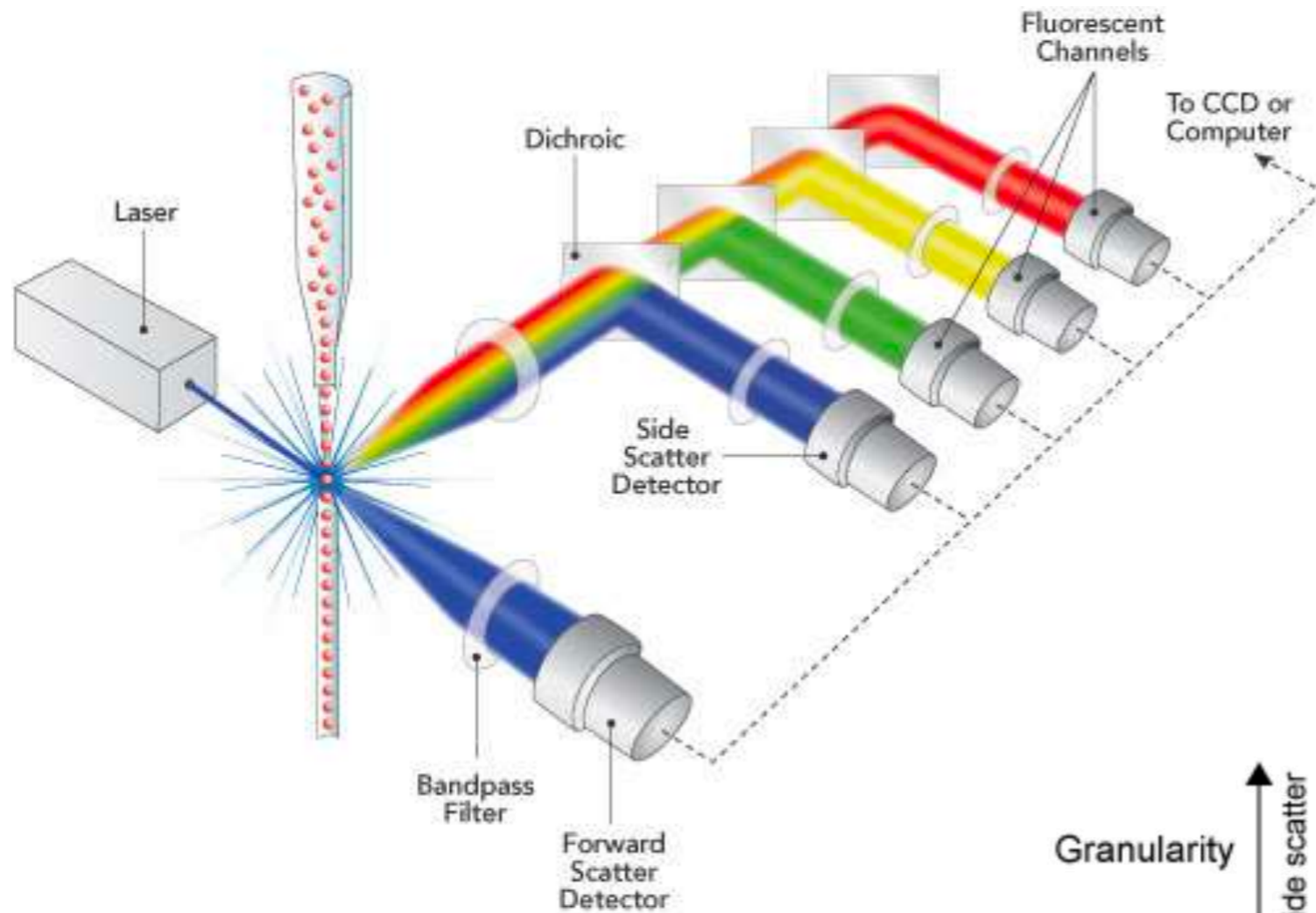
- Transmission electron microscope (TEM) is used to examine cells and cell structure at very high magnification and resolution → resolving power of a TEM is much greater than that of the light microscope → wavelength of electrons is much shorter than the wavelength of visible light → electrons are very poor at penetrating → even a single cell is too thick to penetrate with an electron beam → to view the internal structure of a cell, thin sections need to be made
- A single bacterial cell, for instance, is cut into extremely thin (20–60 nm) slices, which are then examined individually by TEM
- To obtain sufficient contrast, the sections are treated with stains such as osmic acid, or permanganate, uranium, lanthanum, or lead salts. Because these substances are composed of atoms of high atomic weight, they scatter electrons well and thus improve contrast
- Only external features of an organism are to be observed, thin sections are unnecessary. Intact cells or cell components can be observed directly in the TEM by a technique called negative staining
- Scanning Electron Microscopy (SEM) → 3D of the surface
- Specimen is coated with a thin film of a heavy metal, typically gold → an electron beam then scans back and forth across the specimen → Electrons scattered from the metal coating are collected and projected on a monitor to produce an image
- In SEM, even fairly large specimens can be observed, and the depth of field (the portion of the image that remains in sharp focus) is extremely good → wide range of magnifications from as low as 15: up to about 100,000

# Sample preparation

ESEM: environmental SEM not ultra-vacuum



# Flow cytometry



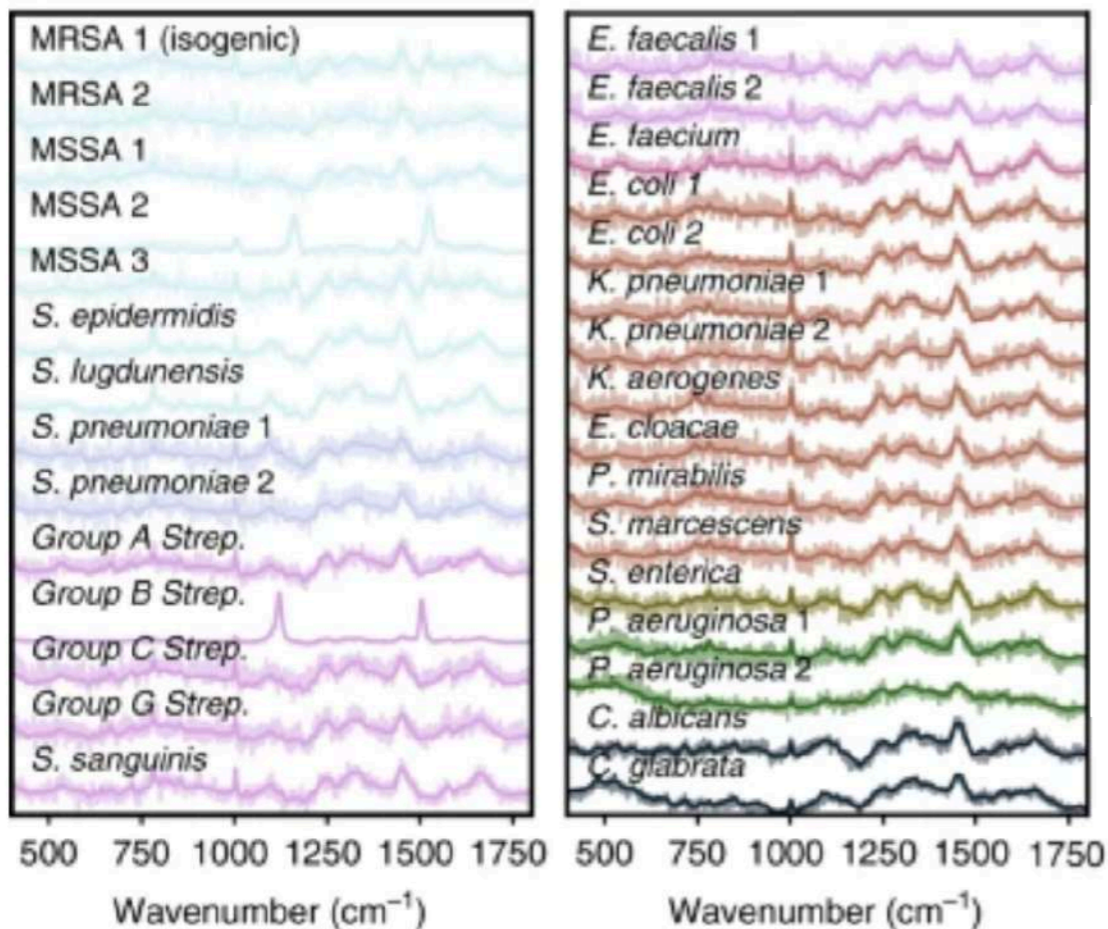
Flow cytometry permits the reliable and rapid detection of single or multiple microbes and can provide information about their distribution within cell populations, activities and identity

# Raman and IR spectroscopy

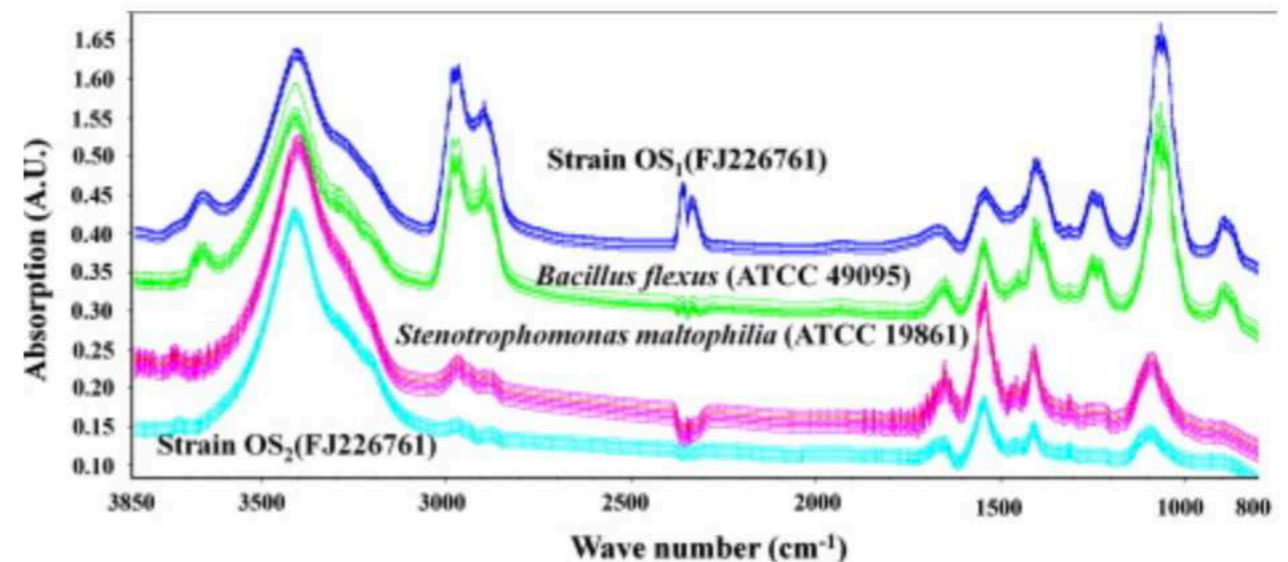
Raman and infrared (IR) are two complementary vibrational spectroscopy techniques that **enable label-free, noninvasive, and nondestructive structural characterization of analyzed specimens**

Infrared spectroscopy (IR) is used in the areas of **determination of molecular structure, identification of chemical species, quantitative/qualitative determination of chemical species, and in a host of other applications.**

## Raman



## IR



# Atomic Force Microscopy, AFM

- Scanning proximity probe microscope
- Atomic resolution
- Invented 1986 → Nobel prize
- Feel local weak forces between the samples and the sharp cantilever
- 3D imaging in vivo, minimal perturbation and artifacts

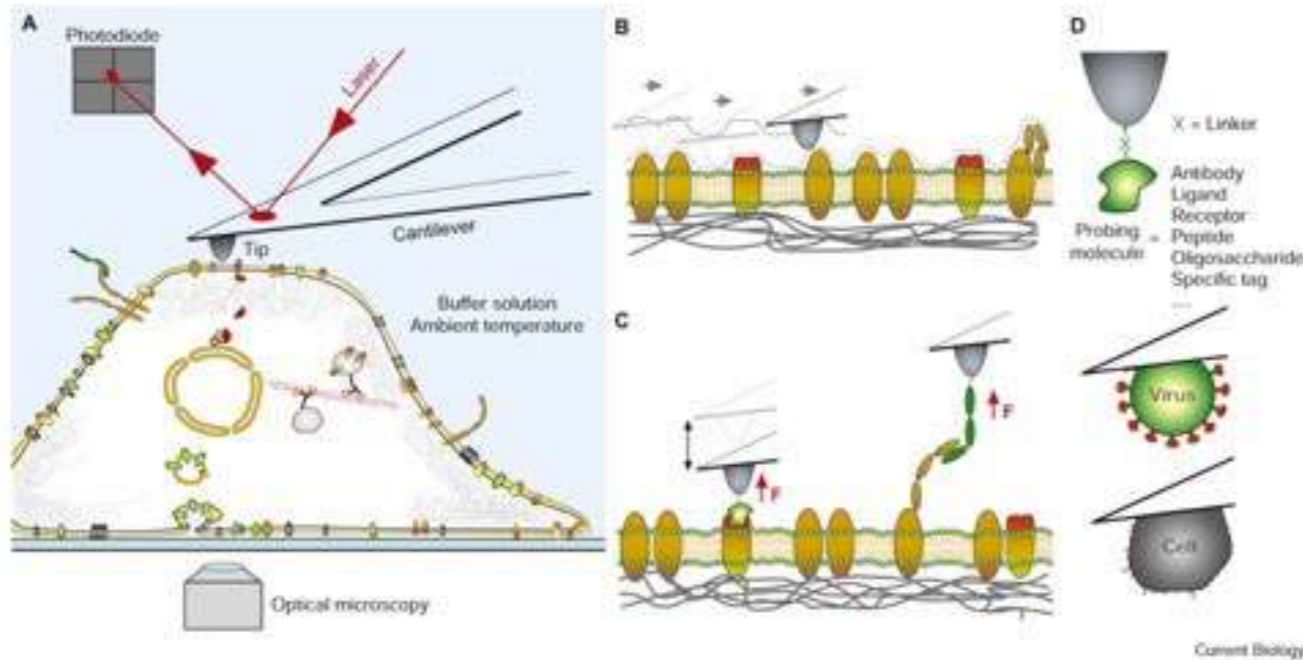
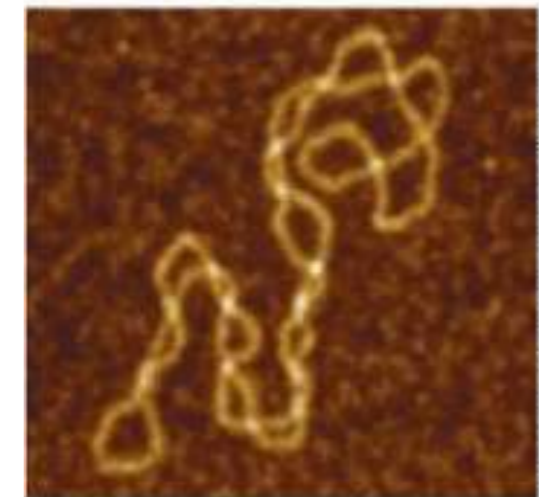
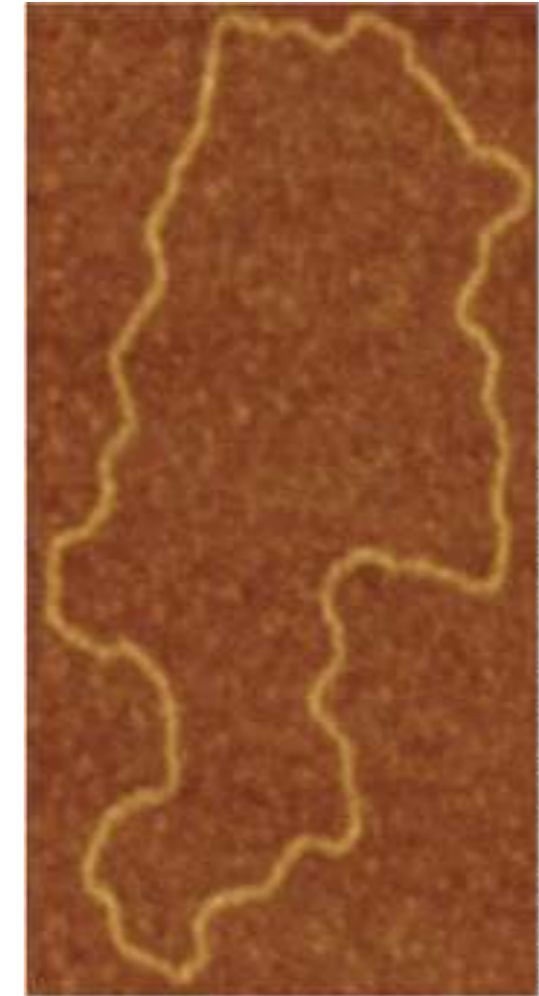
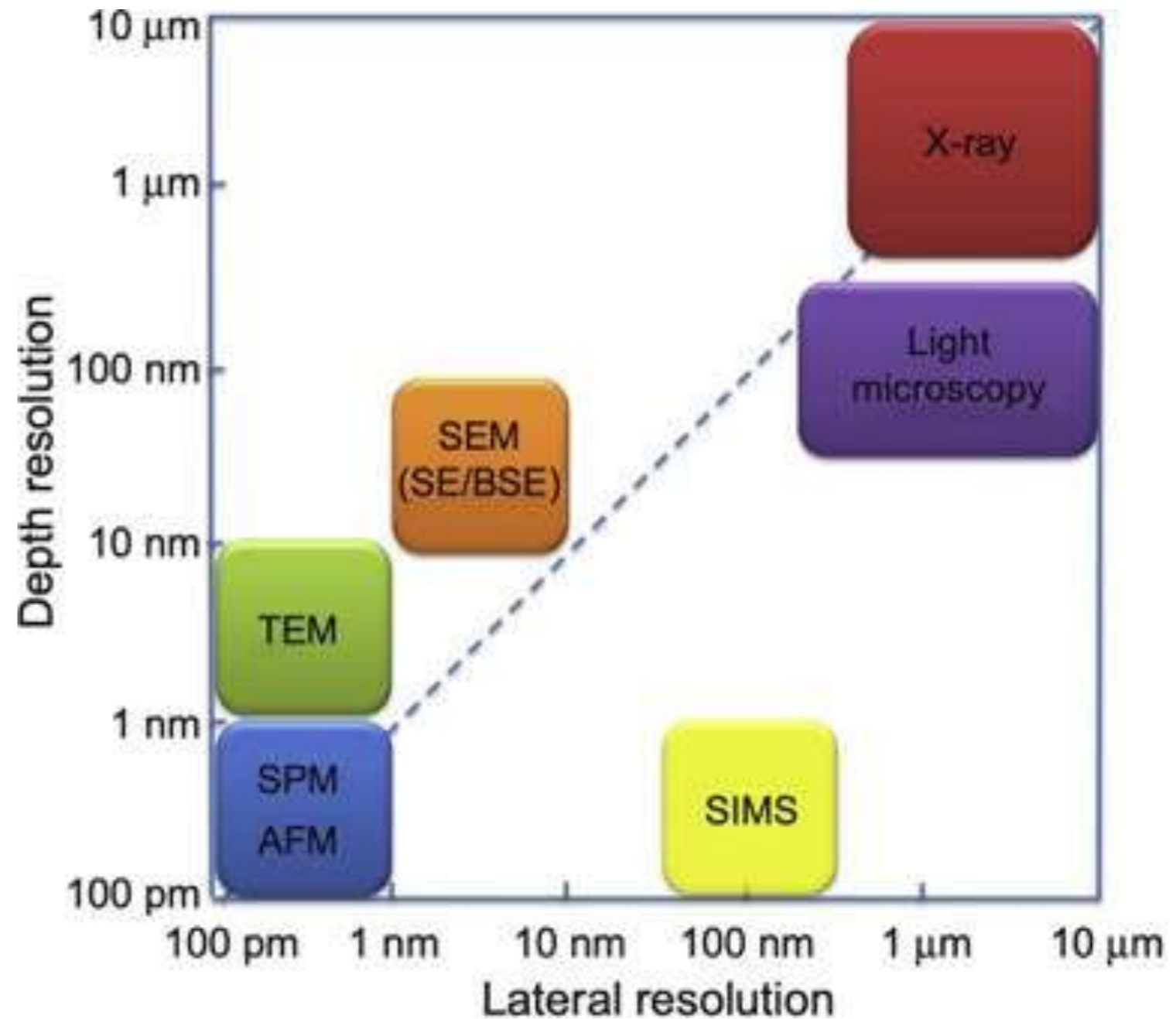


Figure 1. Structural and functional nanoscopy of the living cell. (A) The use of AFM to probe the surface of a living cell, in combination with modern optical microscopy techniques. (B) In the imaging mode, AFM contours the cell surface with nanometer resolution. (C) In force spectroscopy, the small interaction force acting between the AFM tip and the cell surface is measured while the tip approaches the cell and is retracted from the cell. (D) Various force spectroscopy modalities may be used depending on whether the tip is functionalized with biomolecules or viruses (SMFS, MRM), or replaced by a living cell (SCFS).



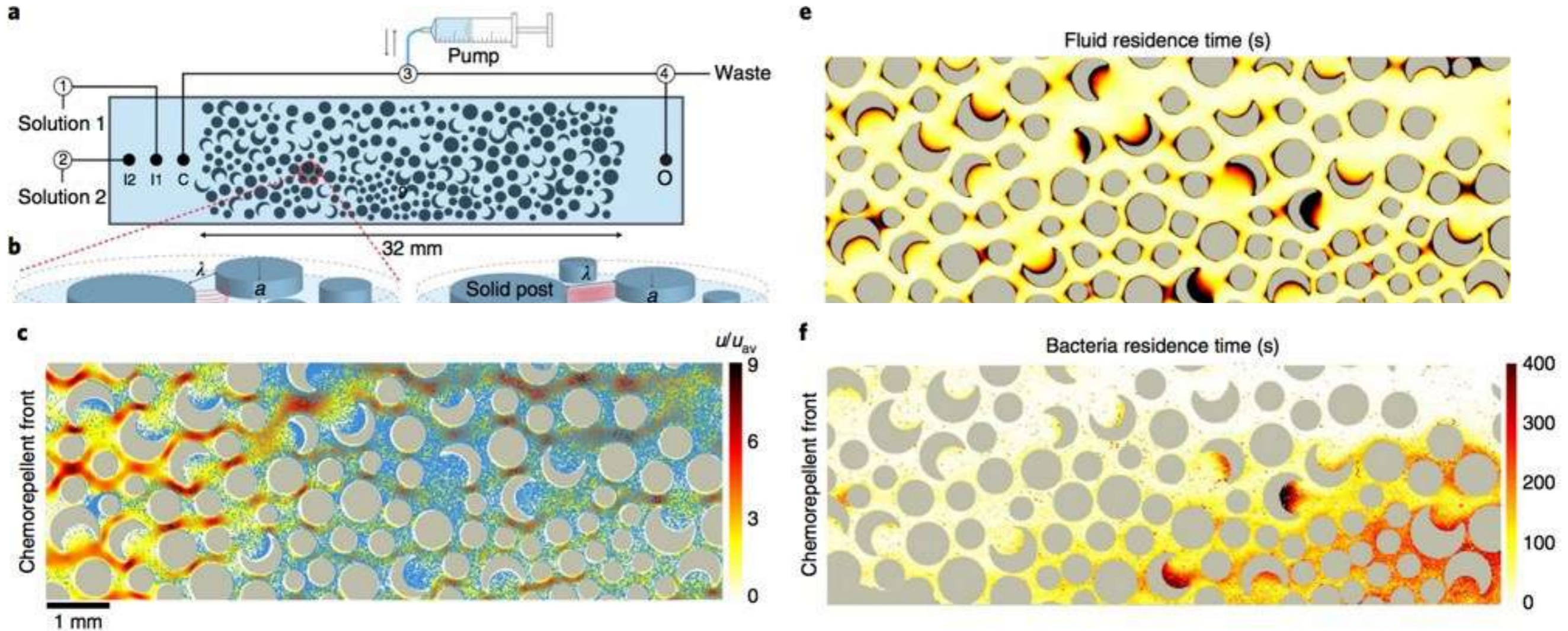
# Resolution: depth & lateral

- Particles can also behave as waves, known as wave-particle duality
- The wavelengths of electrons are dependent on their momentum, which can be changed by accelerating them through a range of voltages
- **Higher accelerating voltages produce high-energy electrons with smaller wavelengths.** Electrons with wavelengths of 40 pm down to 1 pm can be readily achieved using accelerating voltages of 1-300 kV
- Magnification range: Electron microscopes offer a very high magnification range, typically in the range 10-500,000 times for SEM, and 2000 to 1 million times for TEM
- High resolution
- Vacuum → distortion
- A microscope designed to use 200-300 keV electrons thus offers a potential step change in image spatial resolution over optical microscopy, down to less than 0.1 nm (100 pm)
- This atomic level resolution is comparable to that of the nondestructive surface imaging techniques scanning probe microscopy (SPM) and atomic force microscopy (AFM), and to that of destructive secondary ion mass spectrometry (SIMS)



# $\mu$ fluidic devices for mimicking microhabitat

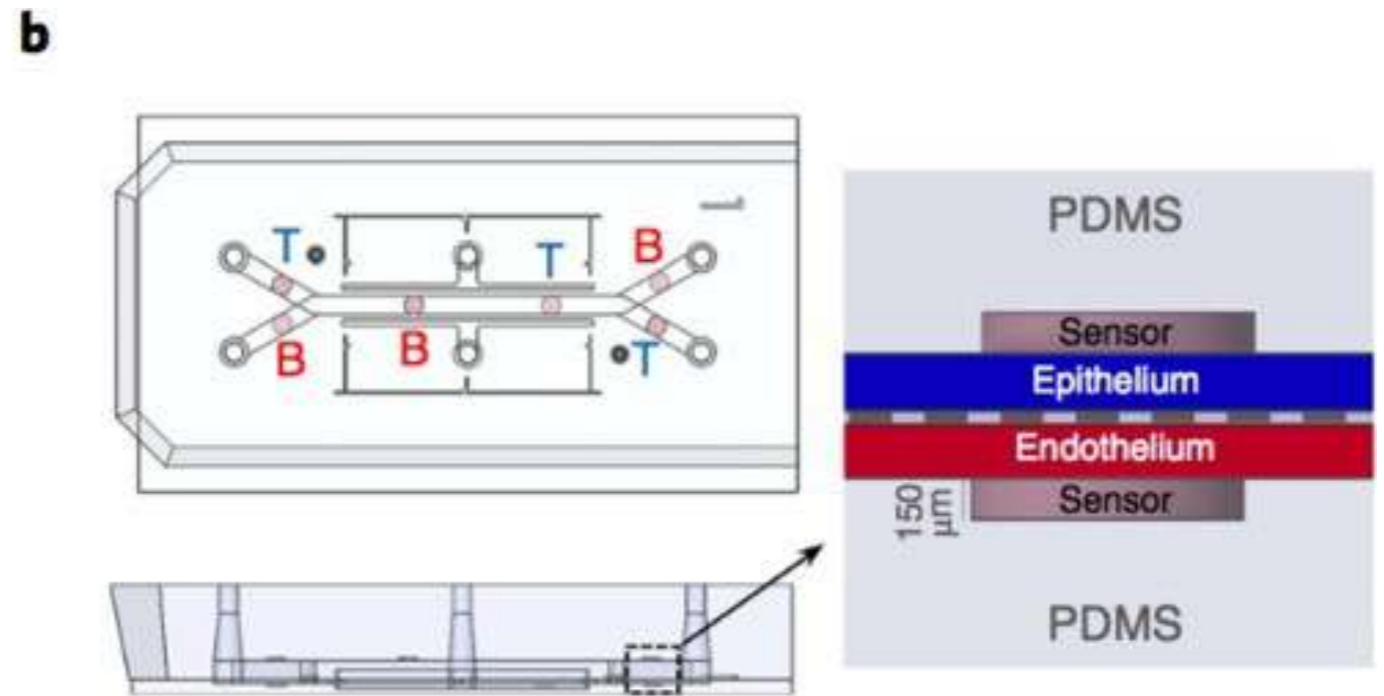
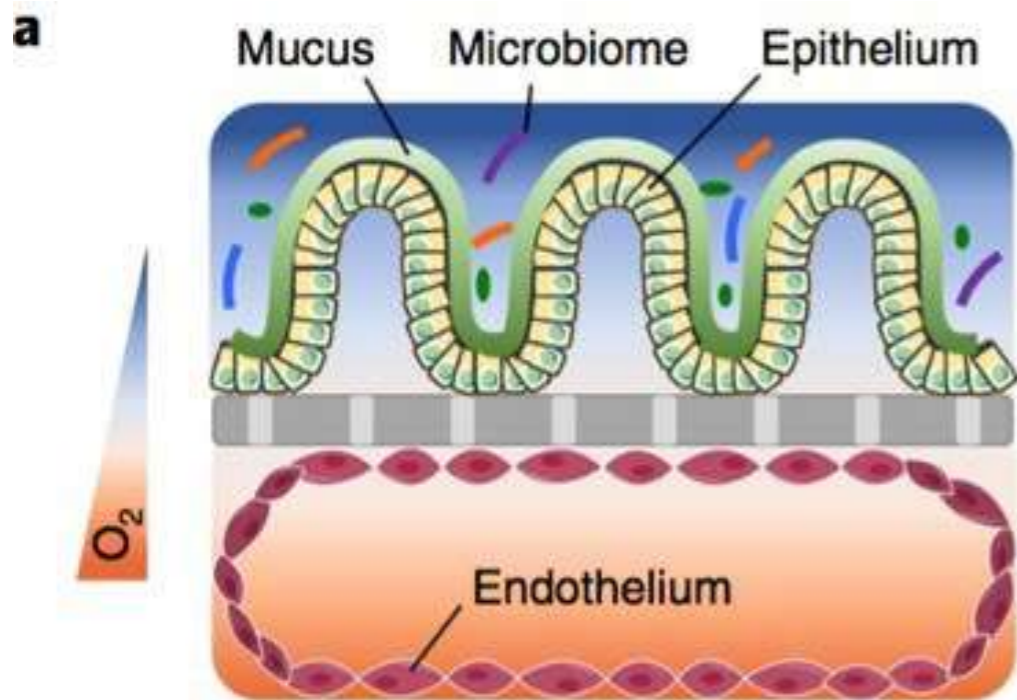
de Anna et al., 2020



*Bacillus subtilis* accumulates in micropockets w. chemorepellent and diffuse in chemoattractant in solution  
 $U_c = 0.9 \pm 0.1 \mu\text{m s}^{-1}$  (chemoattractant, tryptone),  $u_c = 1.6 \pm 0.1 \mu\text{m s}^{-1}$  (chemorepellent, spent medium)

Flow:  $Q = 0.01 \mu\text{l s}^{-1}$

# A complex human gut microbiome cultured in an anaerobic intestine-on-a-chip



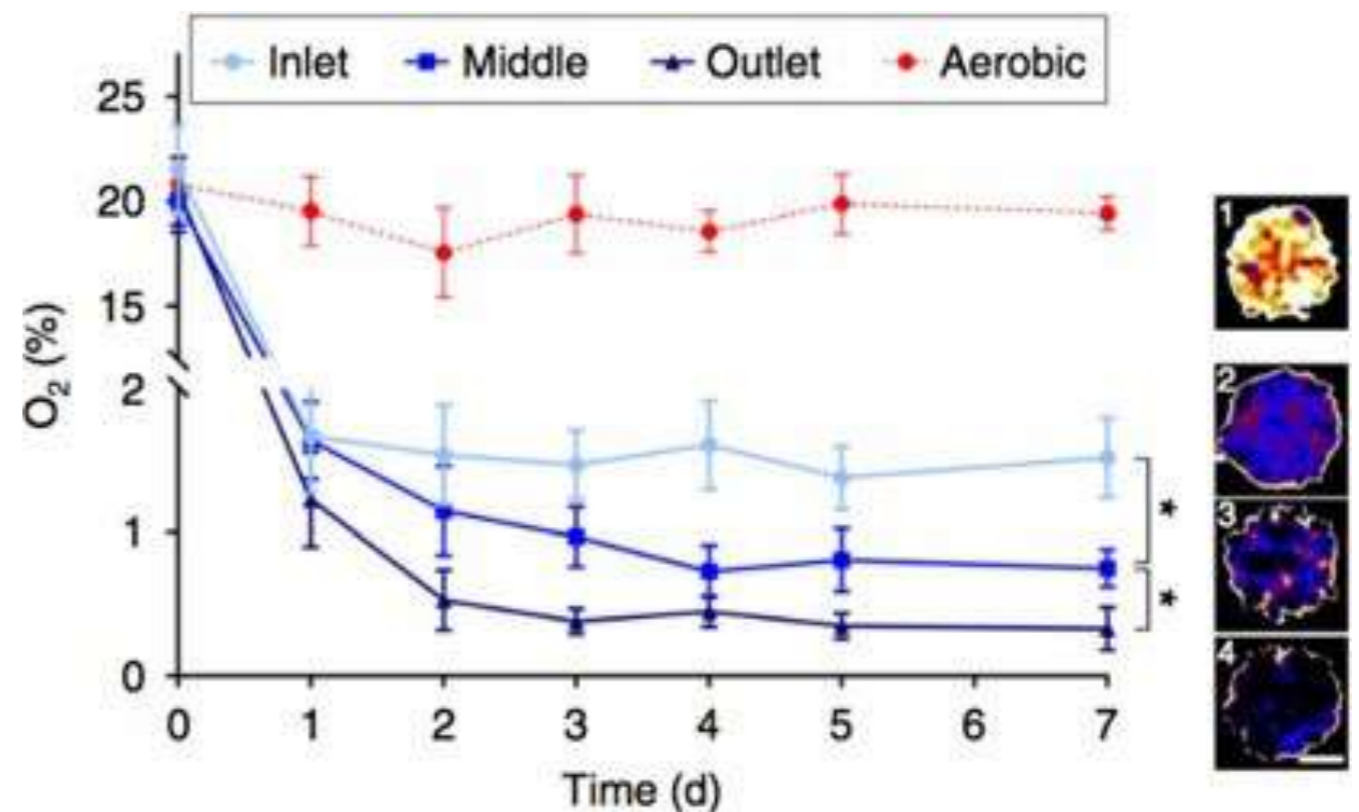
Organoid cultures showed great promise for studying host-microbiome interactions

7 days experiment

Steep gradients of oxygen

Embedded sensors

Imaging



# Controlling microbial growth

**Sterilization**—the killing or removal of all microorganisms (including viruses) —> physical methods heat, radiation, and filtration

**Filter** sterilization

**Heat Sterilization:** heat killing proceeds more rapidly as the temperature rises —> type of heat is also important: moist heat has better penetrating power than dry heat and, at a given temperature, inhibits growth or kills cells more quickly than does dry heat —> **autoclaving** at high pressure and 120°C and pasteurization

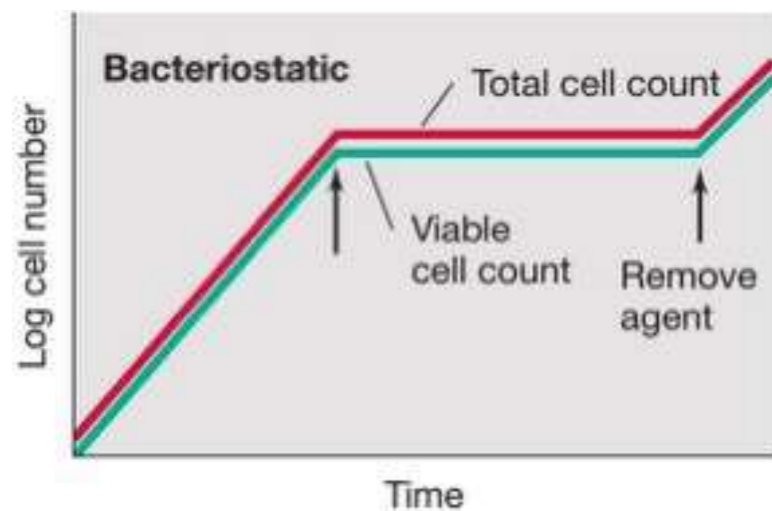
**Radiation**, in particular ultraviolet (UV) light, X-rays, and gamma rays, are also effective microbial killing agents

# Chemical control

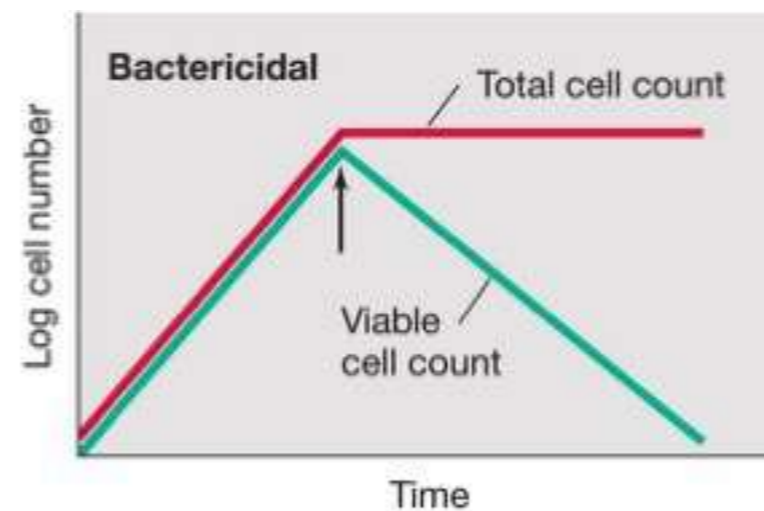
Agents that do not kill but **only inhibit growth** are called: **bacteriostatic**, fungistatic, and viristatic compounds

Agents that actually **kill** microbes: **bactericidal**, fungicidal, and viricidal agents kill bacteria, fungi, and viruses, respectively

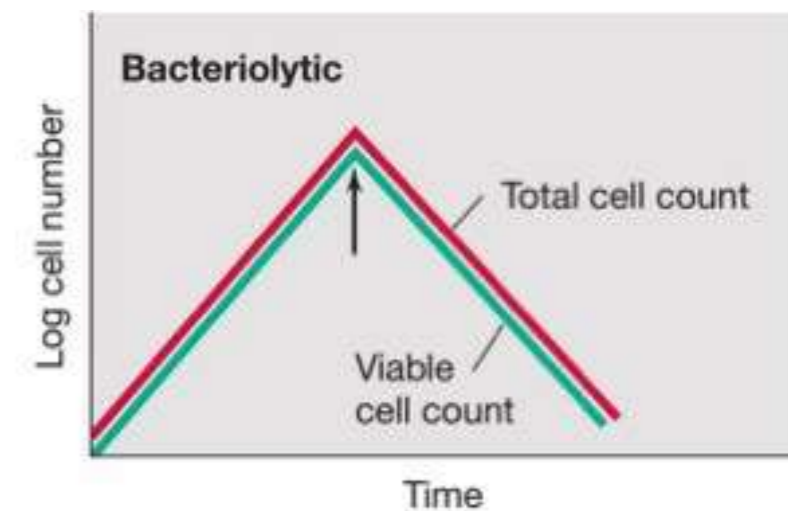
**Antibacterial agents** are classified as -static, -cidal, or -**lytic** (cell lysing) by observing their effects on cultures using viable and turbidimetric growth assays



(a)

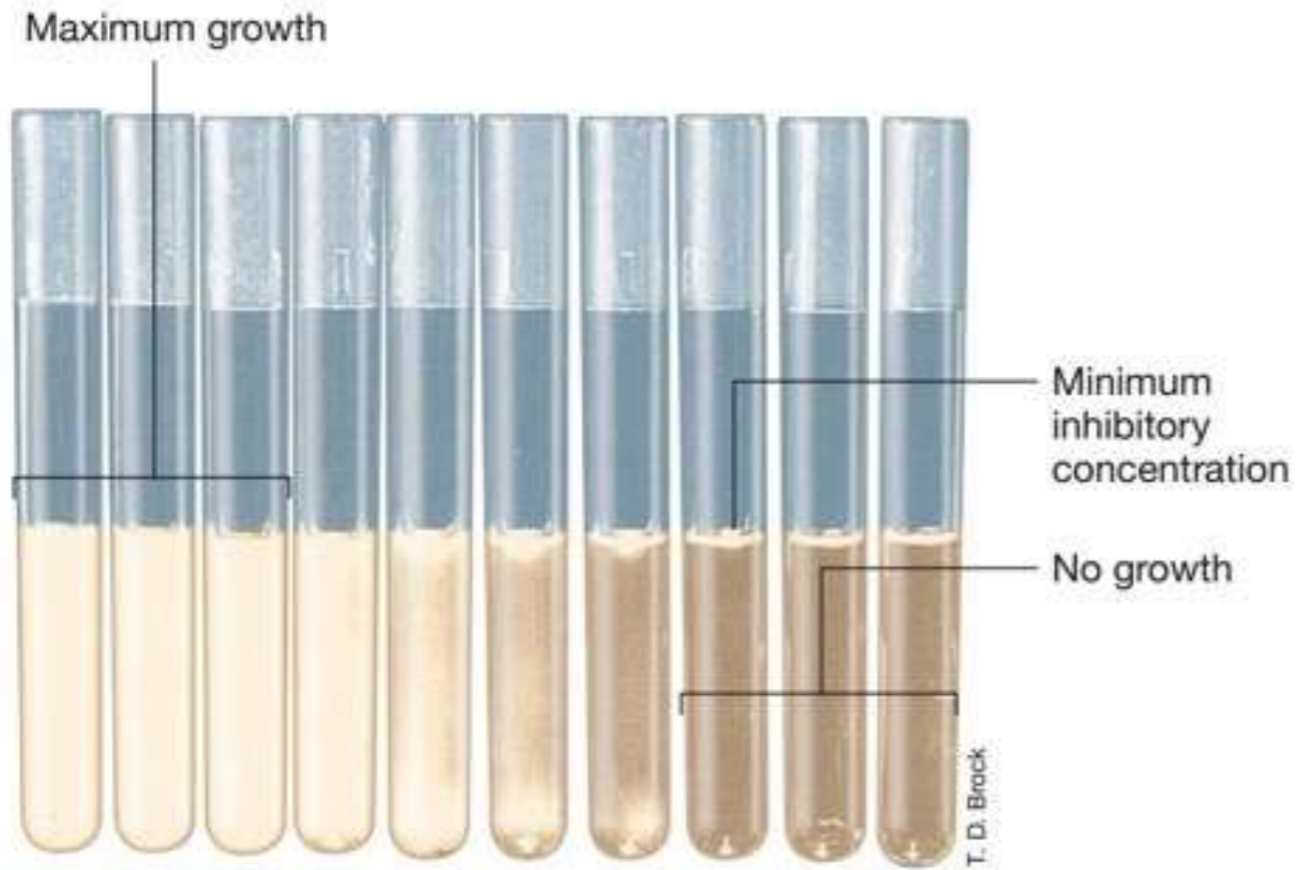


(b)



(c)

# Antibiotic testing

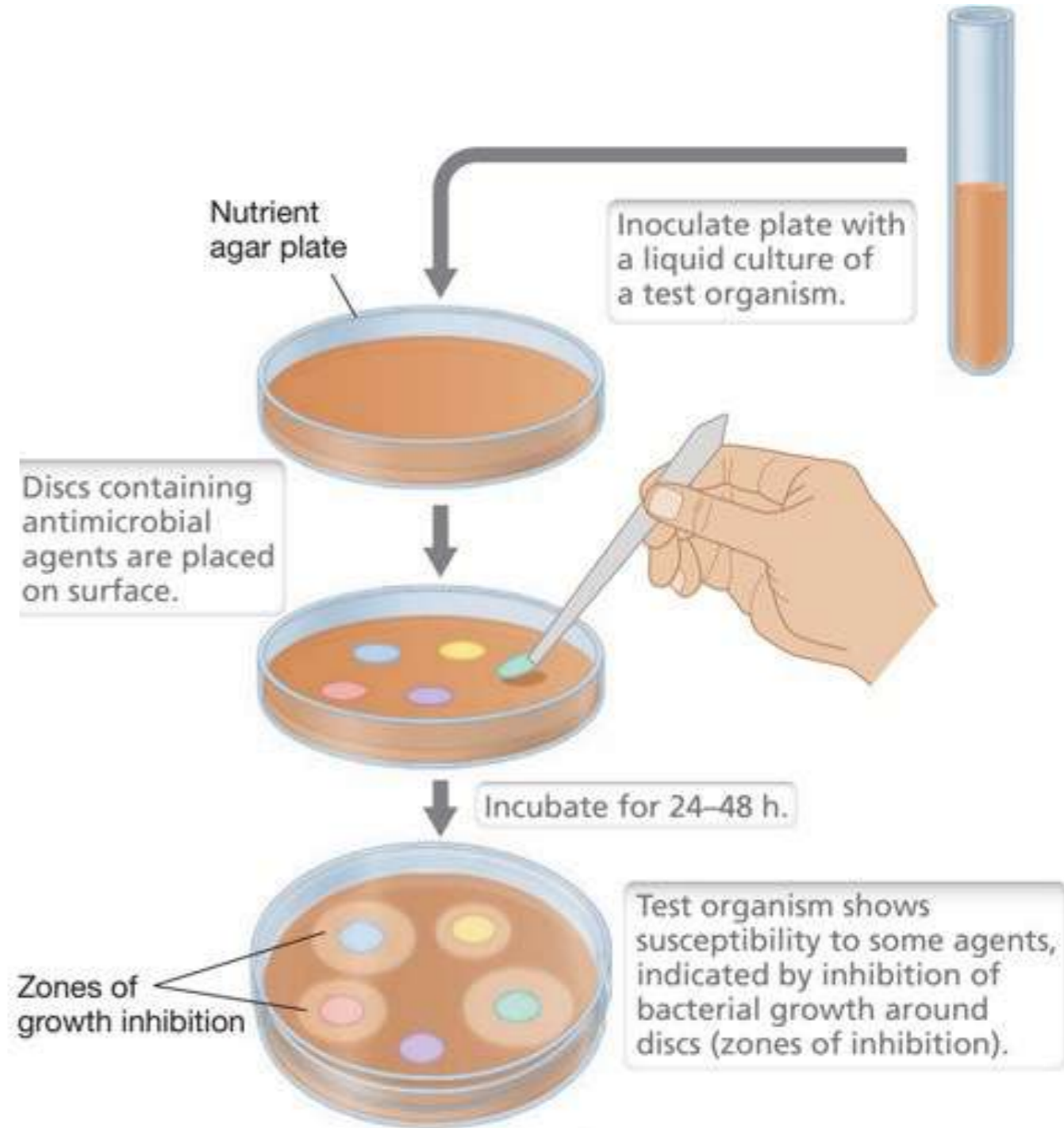


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Antimicrobial activity can be measured by determining the smallest amount to inhibit the growth of a test organism: minimum inhibitory concentration (MIC)

To determine the MIC is to inoculate a series of tubes of liquid growth medium containing a test organism and dilutions antibiotic

Paper discs are loaded with known concentration of antibiotic → over time antibiotic diffuses from the disc into the agar, establishing a gradient; the farther a chemical diffuses away from a disc, the lower its concentration → zone of growth inhibition forms around discs



# Sterilants, disinfectants, sanitizers, and antiseptics

**Sterilants** destroy **all microorganisms**, including endospores —> used for decontamination or sterilization in situations where it is impractical or impossible to use heat or radiation

**Disinfectants** are chemicals that **kill microorganisms** but not necessarily endospores and are primarily used on surfaces: phenol and cationic detergents are used to disinfect floors, tables, bench tops, walls, and so on and are important agents of infection control in hospitals and other medical settings

**Sanitizers**, by contrast, are less harsh than disinfectants and reduce microbial numbers but do not sterilize —> widely used in the food industry to treat surfaces

**Antiseptics**, often called **germicides**, are chemicals that kill or inhibit the growth of microorganisms but are sufficiently nontoxic to animals to be applied to living tissues

Most germicides are used for hand washing or for treating surface wounds

Certain antiseptics are also effective disinfectants: ethanol can be both an antiseptic and a disinfectant, depending on the concentration and exposure time employed

Several factors affect the efficacy of any chemical antimicrobial agent

**The ultimate efficacy of any antimicrobial agent must be determined empirically and under the actual conditions of use**

**Only by actually testing the chemical and assaying for microbial growth both before and after treatment can one be confident that the agent is working as it should**

**TABLE 5.8 Antiseptics, sterilants, disinfectants, and sanitizers<sup>a</sup>**

<i>Agent</i>	<i>Mode of action</i>	<i>Use</i>
<b>Antiseptics (germicides)</b>		
Alcohol (60–85% ethanol or isopropanol in water)	Lipid solvent and protein denaturant	Topical antiseptic
Phenol-containing compounds (hexachlorophene, triclosan, chloroxylenol, chlorhexidine)	Disrupts cytoplasmic membrane	Soaps, lotions, cosmetics, deodorants, topical disinfectants; paper, leather, and textile industries
Cationic detergents, especially quaternary ammonium compounds (benzalkonium chloride)	Disrupts cytoplasmic membrane	Soaps, lotions, topical disinfectants; metal and petroleum industries
Hydrogen peroxide (3% solution)	Oxidizing agent	Topical antiseptic
Iodophors (Betadine <sup>®</sup> )	Iodates proteins, rendering them nonfunctional; oxidizing agent	Topical antiseptic
Octenidine	Cationic surfactant, disrupts cytoplasmic membrane	Topical antiseptic
<b>Sterilants, disinfectants, and sanitizers</b>		
Alcohol (60–85% ethanol or isopropanol in water)	Lipid solvent and protein denaturant	General-purpose disinfectant for virtually any surface
Cationic detergents (quaternary ammonium compounds, Lysol <sup>®</sup> and many related disinfectants)	Interact with phospholipids	Disinfectant/sanitizer for medical instruments, food and dairy equipment
Chlorine gas	Oxidizing agent	Disinfectant for drinking water and electrical/nuclear cooling towers
Chlorine compounds (chloramines, sodium hypochlorite, sodium chlorite, chlorine dioxide)	Oxidizing agent	Disinfectant/sanitizer for medical instruments, food/dairy equipment, and in water purification
Copper sulfate	Protein precipitant	Algicide in swimming pools
Ethylene oxide (gas)	Alkylating agent	Sterilant for temperature-sensitive materials such as plastics
Formaldehyde	Alkylating agent	Diluted (3% solution) as surface disinfectant/sterilant; concentrated (37% solution) as sterilant
Glutaraldehyde	Alkylating agent	Disinfectant or sterilant as 2% solution
Hydrogen peroxide	Oxidizing agent	Vapor used as sterilant
Iodophors (Wescodyne <sup>®</sup> )	Iodates proteins; oxidizing agent	General disinfectant
OPA (ortho-phthalaldehyde)	Alkylating agent	Powerful disinfectant used for sterilizing medical instruments
Ozone	Strong oxidizing agent	Disinfectant for drinking water
Peroxyacetic acid	Strong oxidizing agent	Disinfectant/sterilant
Phenolic compounds	Protein denaturant	General-purpose disinfectant
Pine oils (Pine-Sol <sup>®</sup> ) (contains phenolics and detergents)	Protein denaturant	General-purpose disinfectant for household surfaces


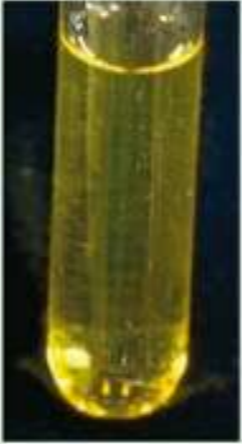
<sup>a</sup>Alcohols, hydrogen peroxide, and iodophors can be antiseptics, disinfectants, sanitizers, or sterilants depending on concentration, length of exposure, and form of delivery.



# Culture media liquid & solid

## Autoclaving of media

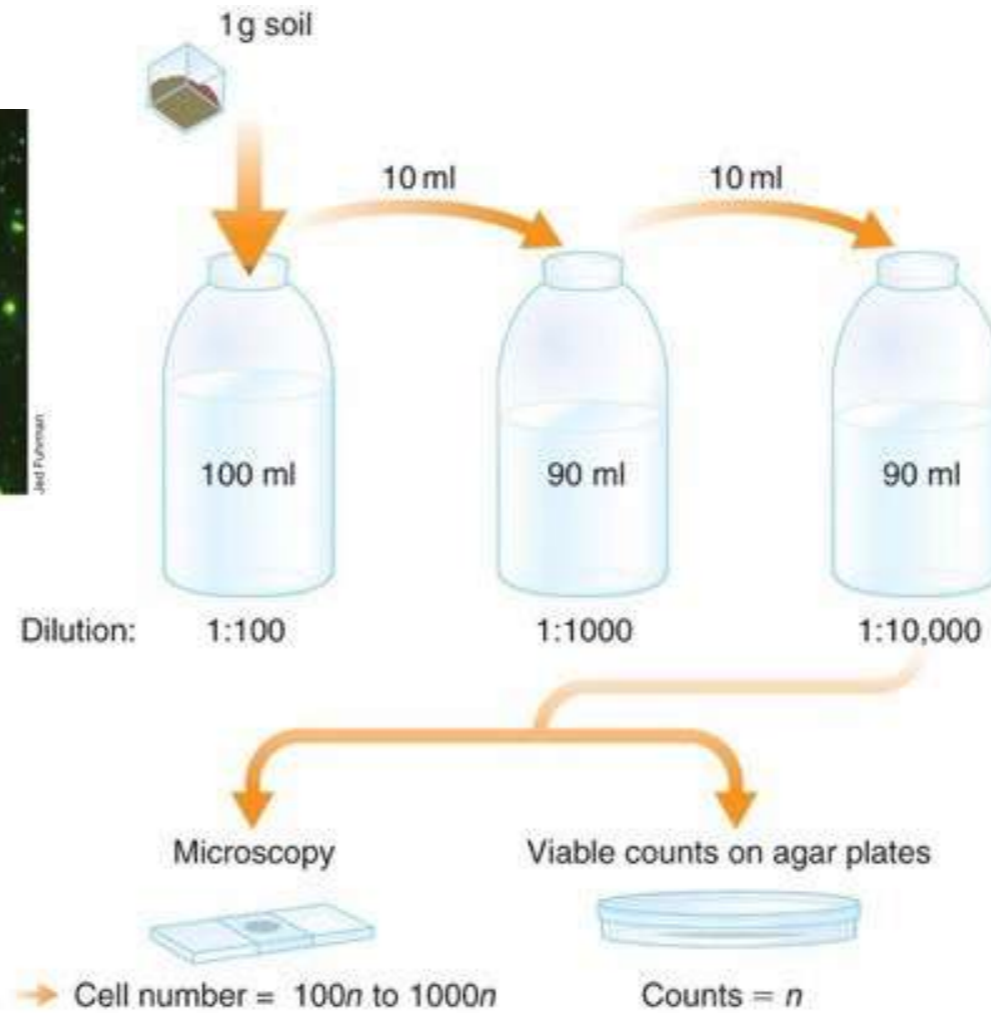
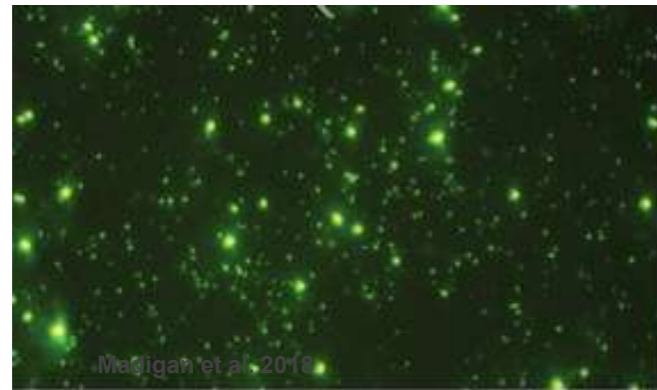
**TABLE 5.1** Examples of culture media for microorganisms with simple and demanding nutritional requirements<sup>a</sup>

Defined culture medium for <i>Escherichia coli</i>	Defined culture medium for <i>Leuconostoc mesenteroides</i>	Complex culture medium for either <i>E. coli</i> or <i>L. mesenteroides</i>	Defined culture medium for <i>Thiobacillus thioparus</i>
<p>K<sub>2</sub>HPO<sub>4</sub> 7 g                      KH<sub>2</sub>PO<sub>4</sub> 2 g                      (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> 1 g                      MgSO<sub>4</sub> 0.1 g                      CaCl<sub>2</sub> 0.02 g                      Glucose 4–10 g                      Trace elements (Fe, Co, Mn, Zn, Cu, Ni, Mo) 2–10 µg each                      Distilled water 1000 ml                      pH 7</p>  <p>(a)</p>	<p>K<sub>2</sub>HPO<sub>4</sub> 0.6 g                      KH<sub>2</sub>PO<sub>4</sub> 0.6 g                      NH<sub>4</sub>Cl 3 g                      MgSO<sub>4</sub> 0.1 g                      Glucose 25 g                      Sodium acetate 25 g                      Amino acids (alanine, arginine, asparagine, aspartate, cysteine, glutamate, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine) 100–200 µg of each                      Purines and pyrimidines (adenine, guanine, uracil, xanthine) 10 mg of each                      Vitamins (biotin, folate, nicotinic acid, pyridoxal, pyridoxamine, pyridoxine, riboflavin, thiamine, pantothenate, p-aminobenzoic acid) 0.01–1 mg of each                      Trace elements (as in first column) 2–10 µg each                      Distilled water 1000 ml                      pH 7</p>	<p>Glucose 15 g                      Yeast extract 5 g                      Peptone 5 g                      KH<sub>2</sub>PO<sub>4</sub> 2 g                      Distilled water 1000 ml                      pH 7</p>  <p>(b)</p>	<p>KH<sub>2</sub>PO<sub>4</sub> 0.5 g                      NH<sub>4</sub>Cl 0.5 g                      MgSO<sub>4</sub> 0.1 g                      CaCl<sub>2</sub> 0.05 g                      KCl 0.5 g                      Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> 2 g                      Trace elements (as in first column) 2–10 µg each                      Distilled water 1000 ml                      pH 7                      Carbon source: CO<sub>2</sub> from air</p>

<sup>a</sup>The photos are tubes of (a) the defined medium described, and (b) the complex medium described. Note how the complex medium is colored from the various organic extracts and digests that it contains. Photo credits: Cheryl L. Broadie and John Vercillo, Southern Illinois University at Carbondale.

# Cell abundance estimation

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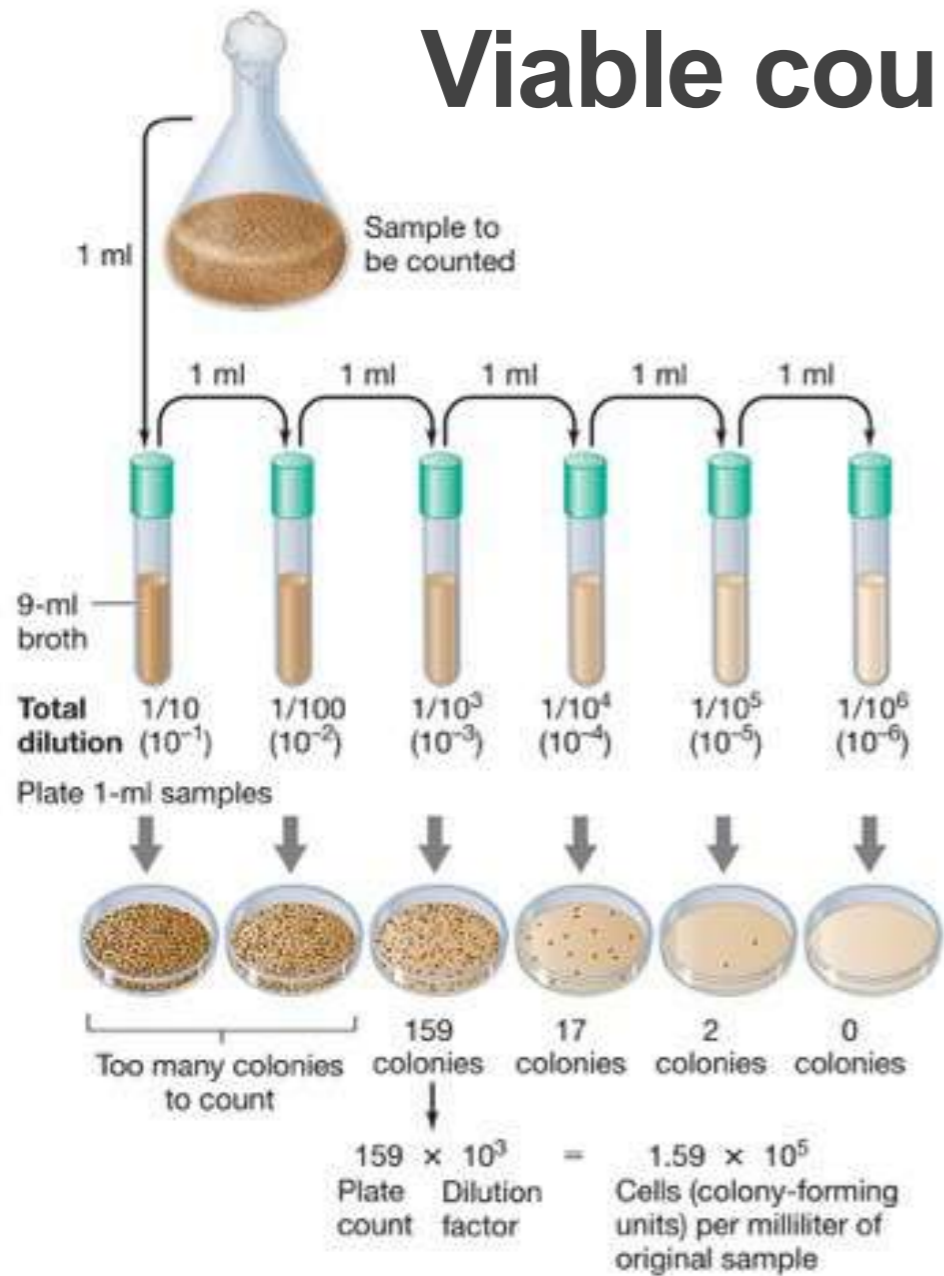
Microscopy-based counting  
Dead/living cells

Plate-based counting Selection  
due to medium, temperature  
and antagonisms

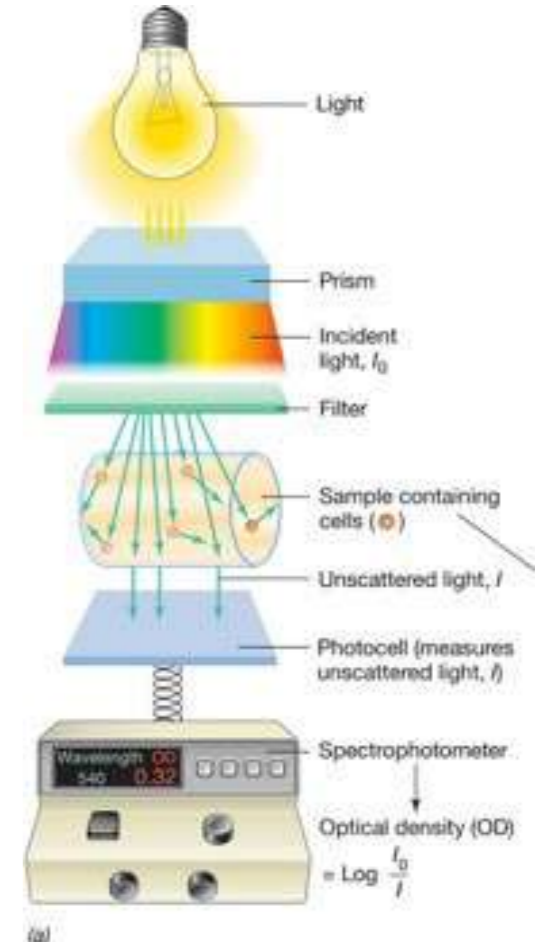
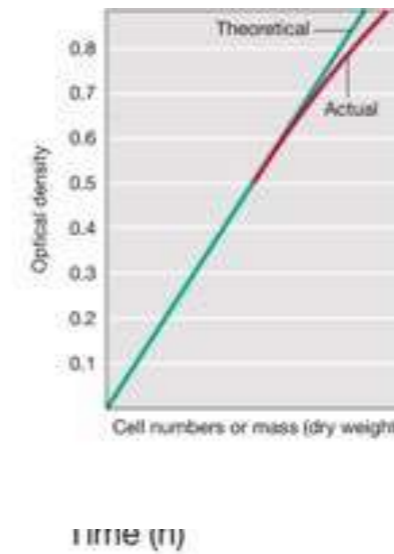
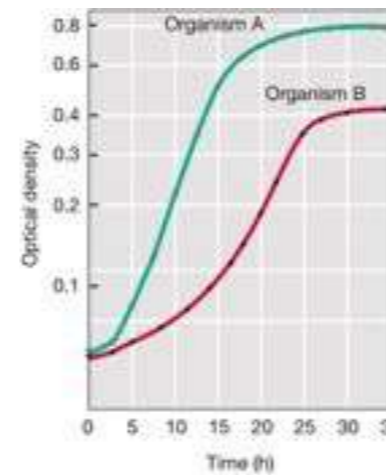
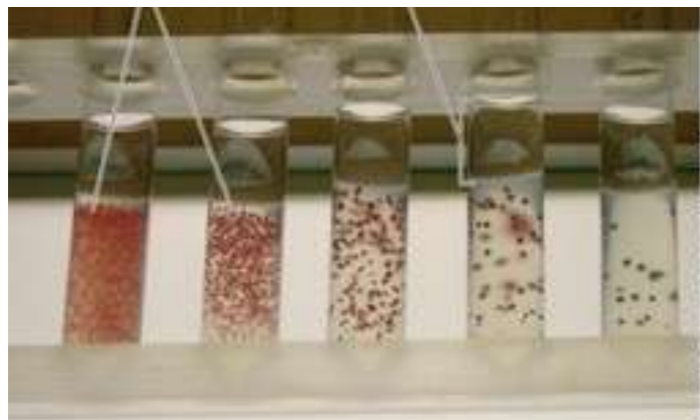
**Figure 5.2** Preparation of soil dilutions in sterile buffer. The contrasts between the number of microscopic microorganisms and the number that grow on solid agar media has been termed the “great plate count anomaly”.

*Environmental Microbiology: From Genomes to Biogeochemistry*, Second Edition, Eugene L. Madsen.  
© 2016 Eugene L. Madsen. Published 2016 by John Wiley & Sons, Inc.  
Companion Website: [www.wiley.com/go/madsen/enviromicrobio2e](http://www.wiley.com/go/madsen/enviromicrobio2e)

# Viable counting of cultural microbes



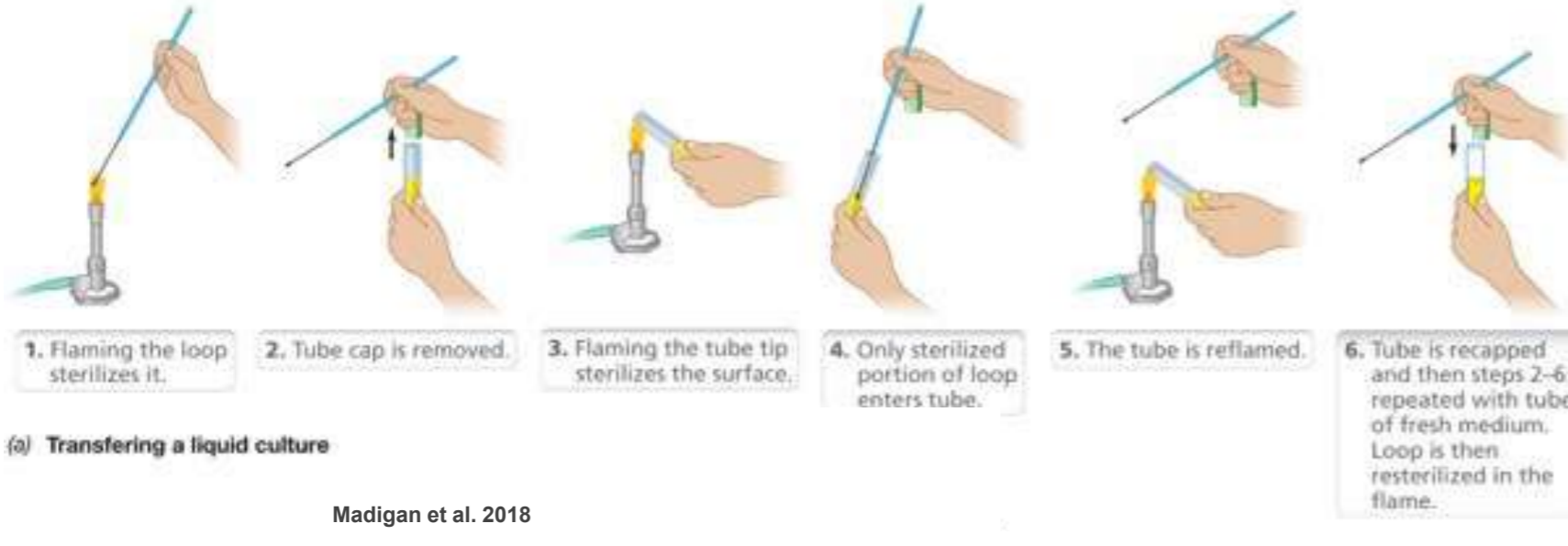
**Figure 5.15** Procedure for viable counting using serial dilutions of the sample and the pour-plate method. The sterile liquid used for making dilutions can simply be water, but a solution of mineral salts or actual growth medium may yield a higher recovery. The dilution factor is the reciprocal of the dilution.



OD <sub>540</sub>	0	0.18	0.45	0.68
Cells/ml (plate count)	0	$1.3 \times 10^8$	$3.3 \times 10^8$	$5 \times 10^8$

(d)

# Working with bacterial cultures

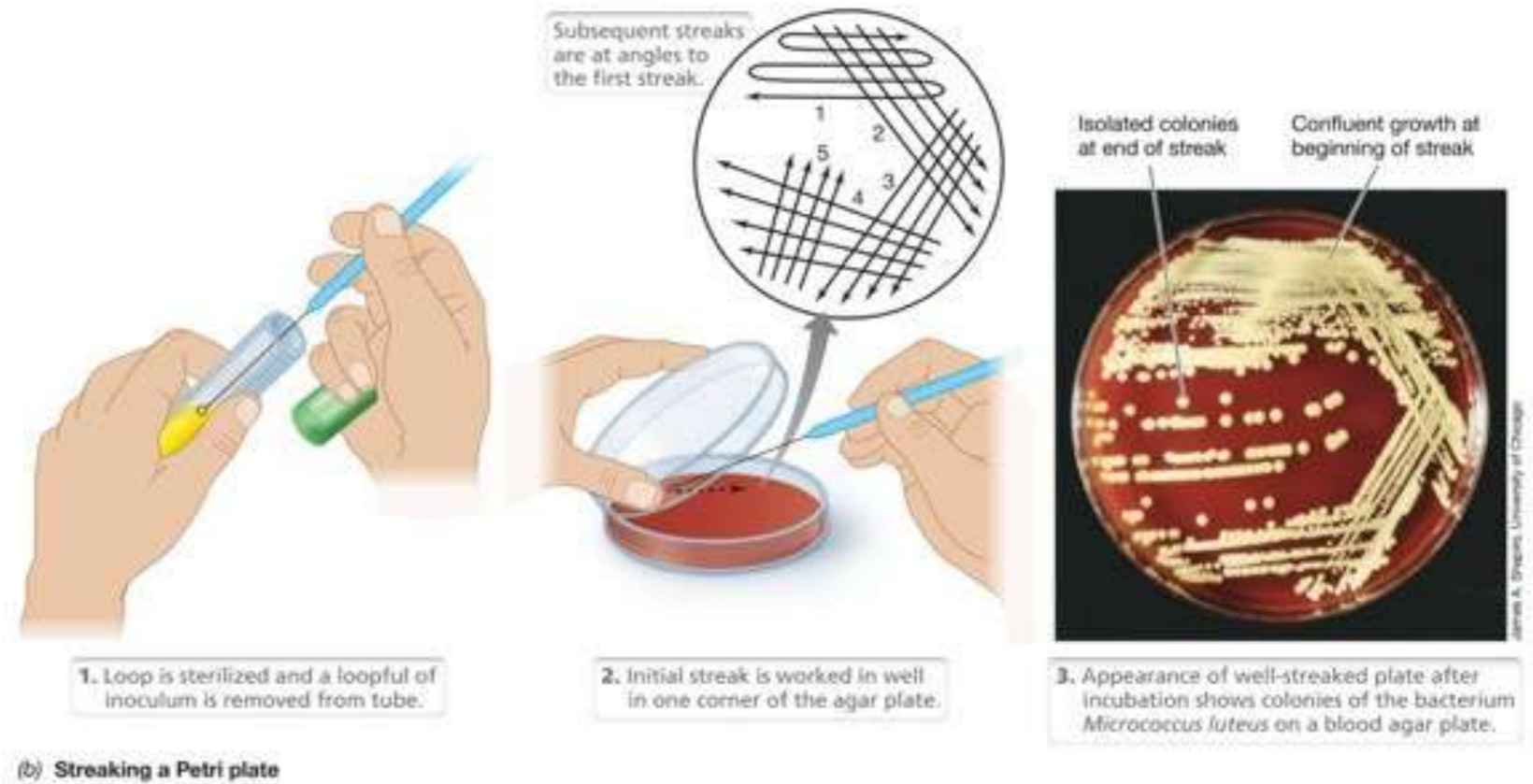


(a) Transferring a liquid culture

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(a) Transferring a liquid culture

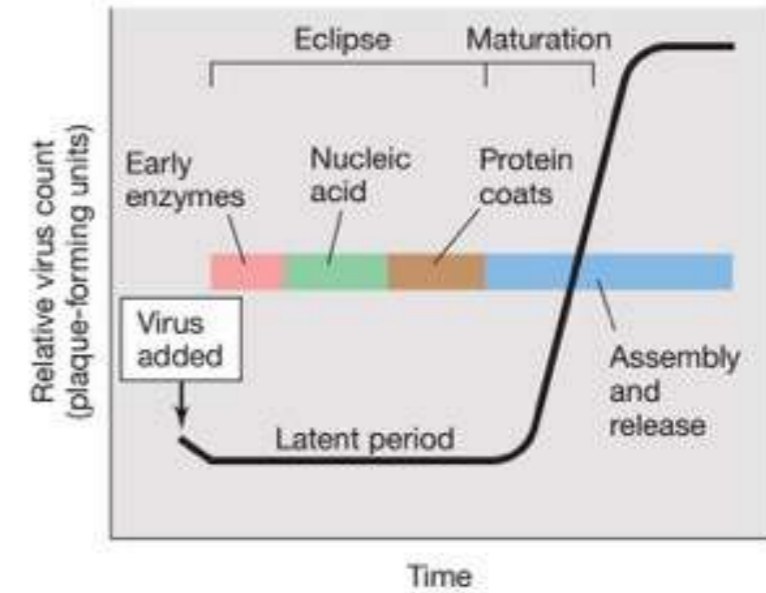
- Frozen bacterial culture stock at -80°C
- Glycerol, cryoprotectant
- Minimizing mutations
- One single colony, one clonal line



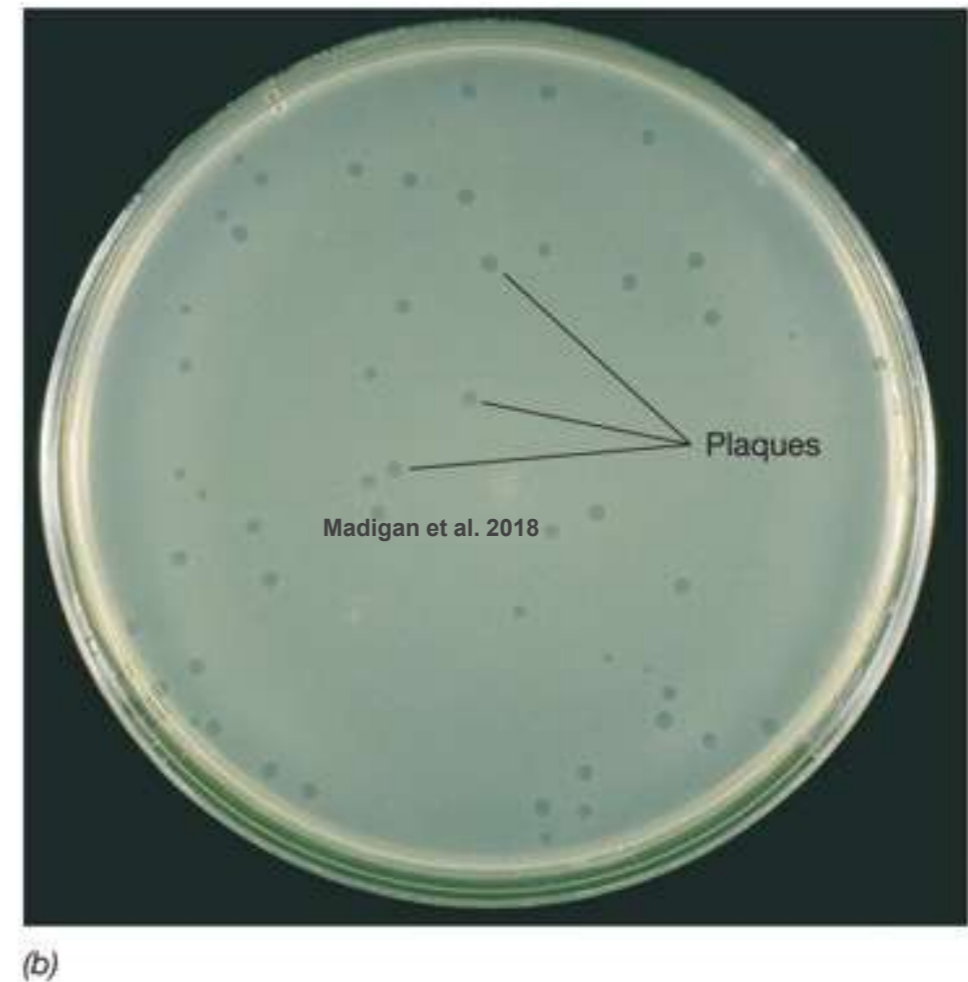
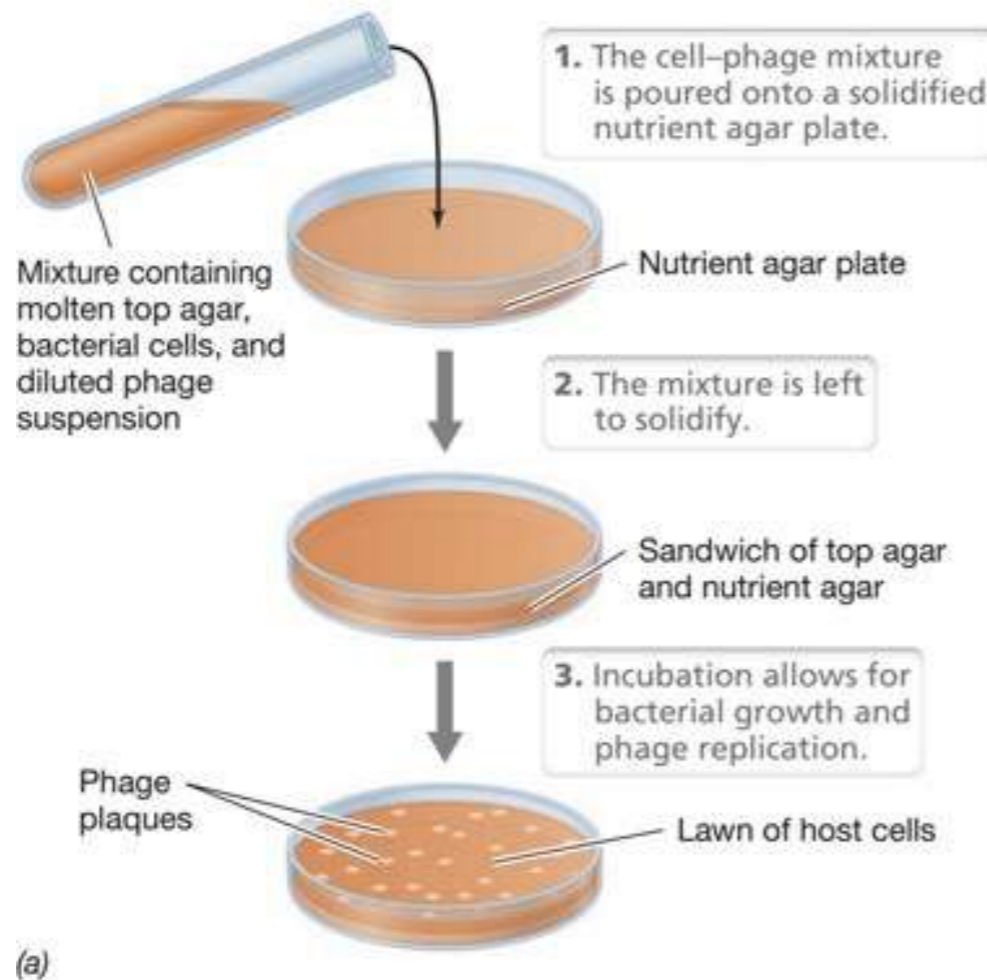
(b) Streaking a Petri plate

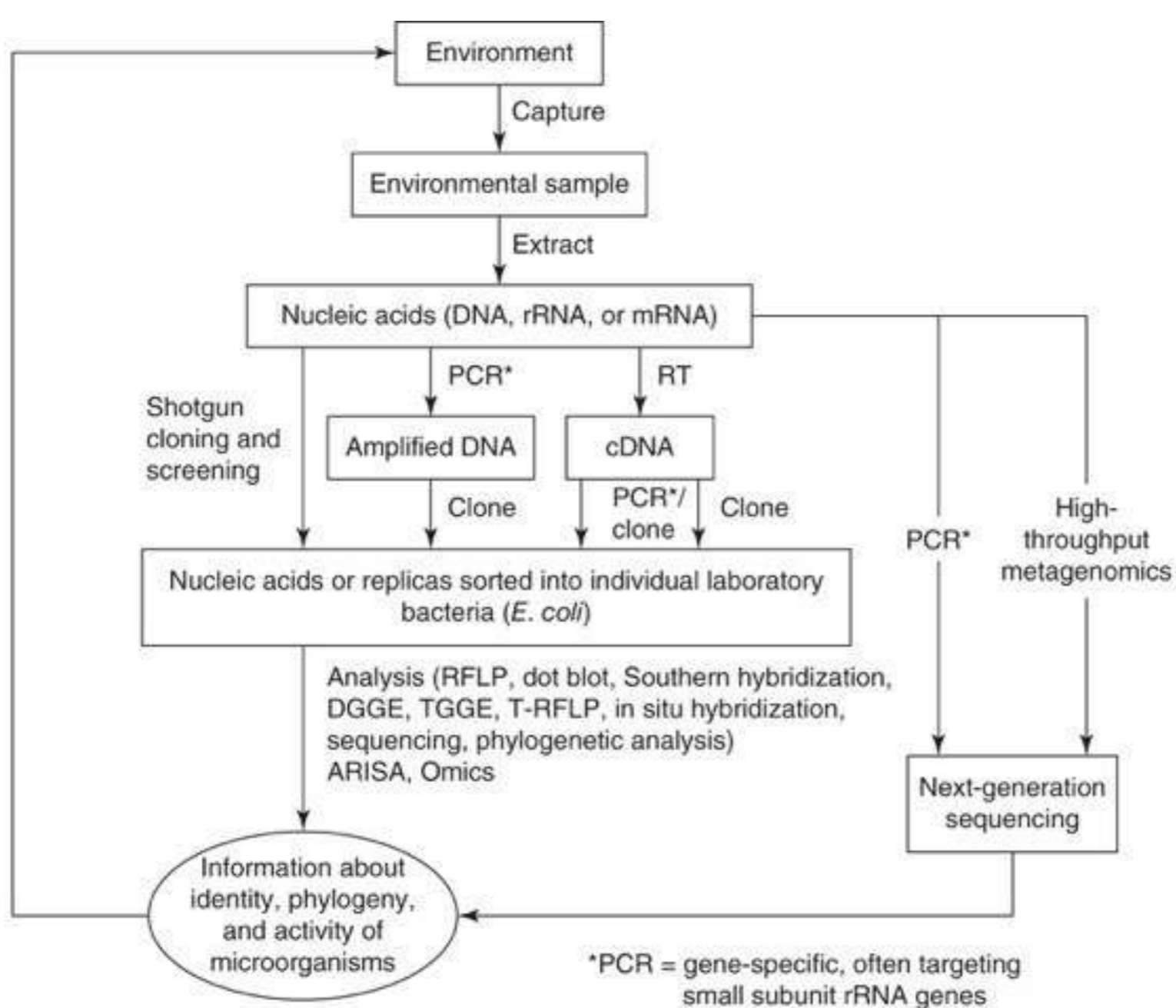
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# Isolating phages



**Figure 8.8 One-step growth curve of virus replication.** Following adsorption, infectious virions cannot be detected in the growth medium, a phenomenon called *eclipse*. During the latent period, which includes the eclipse and early maturation phases, viral nucleic acid replicates and protein synthesis occurs. During the maturation period, virus nucleic acid and protein are assembled into mature virions and then released.



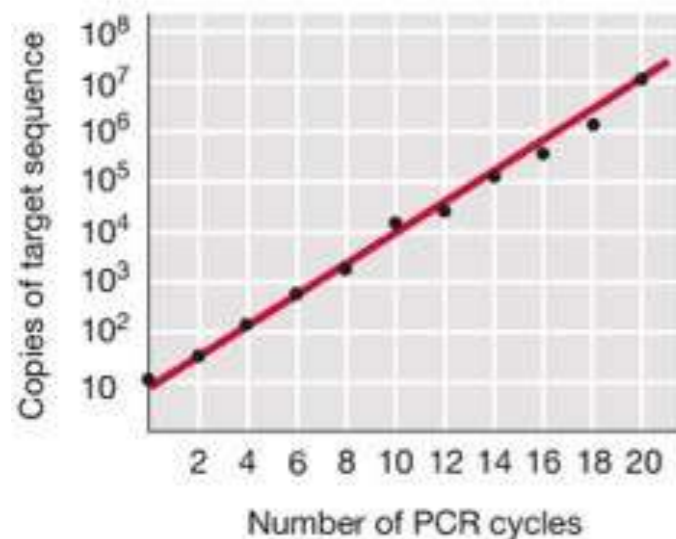
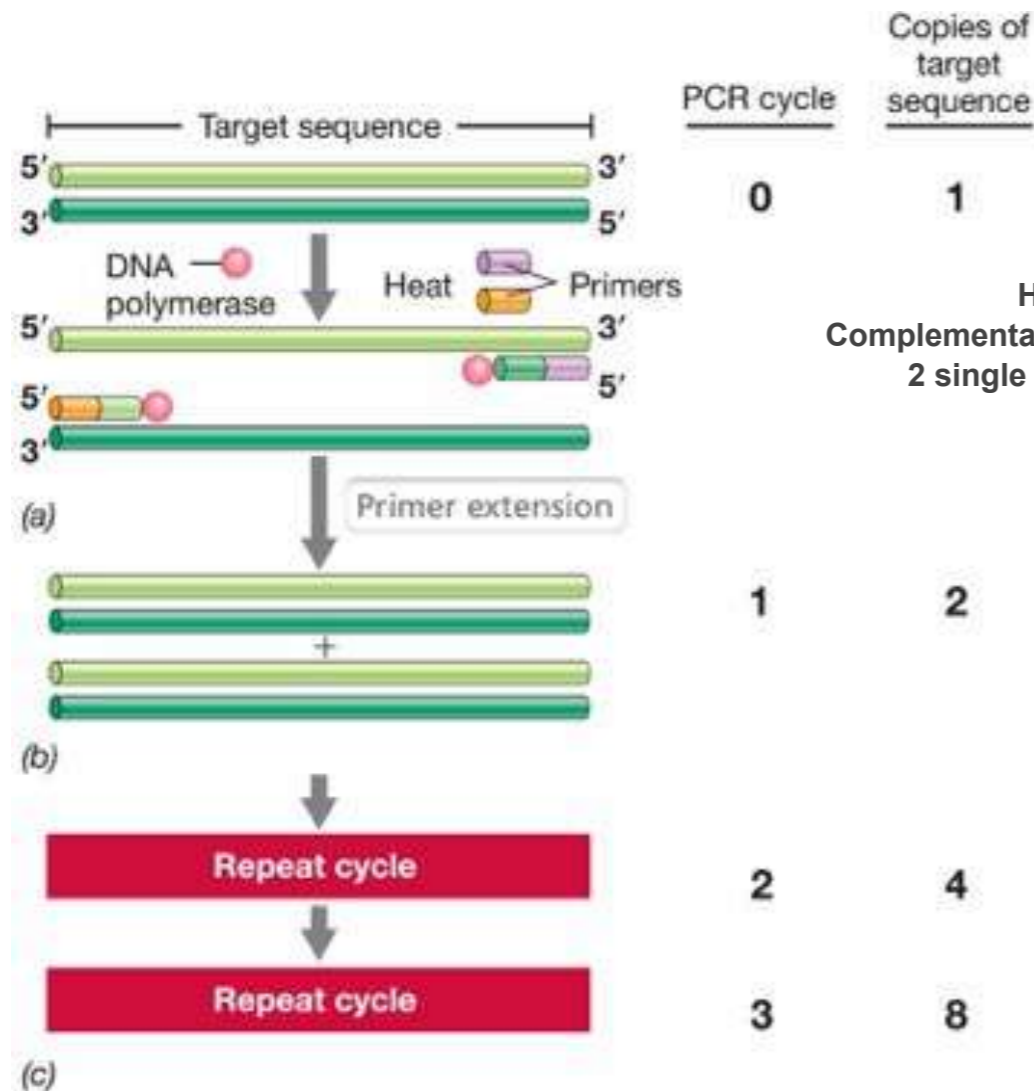


**Figure 6.7** Stepwise scheme for carrying out nucleic acid analysis of naturally occurring microorganisms. For abbreviations, see Table 6.4. (Modified from Madsen, E.L. 2000. Nucleic acid procedures for characterizing the identity and activity of subsurface microorganisms. *Hydrogeol. J.* **8**: 112–125, fig. 1. With kind permission of Springer Science and Business Media.) Note that the high-throughput, next-generation sequencing procedures (right side) are discussed in Section 6.10.

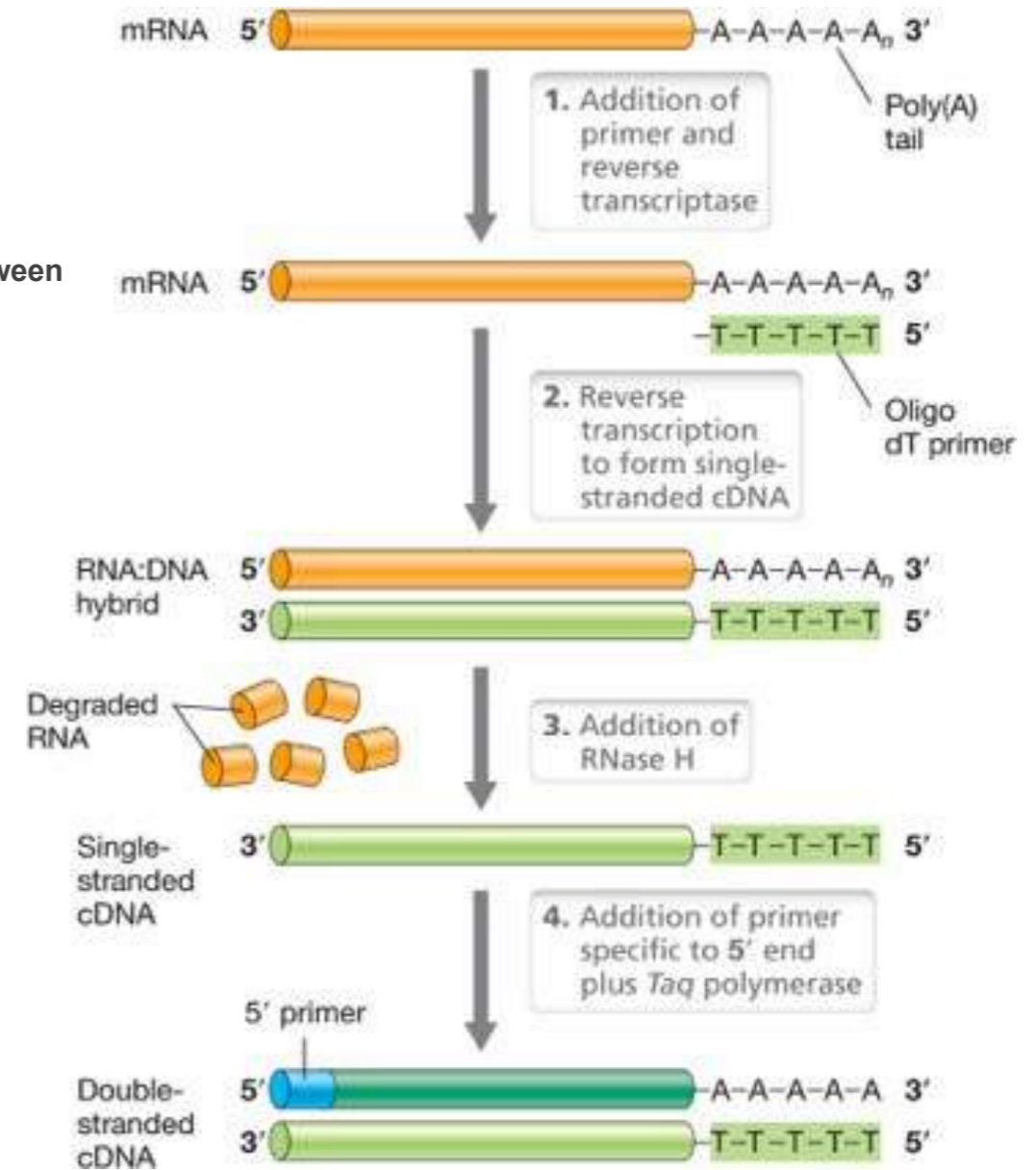
**WILEY** Blackwell

# Polymerase Chain Reaction, PCR

## Amplification and quantification

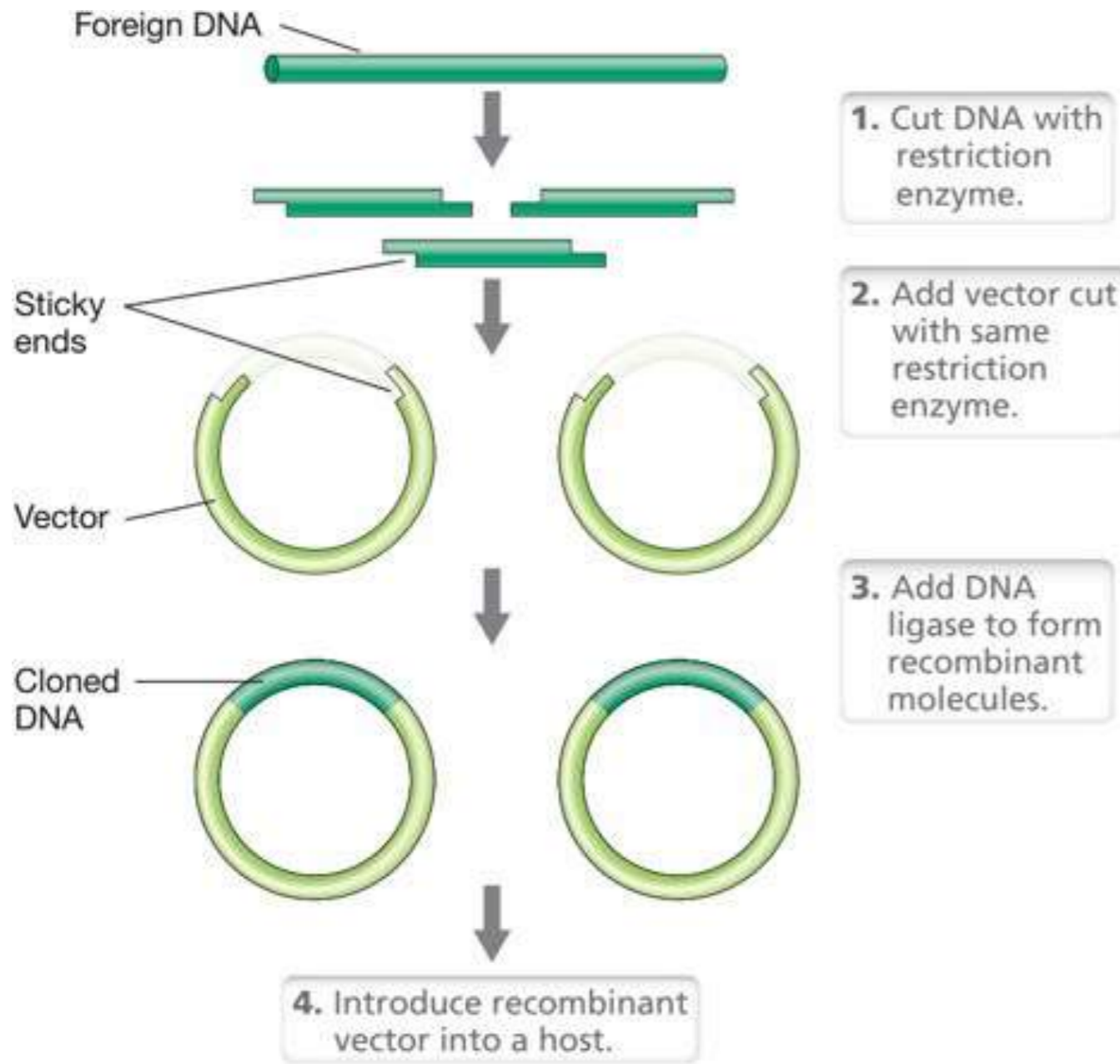


## Quantification of gene expression of specific genes



**Figure 12.2 Reverse transcription PCR.** Steps in the synthesis of cDNA from a eukaryotic mRNA. Reverse transcriptase synthesizes a hybrid molecule containing both RNA and DNA using the mRNA as a template and oligo-dT primer as a substrate. Next, the enzyme RNase H hydrolyzes the RNA portion of the hybrid molecule, yielding a single-stranded molecule of complementary DNA (cDNA). Following the addition of a primer complementary to the 5' end of the cDNA, *Taq* polymerase produces a double-stranded cDNA.

# Cloning

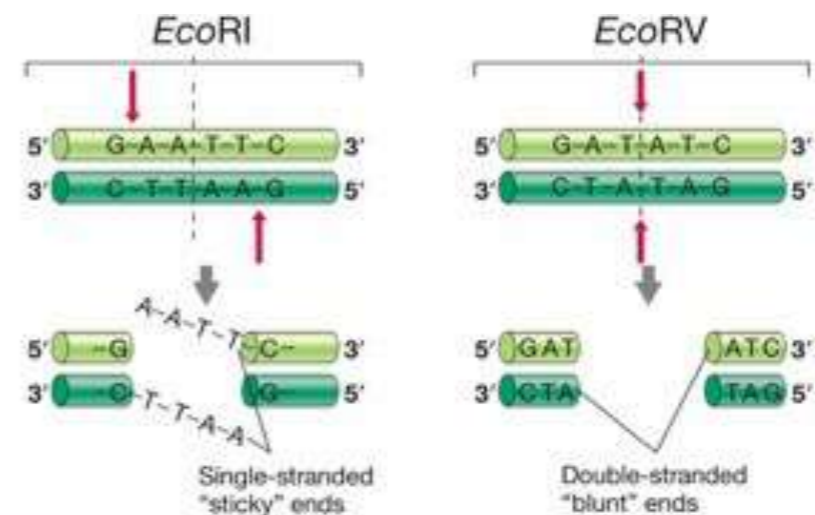


**Figure 12.6 Major steps in gene cloning.** By cutting the foreign DNA and the vector DNA with the same restriction enzyme, complementary sticky ends are generated that allow foreign DNA to be inserted into the vector.

- Insertion of foreign DNA via plasmids —
- > make many copies
  - > Community fingerprint
  - > Expression

Bacteria		Eukaryote
<i>Escherichia coli</i>	<i>Bacillus subtilis</i>	<i>Saccharomyces cerevisiae</i>
Well-developed genetics Many strains available Most-studied bacterium	Easily transformed Nonpathogenic Naturally secretes proteins Endospore formation simplifies culture	Well-developed genetics Nonpathogenic Can process eukaryotic mRNAs Easy to grow
Potentially pathogenic Periplasm traps proteins	Genetically unstable Genetics less developed than in <i>E. coli</i>	Plasmids unstable Will not replicate most bacterial plasmids
Advantages		Disadvantages

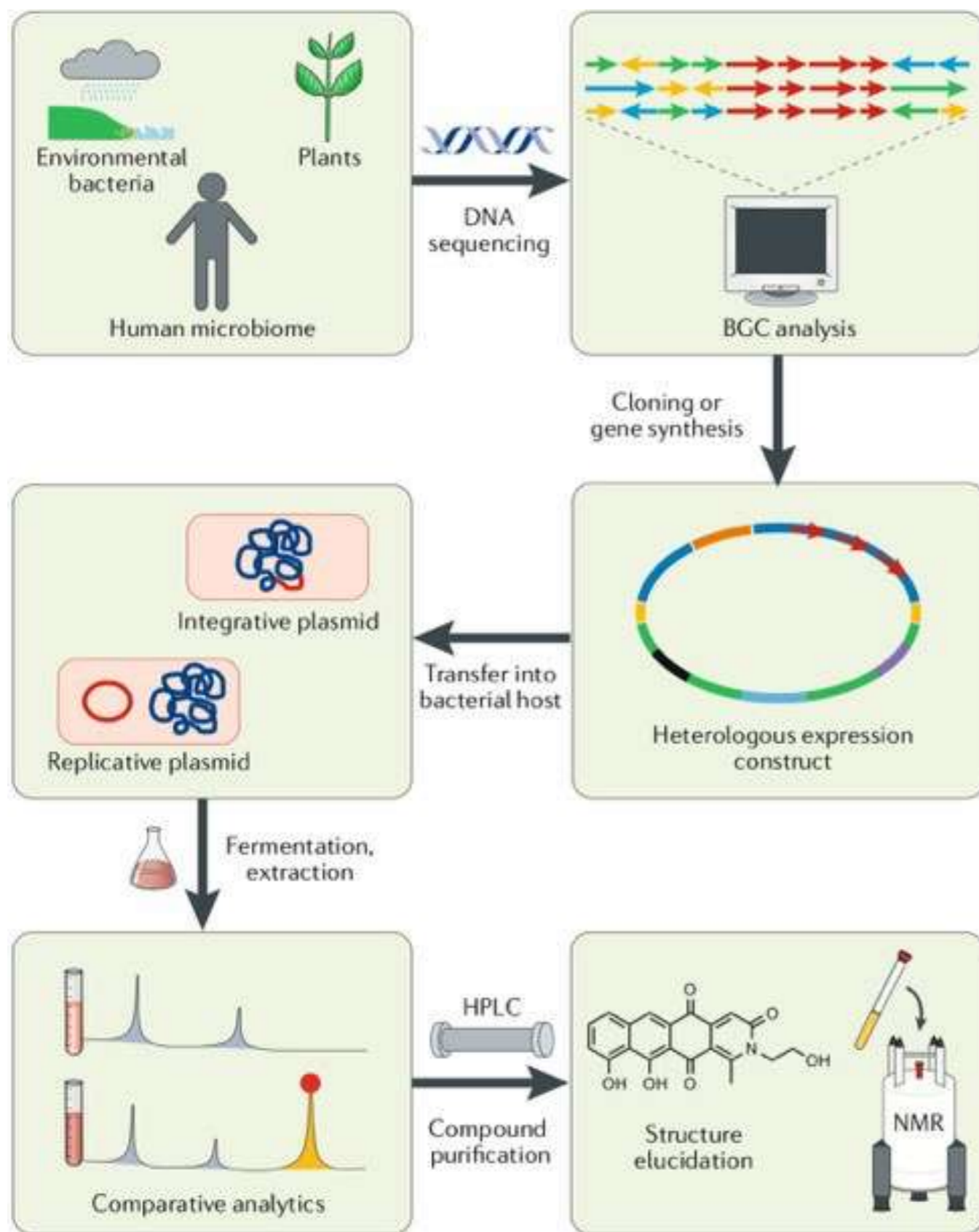
**Figure 12.10 Hosts for molecular cloning.** A summary of the advantages and disadvantages of some common cloning hosts.



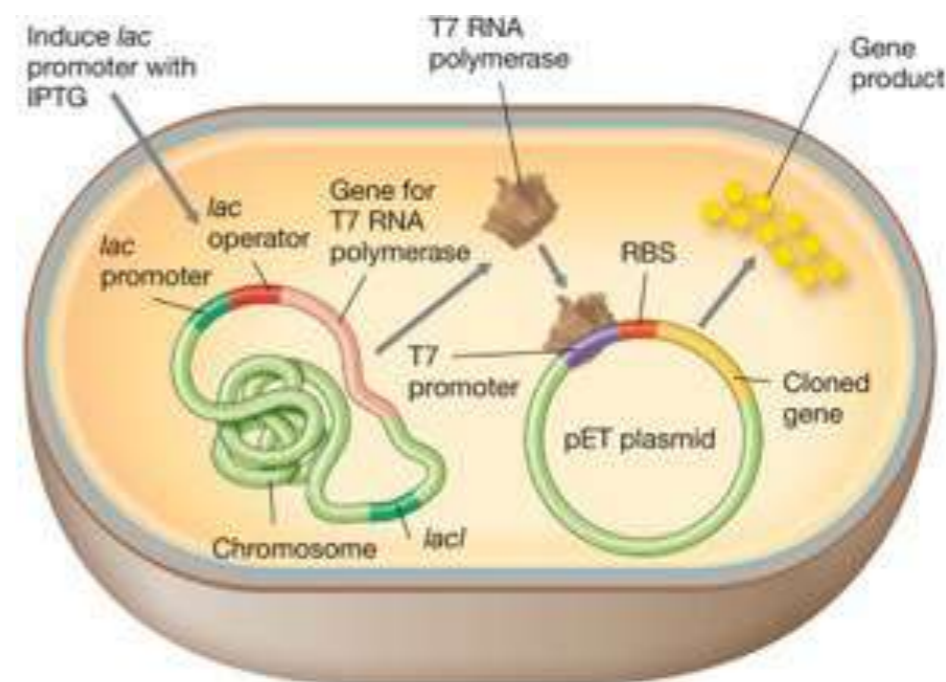
**Figure 12.7 Restriction and modification of DNA.** Sequences of DNA recognized by the restriction endonucleases *EcoRI* and *EcoRV*. The red arrows indicate the bonds cleaved by the enzyme, and the dashed line indicates the axis of symmetry of the sequence. After cutting DNA with these restriction enzymes, note the single-stranded "sticky" ends generated by *EcoRI* versus the "blunt" ends generated by *EcoRV*.



# Expression vector



## Efficient mRNA translation



**Figure 12.11 The T7 expression system.** The gene for T7 RNA polymerase is in a gene fusion under control of the *lac* promoter and is inserted into the chromosome of a special host strain of *Escherichia coli* (BL21). Addition of IPTG induces the *lac* promoter, causing expression of T7 RNA polymerase. This transcribes the cloned gene, which is under control of the T7 promoter and is carried by the pET plasmid. RBS, ribosome-binding site.

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### Heterologous expression workflow

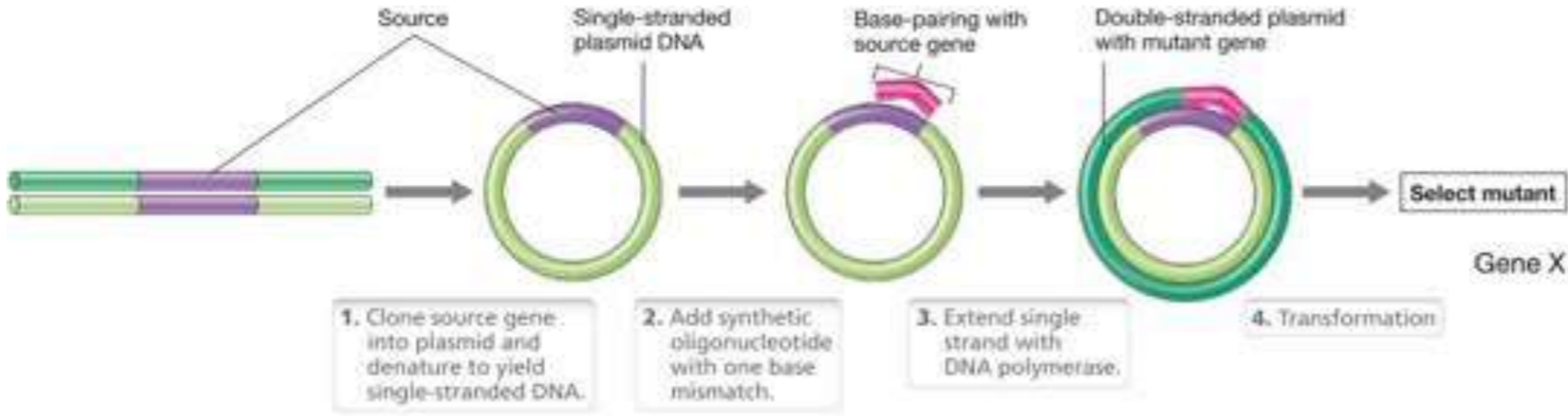
Biosynthetic gene clusters (BGCs) are DNA sequences encoding biosynthetic machineries, which are often co-localized

These BGCs can be subcloned from the original source or acquired via gene synthesis to generate a heterologous expression construct

This construct has to be transferred into a bacterial host and maintained by integration into its genome or as a replicative plasmid, depending on the construct and heterologous host

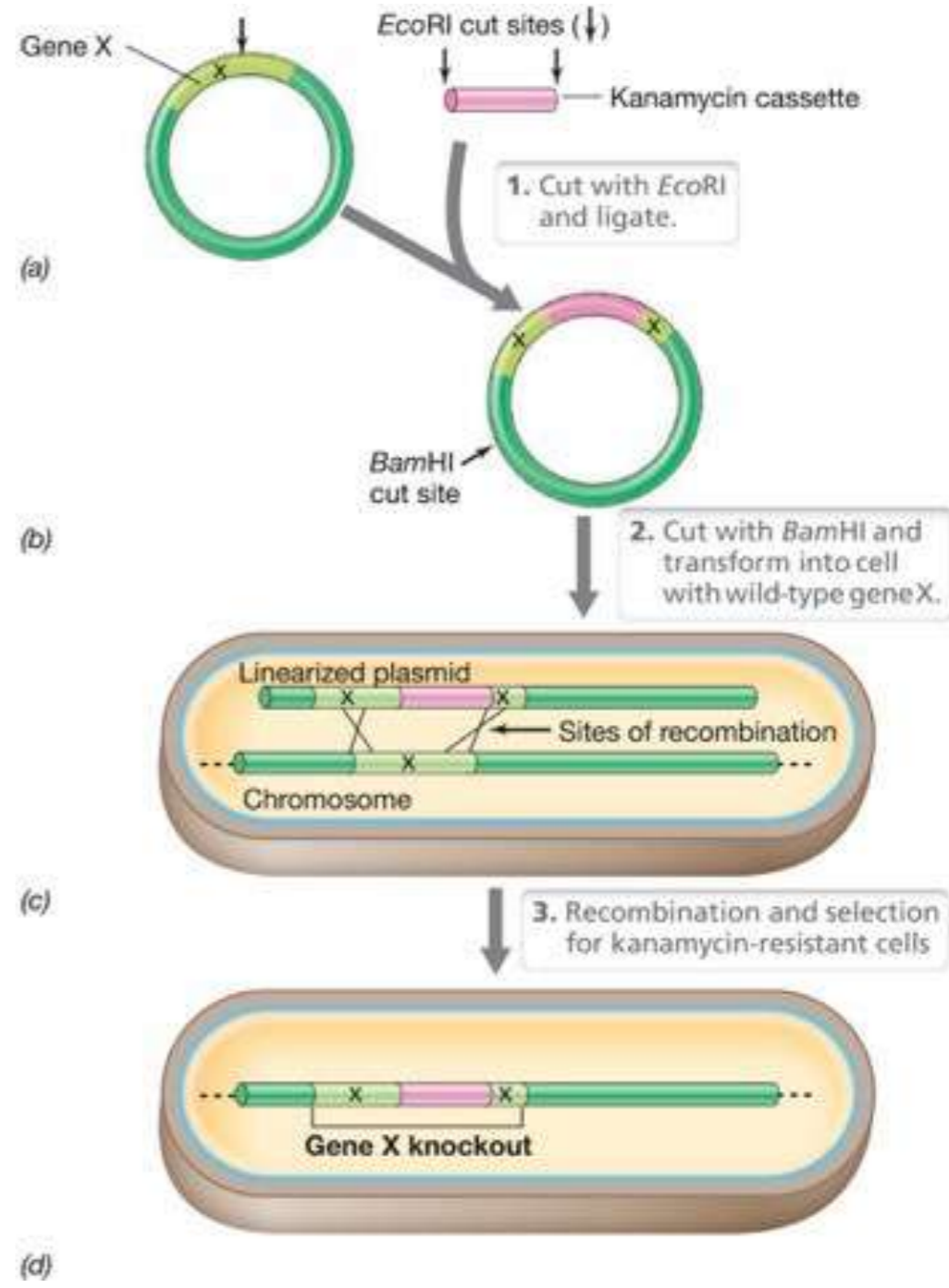
HPLC, high-performance liquid chromatography

# Mutagenesis

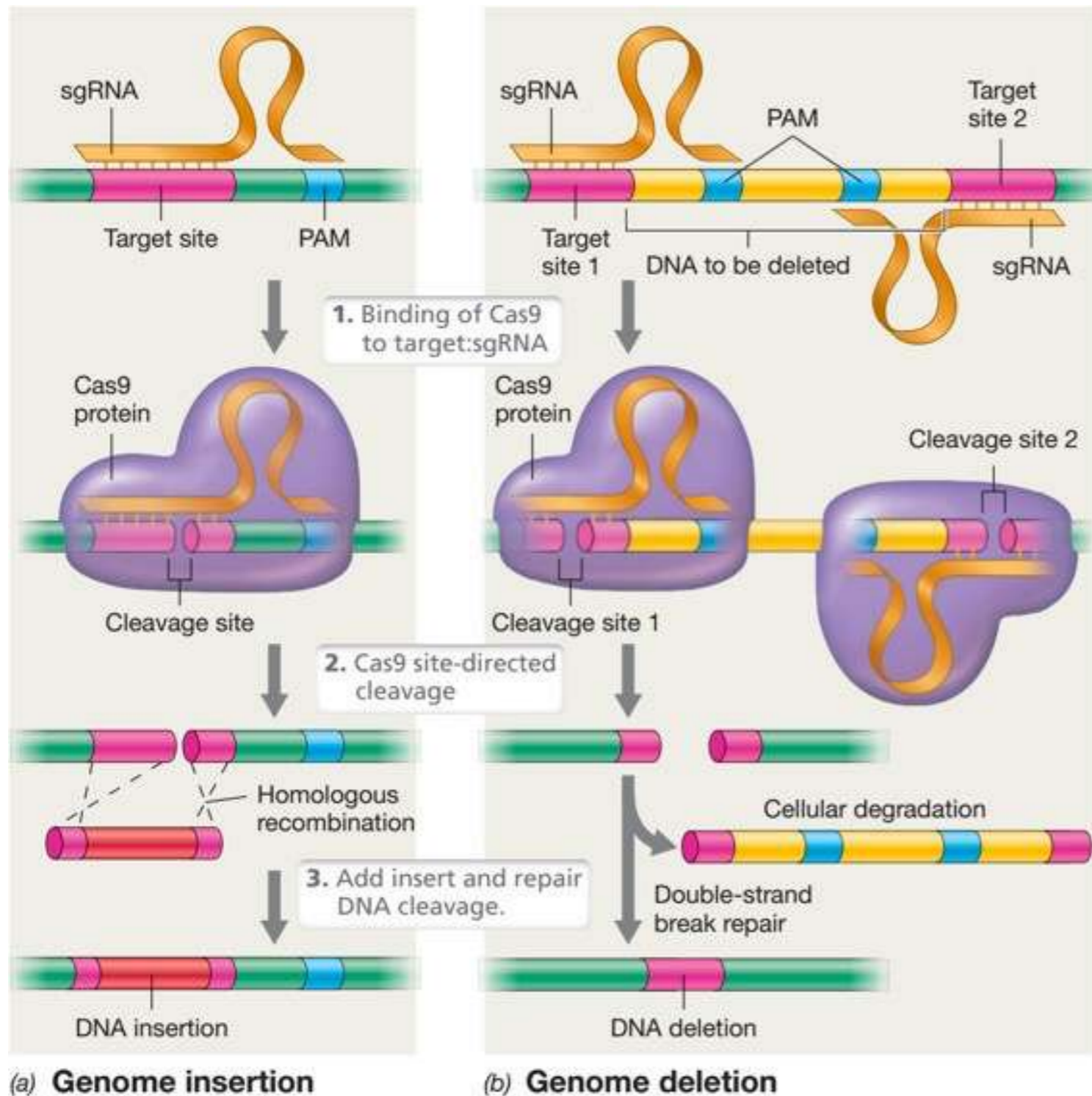


**Figure 12.14 Site-directed mutagenesis using synthetic DNA.** Short synthetic oligonucleotides hybridized to the cloned gene may be used to generate mutations. Cloning the source DNA into a plasmid followed by denaturation yields the single-stranded DNA needed for site-directed mutagenesis to work.

**Figure 12.15 Gene disruption by cassette mutagenesis.** (a) A cloned wild-type copy of gene X, carried on a plasmid, and a kanamycin cassette are cut with *EcoRI* and mixed. (b) The cut plasmid and the cassette are ligated, creating a plasmid with the kanamycin cassette as an insertion mutation within gene X. This new plasmid is cut with *BamHI* and transformed into a cell. (c) The transformed cell contains the linearized plasmid with a disrupted gene X and its own chromosome with a wild-type copy of the gene. (d) In some cells, homologous recombination occurs between the wild-type and mutant forms of gene X. Cells that can grow in the presence of kanamycin have only a single, disrupted copy of gene X.



# Genome editing



**Figure 12.36 CRISPR/Cas9 genome editing.**

sgRNA represents the synthetic guide RNA, while PAM represents a protospacer adjacent motif. Note that each genome target site must possess a PAM sequence for DNA cleavage to occur. (a) Insertion of foreign DNA into a targeted site of the genome. An sgRNA is synthesized to bind to a single target site on the genome through complementarity. This binding of the sgRNA to the DNA

stimulates the Cas9 protein to cleave the genome at the target site. Foreign DNA with ends homologous to the cleavage site can be incorporated into the cut site through homologous recombination. This results in a genomic insertion. (b) Deletion of a genomic region. Two separate target sites flanking the DNA to be deleted are selected. After the design, addition, and binding of sgRNAs corresponding to these regions, Cas9

protein-dependent DNA cleavage occurs. This results in a double-stranded break in the target chromosome and a free piece of DNA. The double-strand break is then ligated by the cell's DNA double-strand break repair pathway, while the free piece of genomic DNA is degraded. This results in a genomic deletion. (c) Crystal structure of the *Streptococcus pyogenes* Cas9 protein. The target DNA is shown in green and the sgRNA in orange.

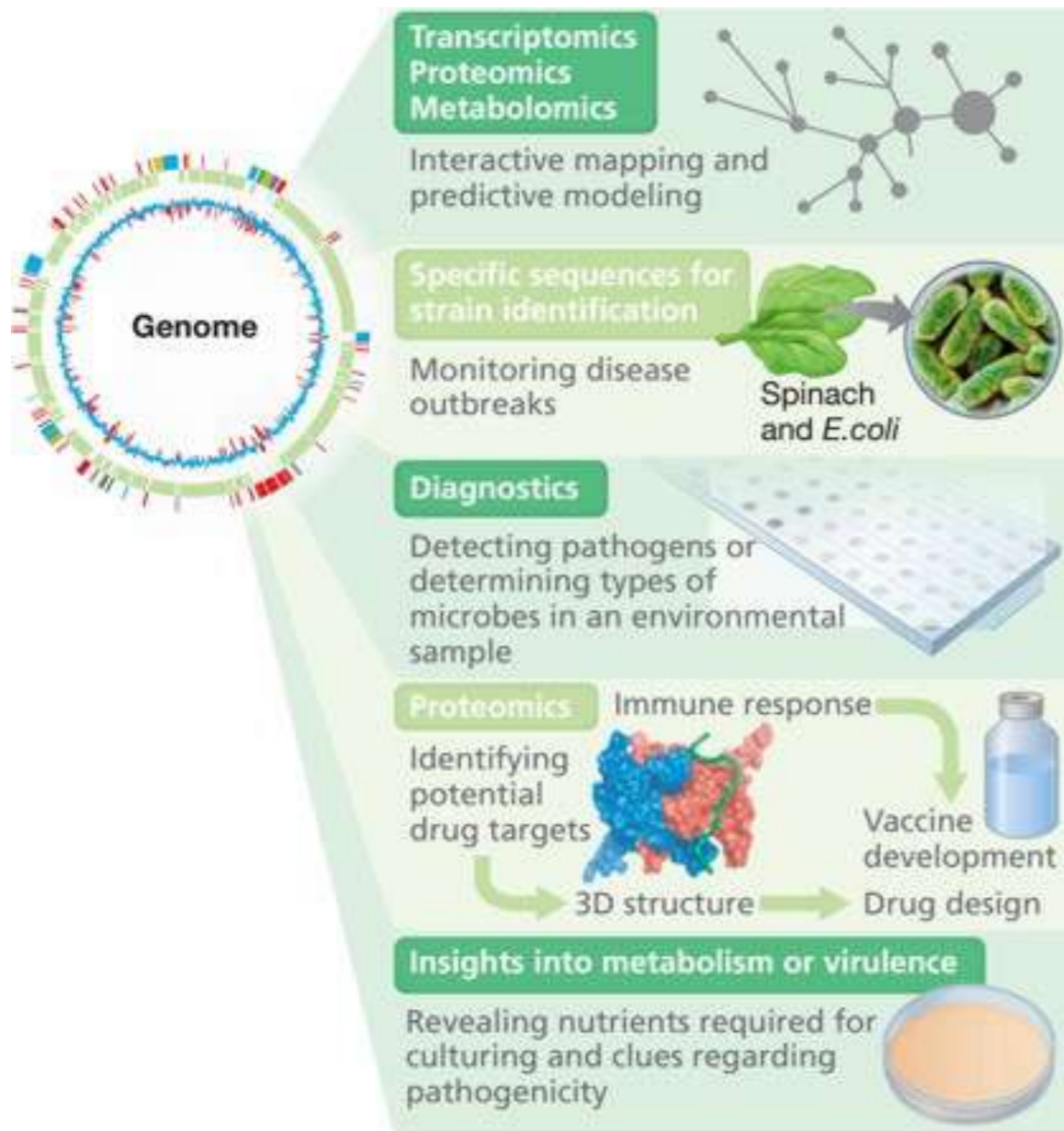
# Sequencing

**TABLE 9.2 DNA sequencing methods**

<i>Generation</i>	<i>Method</i>	<i>Features</i>
<b>First generation</b>	Sanger dideoxy method (radioactivity or fluorescence; DNA amplification)	Read length: 700–900 bases Used for the Human Genome Project
<b>Second generation</b>	454 Pyrosequencing (fluorescence; DNA amplification; massively parallel) Illumina/Solexa method (fluorescence; DNA amplification; massively parallel)  SOLiD method (fluorescence; DNA amplification; massively parallel)	Read length: 400–500 bases Used to sequence genome of James Watson (completed 2007) Read length: 50–100 bases Giant panda genome (2009; Beijing Genome Institute); Denisovan genome (2010) Read length 50–100 bases
<b>Third generation</b>	HeliScope Single Molecule Sequencer (fluorescence; single molecule) Pacific Biosciences SMRT (fluorescence; single molecule; zero mode waveguide)	Read length: up to 55 bases Fossil DNA accuracy greatly improved Read length: 2500–3000 bases
<b>Fourth generation</b>	Ion torrent (electronic—pH; DNA amplification)  Oxford nanopore (electronic—current; single molecule; real time)	Read length: 100–200 bases Sequenced genome of Intel cofounder Gordon Moore (originator of Moore’s law), 2011 Read length: thousands of bases Portable MinION unit is approximately the size of a flash drive

- From many cells of the same “species” to one cell
- From monoculture to complex and diverse environmental communities

# OMICS

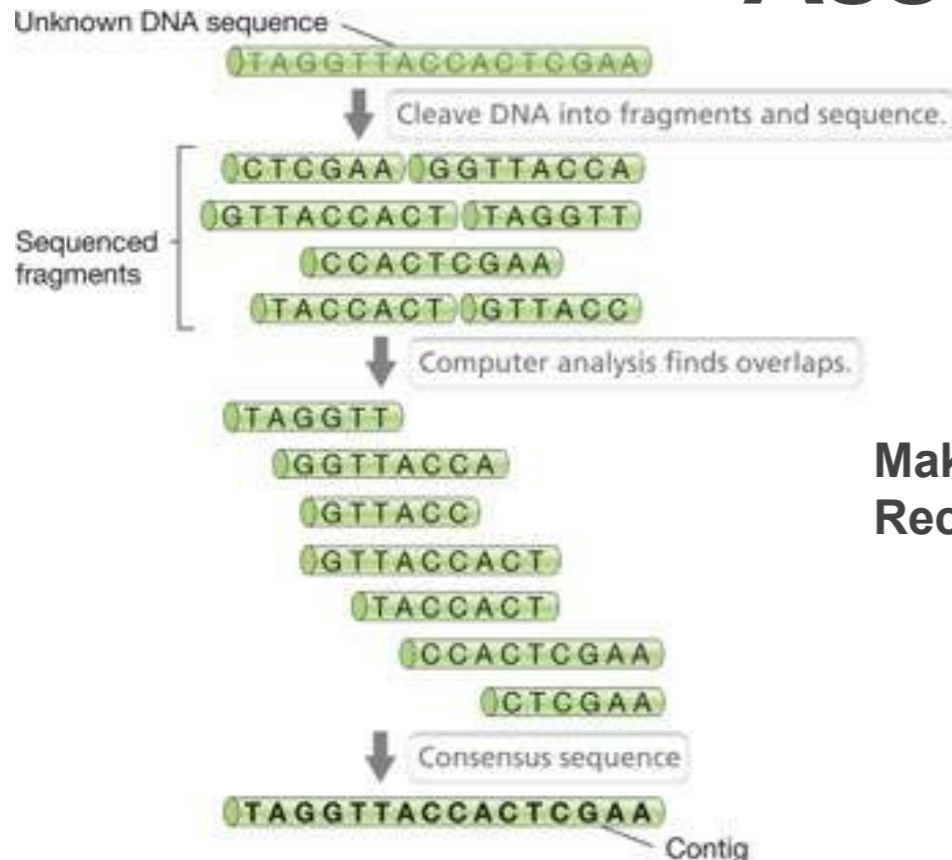


**Figure 9.1 Utility of microbial genome sequences.** A genome sequence allows for the development of omics approaches and tools for understanding, investigating, and monitoring microorganisms. It can also provide targets for drug and vaccine design.

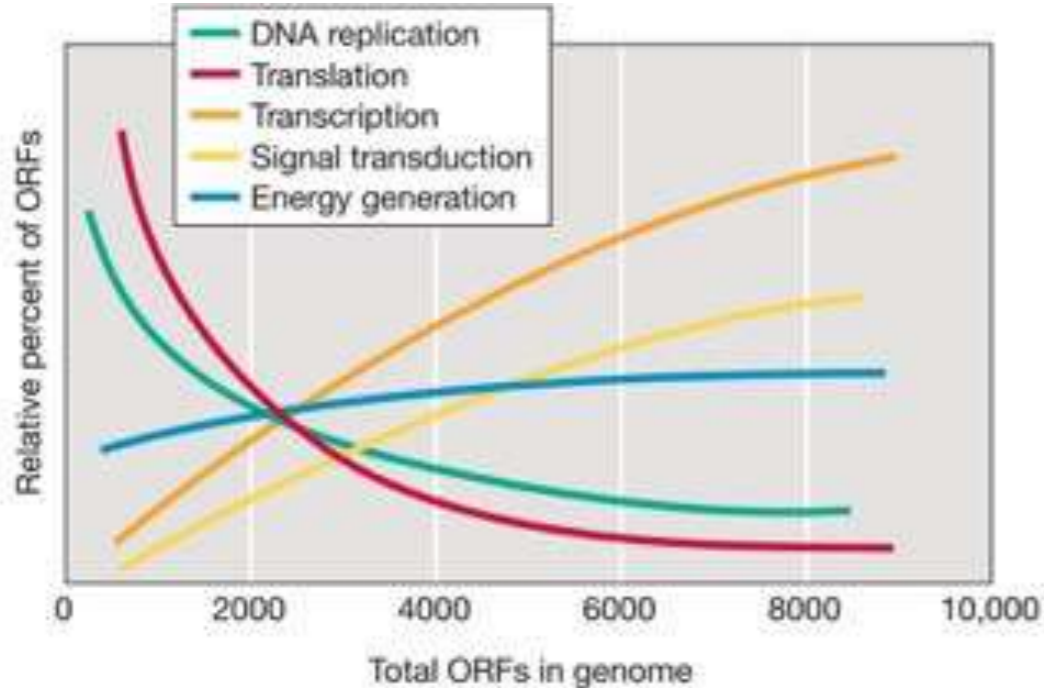
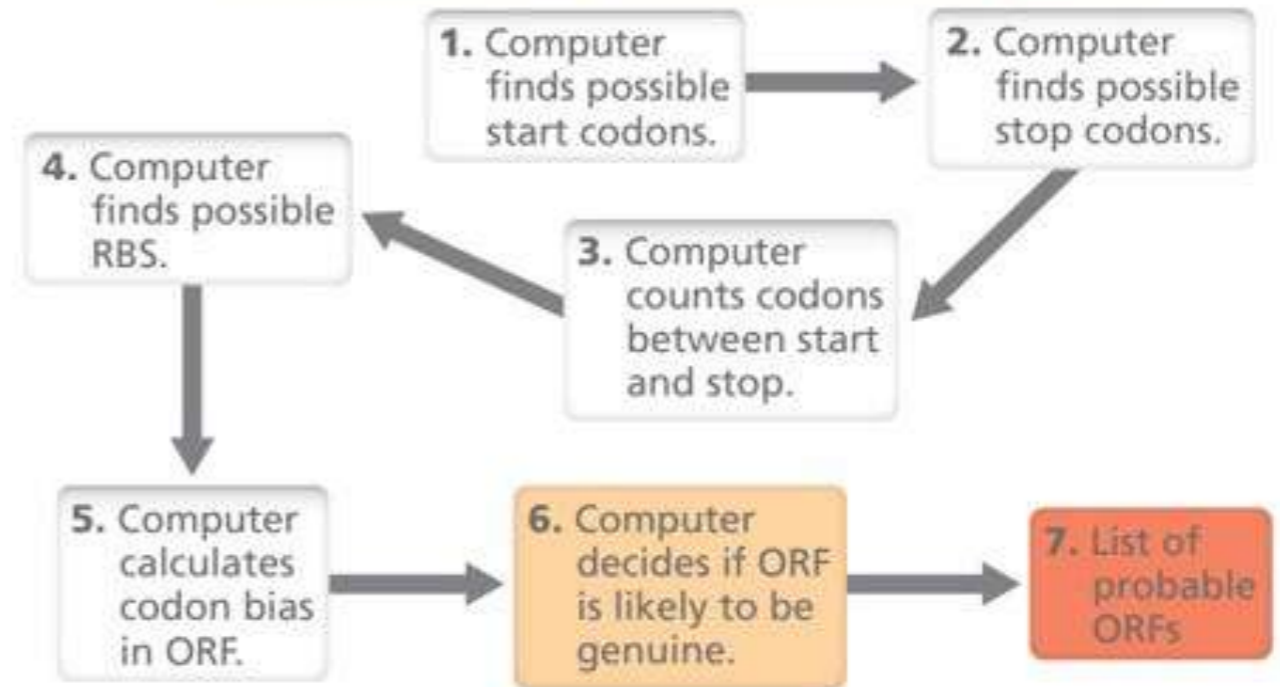
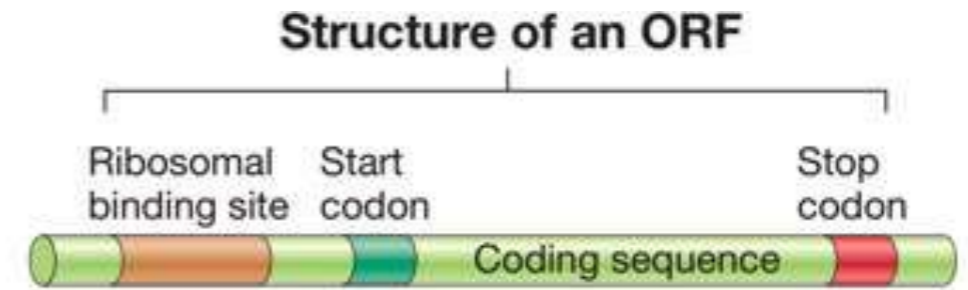
**TABLE 9.6 Some omics terminology**

<b>DNA</b>	<p><b>Genome</b> the total complement of genetic information of a cell or a virus</p> <p><b>Metagenome</b> the total genetic complement of all the cells present in a particular environment</p> <p><b>Epigenome</b> the total number of possible epigenetic changes</p> <p><b>Methylome</b> the total number of methylated sites on the DNA (whether epigenetic or not)</p> <p><b>Mobilome</b> the total number of mobile genetic elements in a cell</p>
<b>RNA</b>	<p><b>Transcriptome</b> the total RNA produced in an organism under a specific set of conditions</p>
<b>Protein</b>	<p><b>Proteome</b> the total set of proteins encoded by a genome; sometimes also used in place of <i>translatome</i></p> <p><b>Translatome</b> the total set of proteins present under specified conditions</p> <p><b>Interactome</b> the total set of interactions between proteins (or other macromolecules)</p> <p><b>Secretome</b> the total set of proteins secreted by a cell</p>
<b>Metabolites</b>	<p><b>Metabolome</b> the total complement of small molecules and metabolic intermediates</p> <p><b>Glycome</b> the total complement of sugars and other carbohydrates</p>
<b>Organisms</b>	<p><b>Microbiome</b> the total complement of microorganisms in an environment (including those associated with a higher organism)</p> <p><b>Virome</b> the total complement of viruses in an environment</p> <p><b>Mycobiome</b> the total complement of fungi in a natural environment</p>

# Assembling genomes



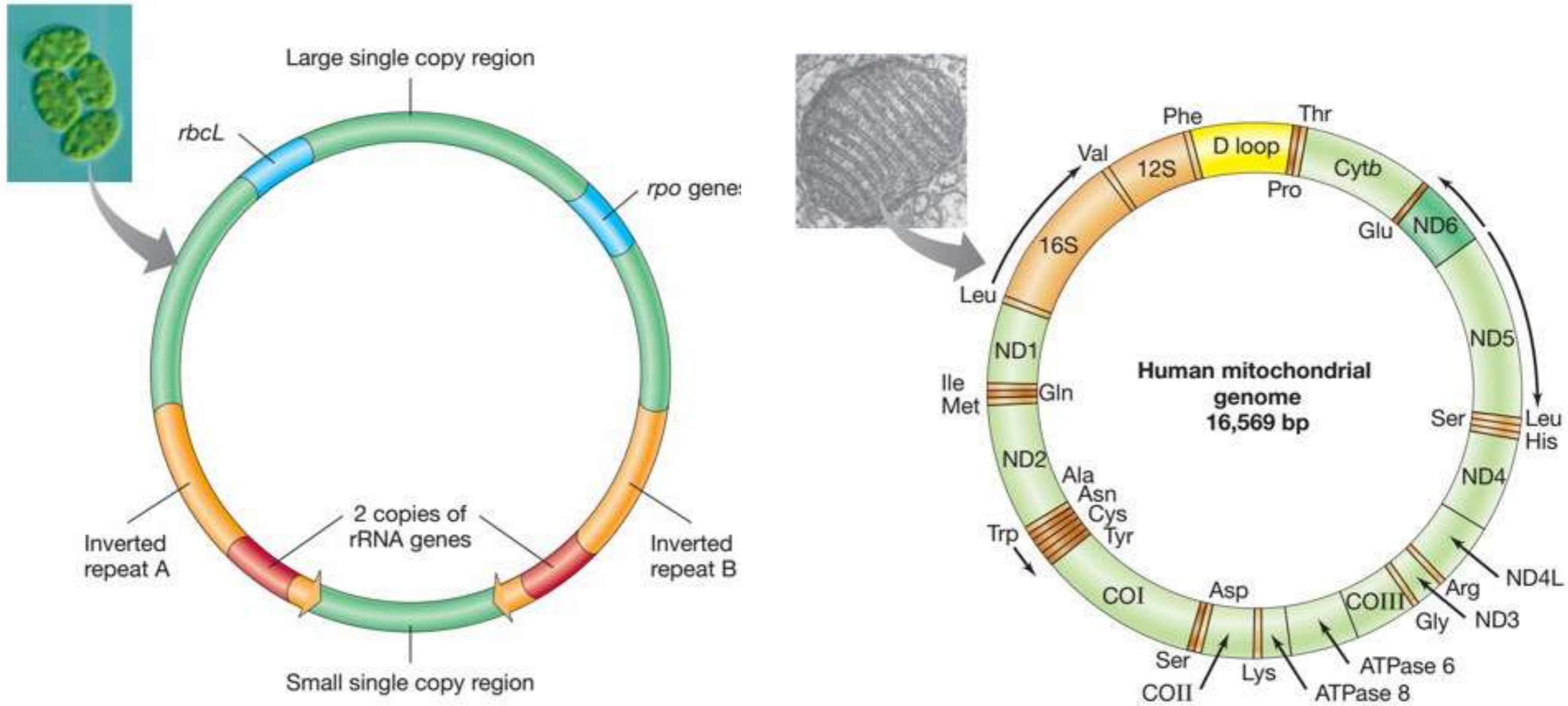
Making sense  
 Recognizing patterns



**Figure 9.10 Functional category of genes as a percentage of the genome.** The percentage of genes encoding products for translation or DNA replication is greater in organisms with small genomes, whereas the percentage of transcriptional regulatory genes is greater in organisms with large genomes.

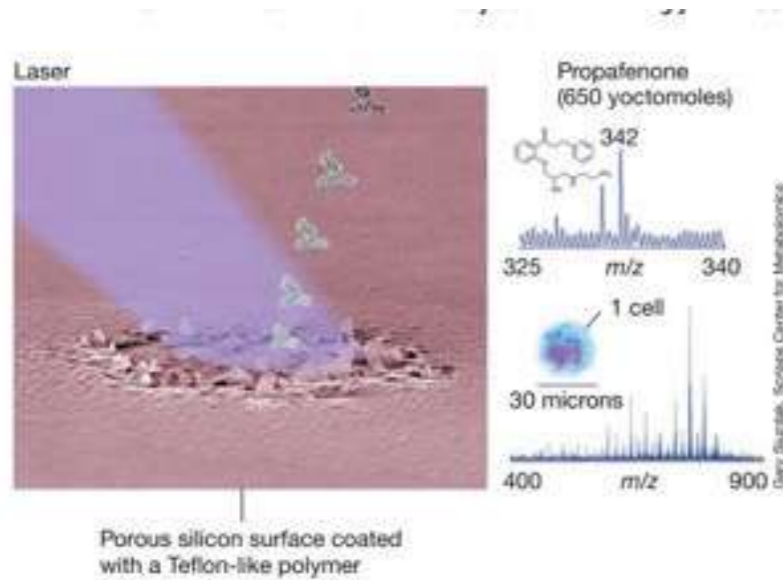
**Figure 9.6 Computer identification of possible ORFs.** The computer scans the DNA sequence looking first for start and stop codons. It then counts the number of codons in each uninterrupted reading frame and rejects those that are too short. The probability of a genuine ORF is made stronger if a likely ribosomal binding site (RBS) is found the correct distance in front of the reading frame. Codon bias calculations are used to test whether an ORF complies with the codon usage of the organism being examined.

# Chloroplast and Mitochondrial Genomes



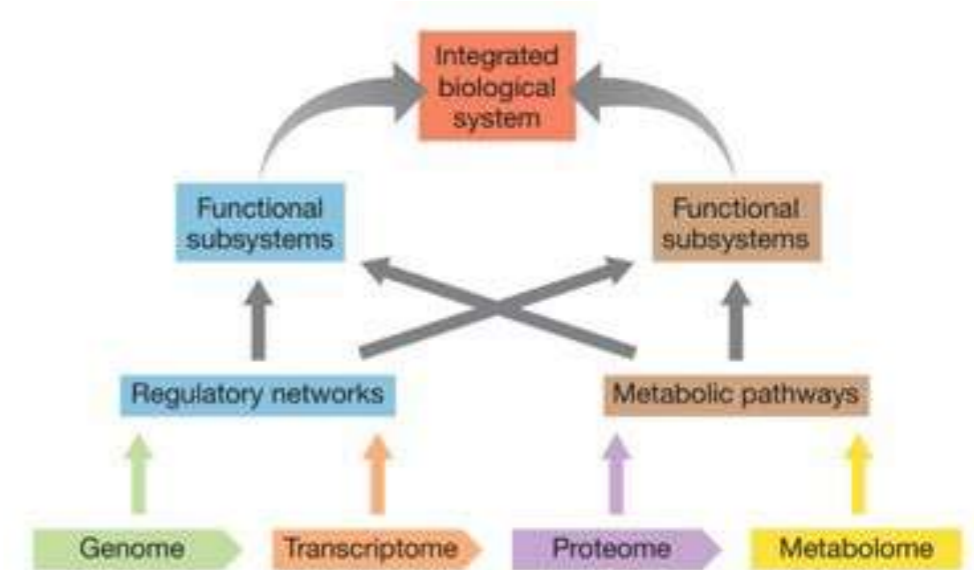
**Figure 9.11** Map of a typical chloroplast genome. The inverted repeats each contain a copy of the three genes for rRNA (5S, 16S, and 23S). The large subunit of RubisCO is encoded by *rbcL* and the chloroplast RNA polymerase by *rpo* genes. Inset: Photo of four cells of the green alga *Makinoella* with chloroplasts clearly visible.

# Mass spectrometry

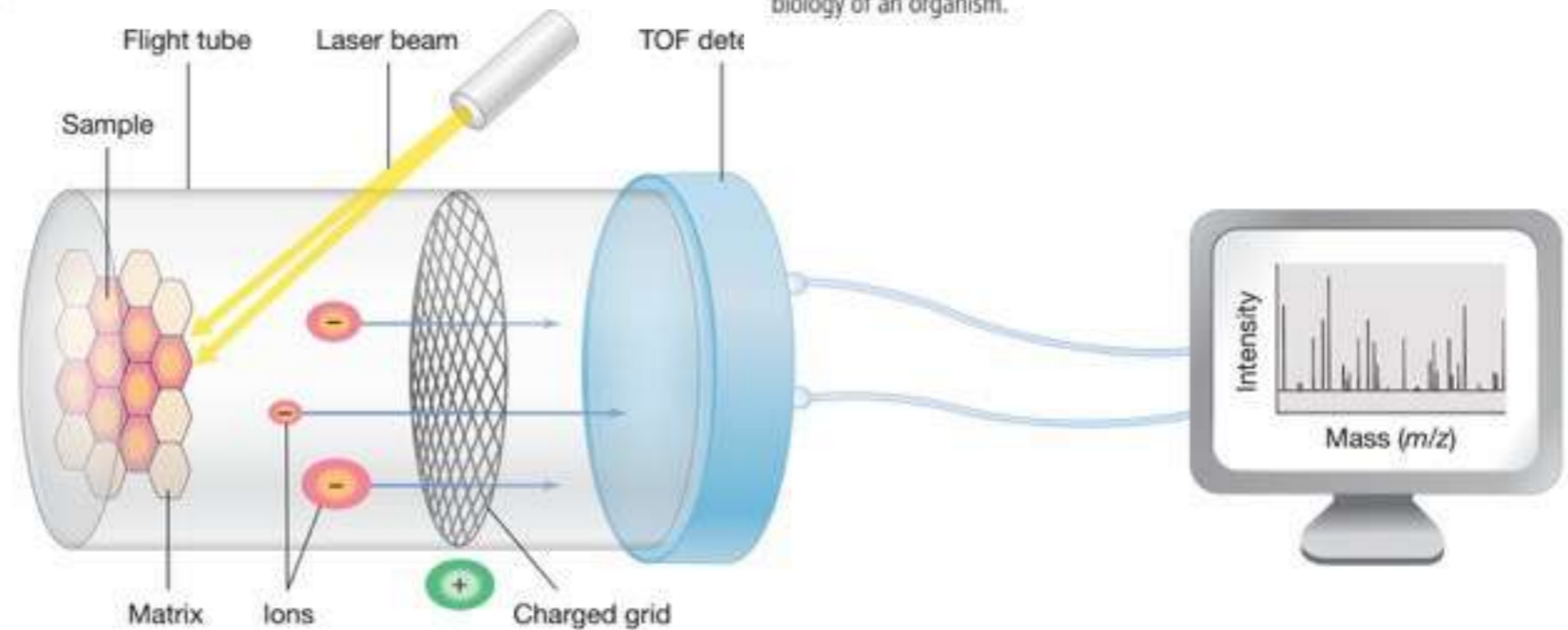


**Figure 9.30 NIMS identification of metabolites.** In nanostructure-initiator mass spectrometry (NIMS), a cell is placed on the silicon initiator surface and vaporized using a laser. The resulting ionized metabolites within a cell are then detected using mass spectrometry. Because NIMS lacks a matrix, it has extremely high sensitivity and resolution. Ionized metabolites are represented rising from the surface.

Proteomics  
Metabolomics



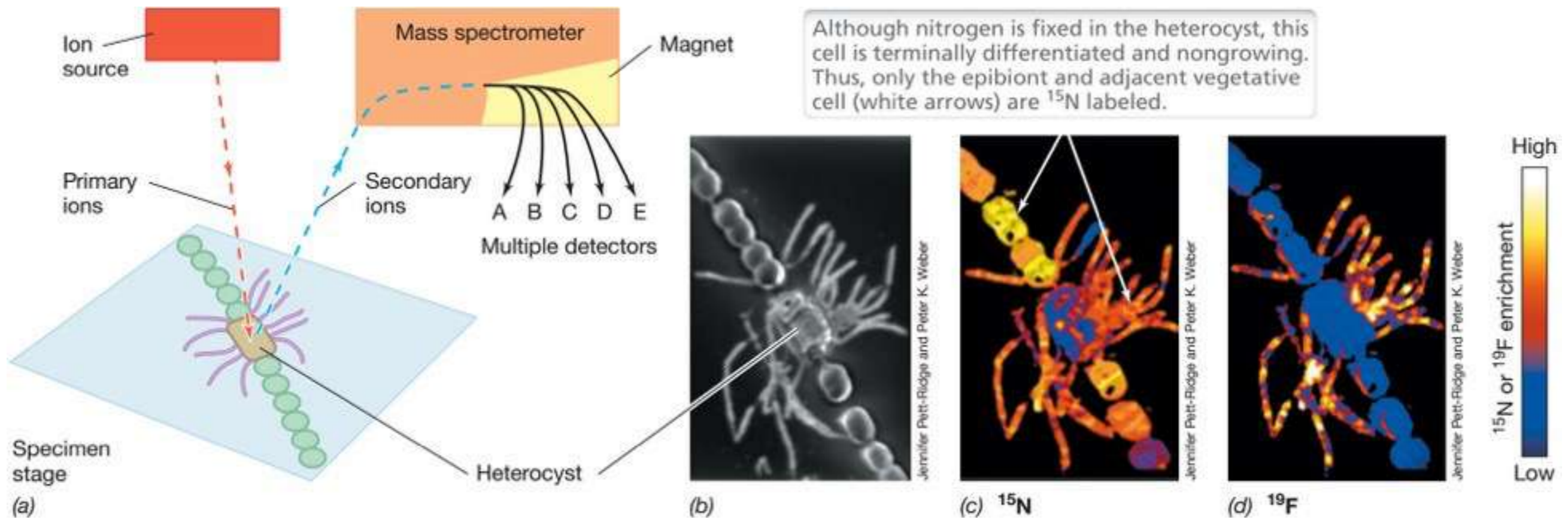
**Figure 9.32 The components of systems biology.** The results of various "omics" analyses are combined and successively integrated into higher-level views of the entire biology of an organism.



**Figure 9.27 MALDI-TOF mass spectrometry.** In matrix-assisted laser desorption ionization (MALDI) spectroscopy, the sample is ionized by a laser and the ions travel down the tube to the detector. The time of flight (TOF) depends on the mass/charge ( $m/z$ ) ratio of the ion. The computer identifies the ions based on their time of flight, that is, the time it takes to reach the detector.



# Mass Spectroscopy-based imaging



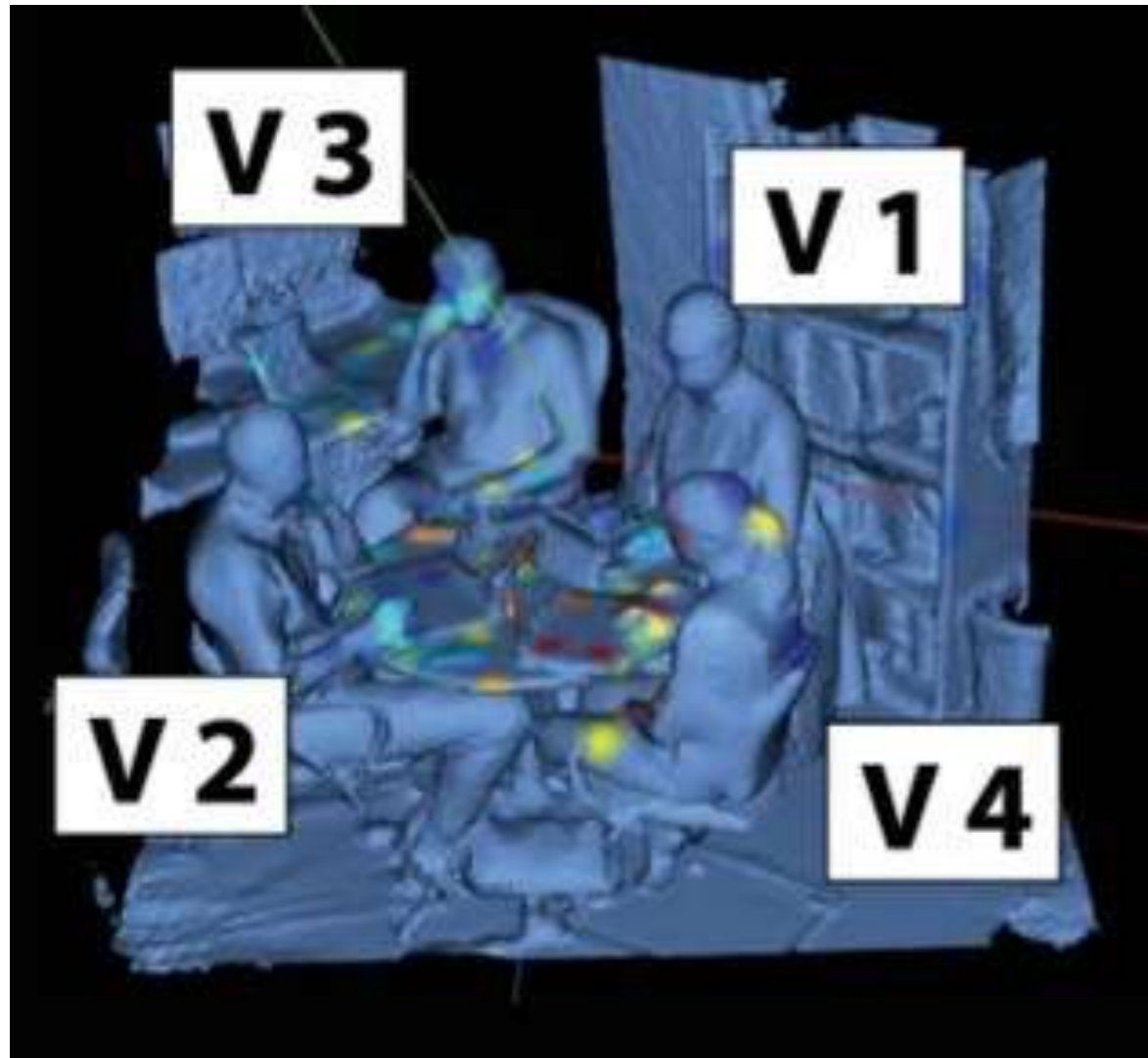
**Figure 19.33 NanoSIMS technology.** (a) Schematic of NanoSIMS operation showing the beams of primary (red) and secondary (blue) ions and five different detectors, each of which identifies ions of a different mass-to-charge ratio. (b–d) Demonstration of interspecies

nutrient transfer from a filamentous cyanobacterium (*Anabaena*) to a *Rhizobium* species attached to the cyanobacterial heterocyst. The coculture was incubated with  $^{15}\text{N}_2$ , and the transfer of  $^{15}\text{N}$ -labeled compounds from *Anabaena* to *Rhizobium* was imaged using a

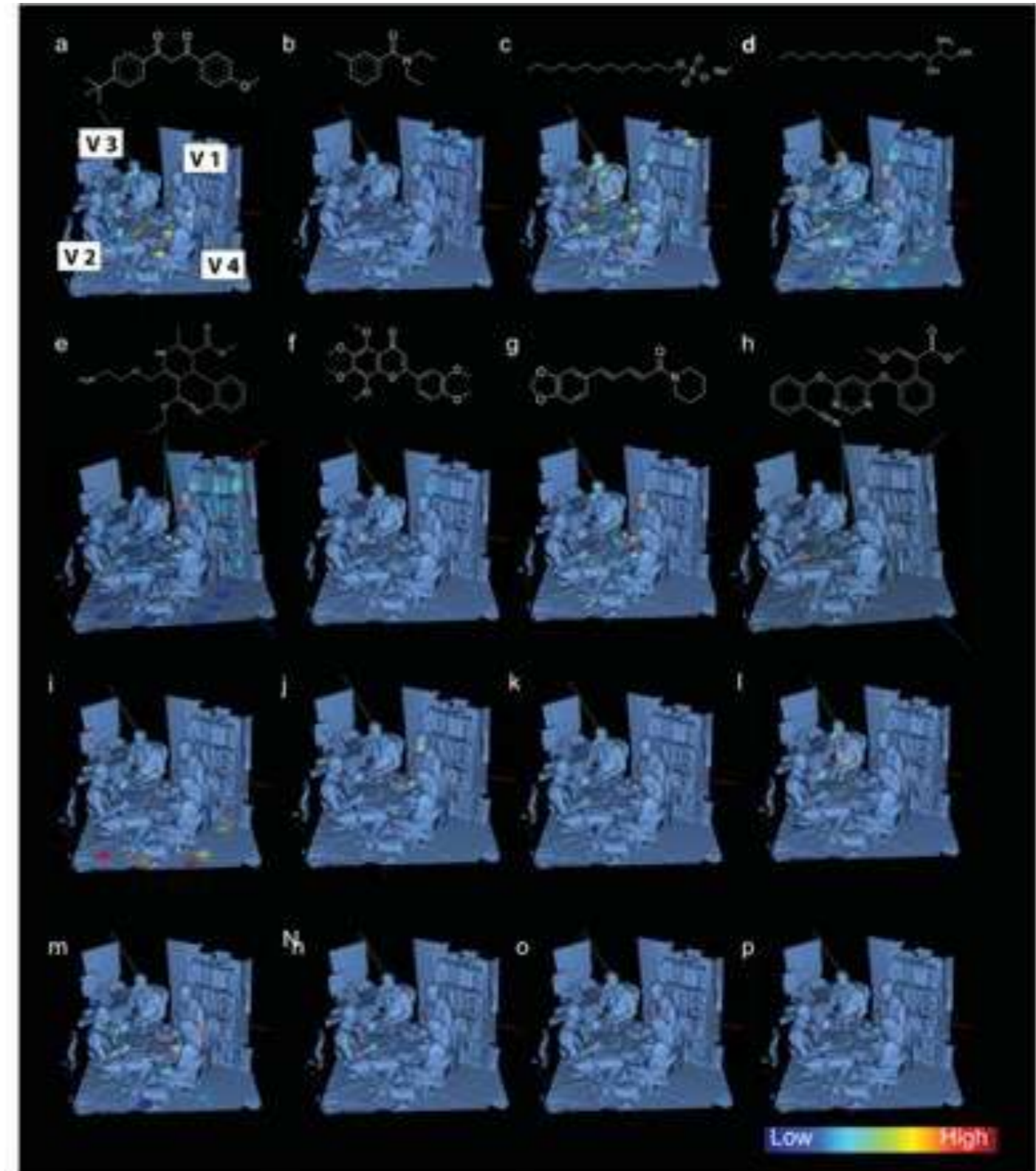
combination of EL-FISH and NanoSIMS. (b) Total  $^{12}\text{C}$  abundance shown by gray tones. (c)  $^{15}\text{N}$  enrichment. (d)  $^{19}\text{F}$  abundance conferred by a probe that hybridizes only to the attached rhizobial cells (EL-FISH).

- Stable isotope for metabolism studies
- Fluorescence In Situ Hybridization for microbial identification

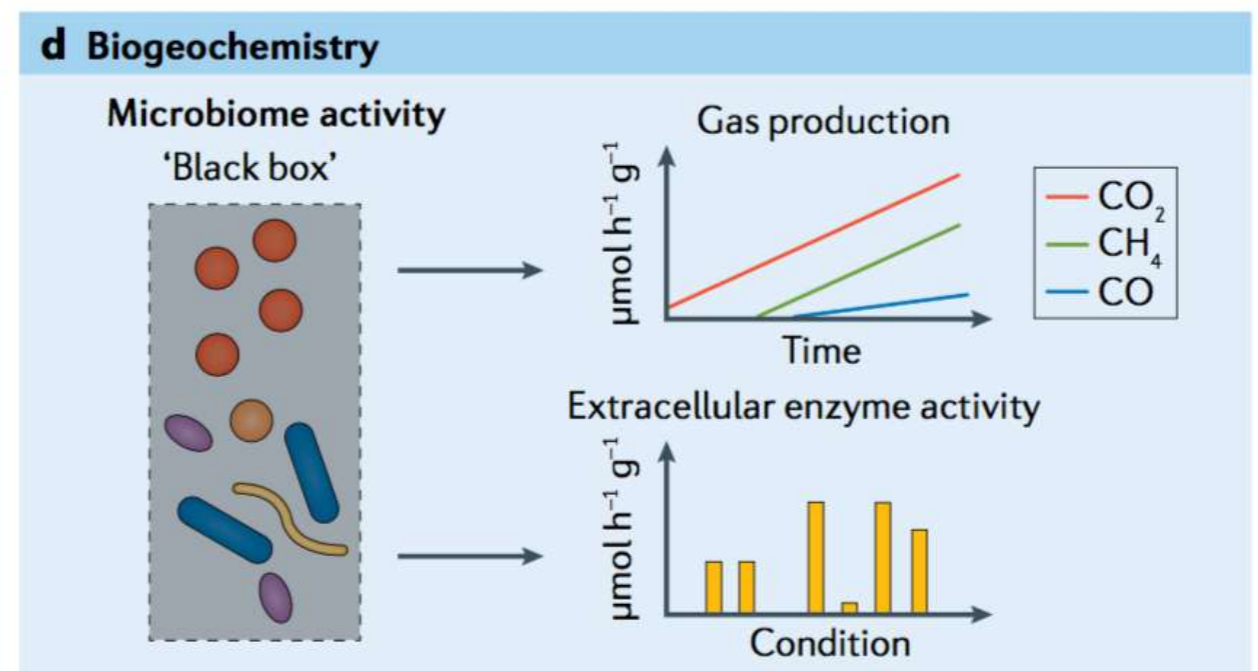
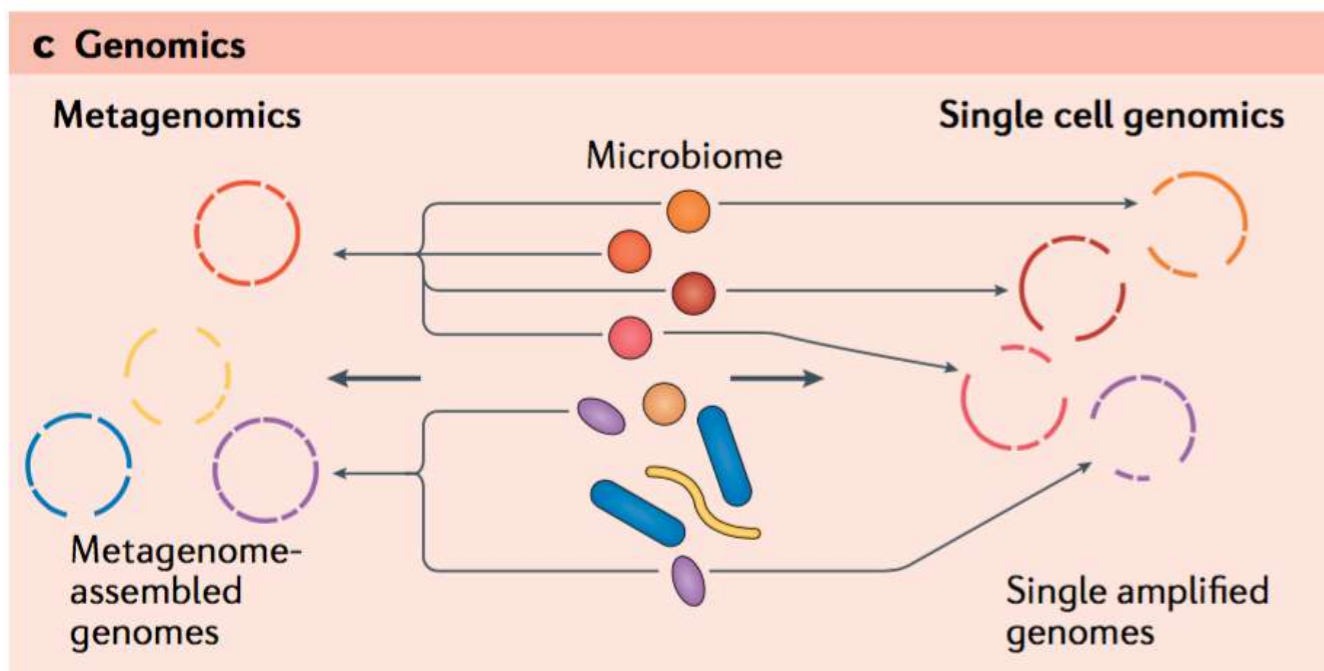
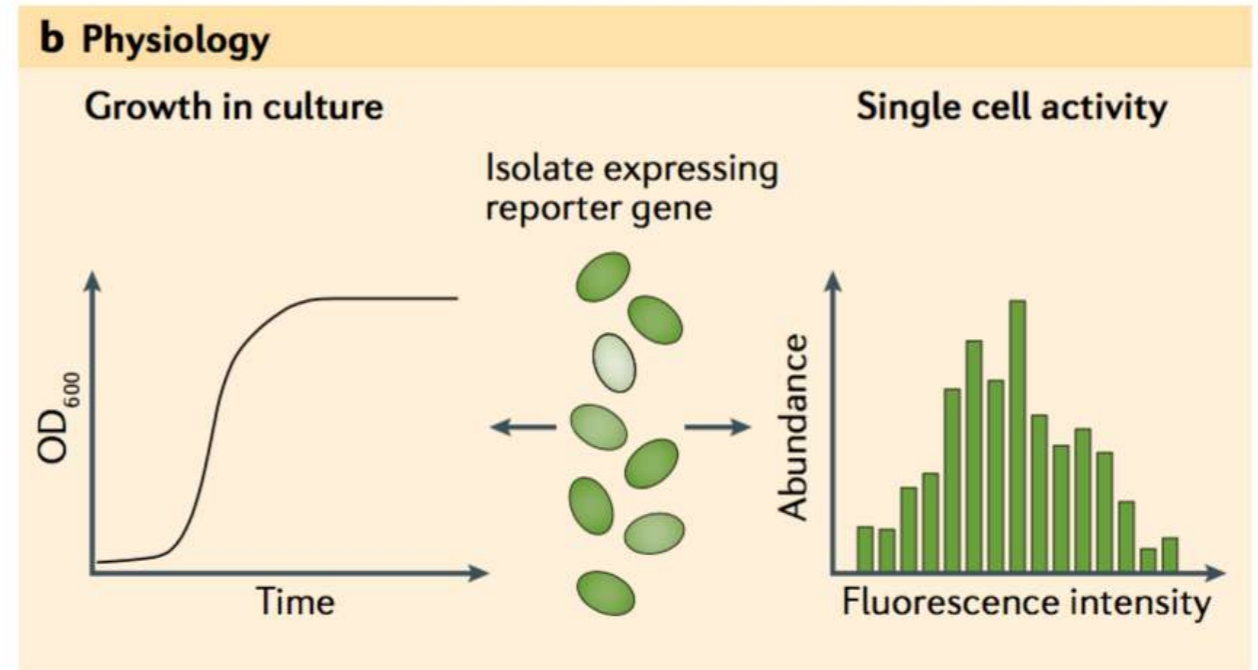
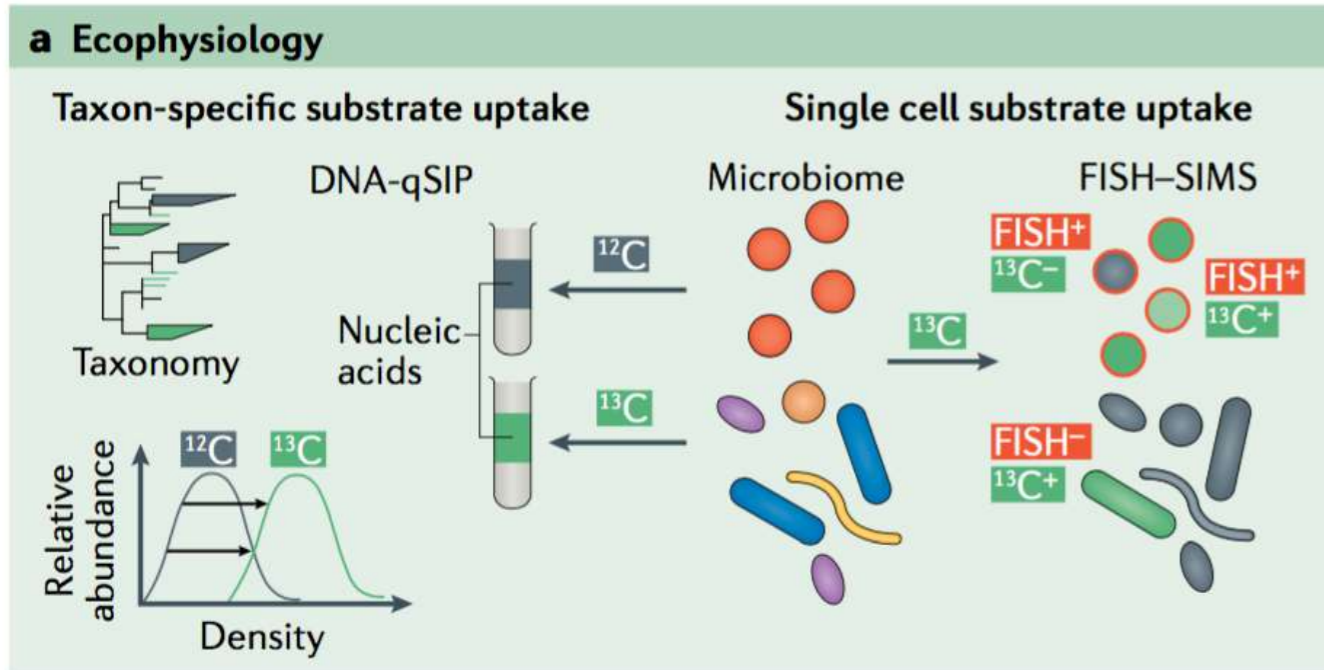
# Creating a 3D microbial and chemical snapshot of a human habitat



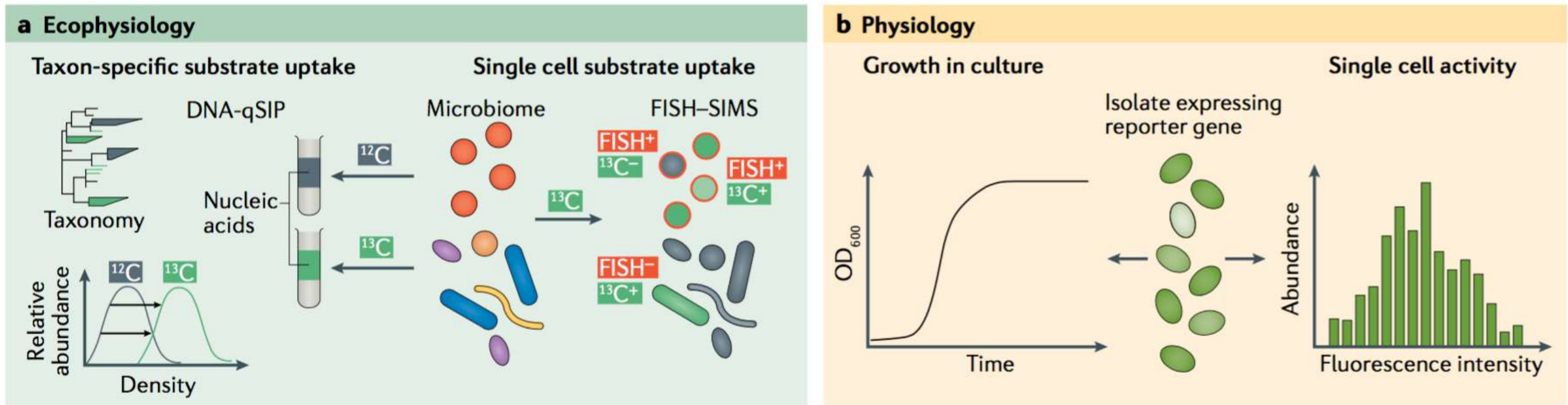
The MS data of the office environment sampling locations and relationships to the microbes in those same locations. (a) Avobenzone, (b) DEET, (c) Sodium Laureth Sulfate, (d) C17 Sphingosine, (e) Amlodipine (Norvasc), (f) Nobiletin, (g) Theophylline, (h) Azoxystrobin, (i) Nocardiaceae, (j) *Acinetobacter guillouiae*, (k) Rhizobiales, (l) *Synechococcus*, (m) Actinomycetales, (n) *Staphylococcus*, (o) *Veillonella parvula*, (p) Chitinophagaceae



# Traditional approaches to study microbial physiology



# Methods for ecophysiology and physiology



Hatzenpichler et al., 2020

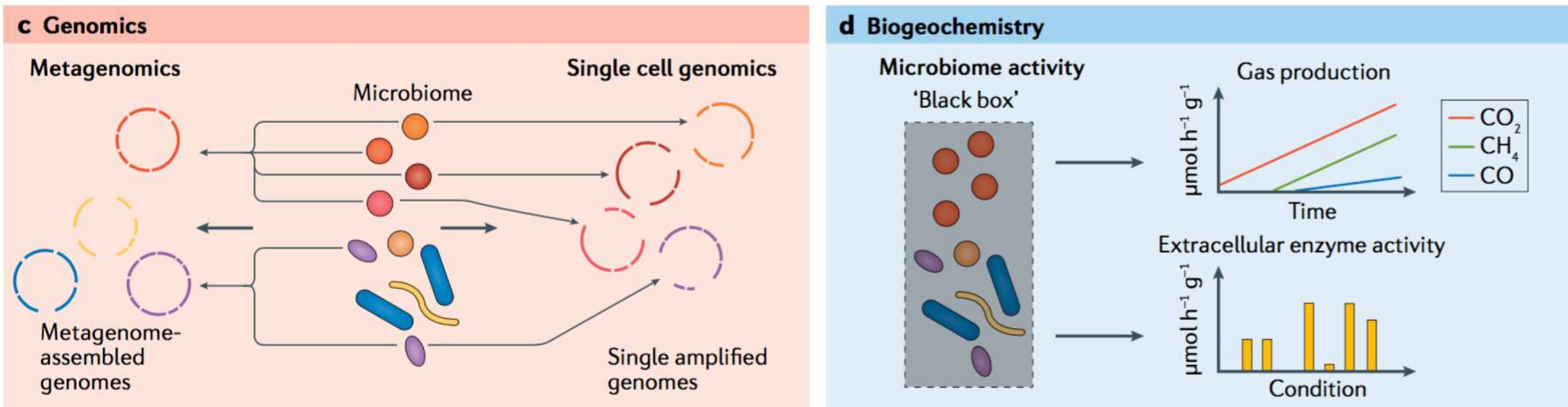
**a** | Stable isotope probing can be coupled to secondary ion mass spectrometry (SIMS) and fluorescence in situ hybridization (FISH) to link cell function and identity.

Isotopically heavy DNA can be separated from light DNA via buoyant density centrifugation.

In quantitative stable isotope probing (qSIP), multiple density fractions are collected and analysed by 16S ribosomal RNA gene sequencing or metagenomics.

**b** | If genetically tractable microorganisms are available, they can be studied using reporter–gene constructs, which enable direct insights into variation of metabolic and anabolic activity between cells.

# Methods for genomics and biogeochemistry



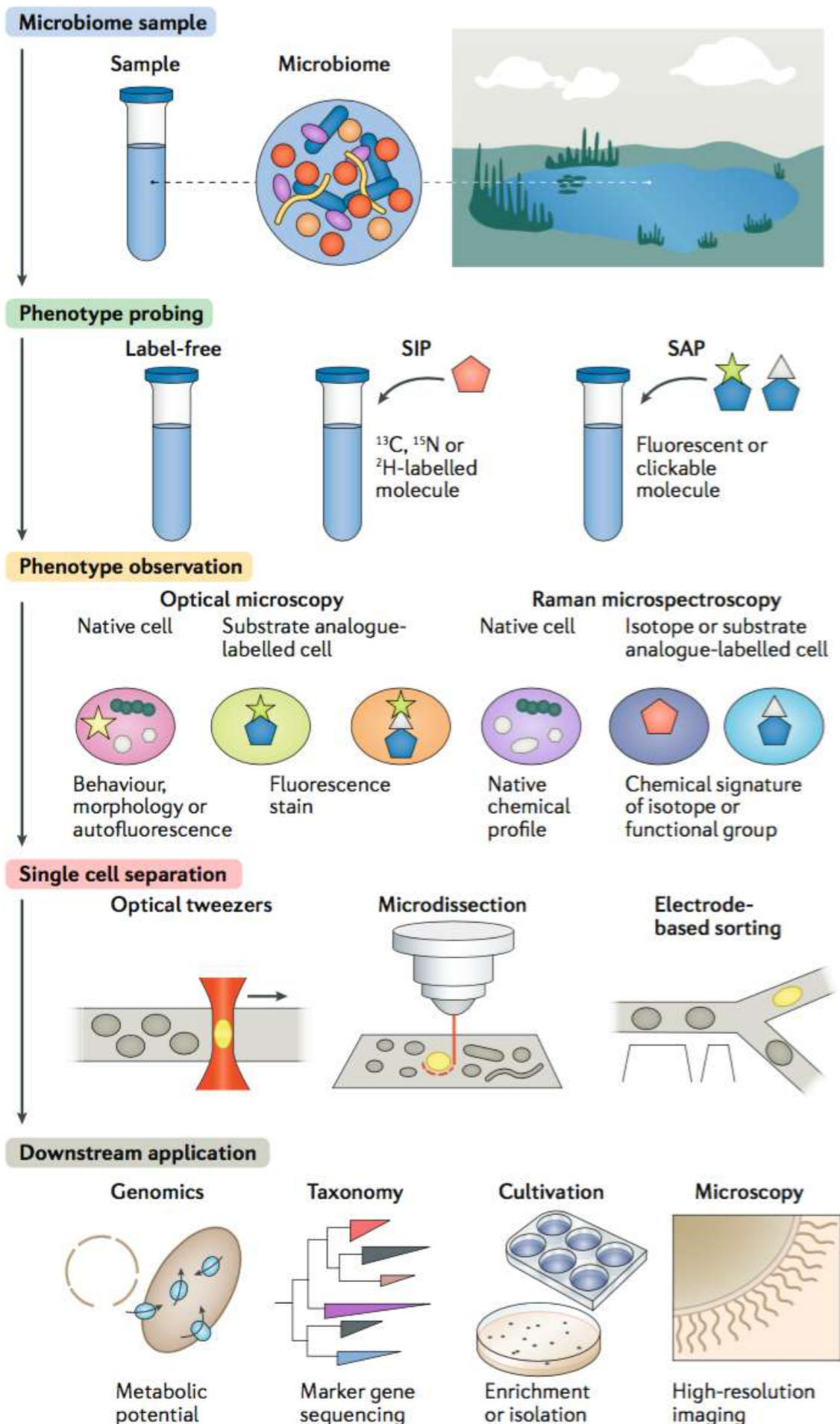
Hatzenpichler et al., 2020

**c** | The genetic make-up of entire microbial communities or individual cells can be studied by metagenomics or single cell genomics.

Whereas single cell genomics typically captures only the most abundant members of a microbial community, metagenomics integrates the genomic information obtained from many individual cells into population genomes, that is, metagenome-assembled genomes.

**d** | Many biogeochemical approaches treat microbiome samples as an undefined 'black box' but provide highly sensitive and precise measurements of overall community activity. OD<sub>600</sub>, optical density at 600 nm.

# Next-generation physiology workflow to study microorganisms



Hatzenpichler et al., 2020

A microbiome sample is obtained using minimally invasive protocols, and a phenotype of interest is detected using non-destructive methodology, for example by light or fluorescence microscopy or Raman microspectroscopy.

Label-free approaches are directed at intrinsic properties of a cell, including chemotactic behaviour, the expression of cofactors or pigments, or the presence of storage compounds.

Label-based approaches introduce a chemical reporter into the cell that provides information about dynamic processes.

Stable isotope probing (SIP) in combination with Raman microspectroscopy reveals substrate assimilation.

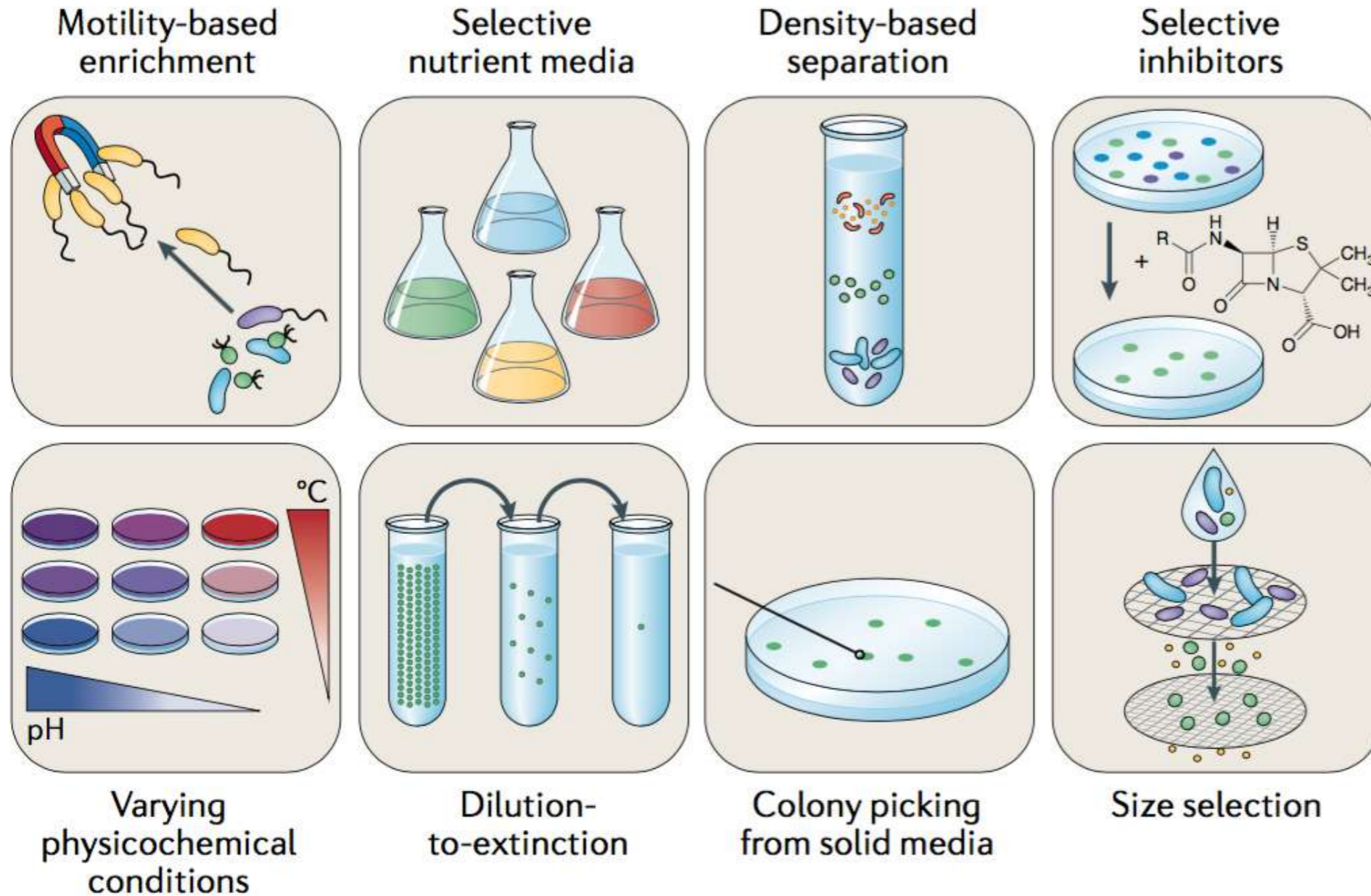
Substrate analogue probing (SAP) uses molecules that carry either a fluorescence tag or a side group amenable to azide-alkyne click chemistry to obtain information on the overall biosynthetic activity or specific enzymatic function of the cell.

After identifying a cell expressing the phenotype of interest, that same cell is separated from the sample using, for example, optical tweezers, laser microdissection or electrostatic deflection.

The unaltered, sorted cell is then committed to downstream applications, which could include whole-genome sequencing, targeted cultivation or complementary microscopic analyses.

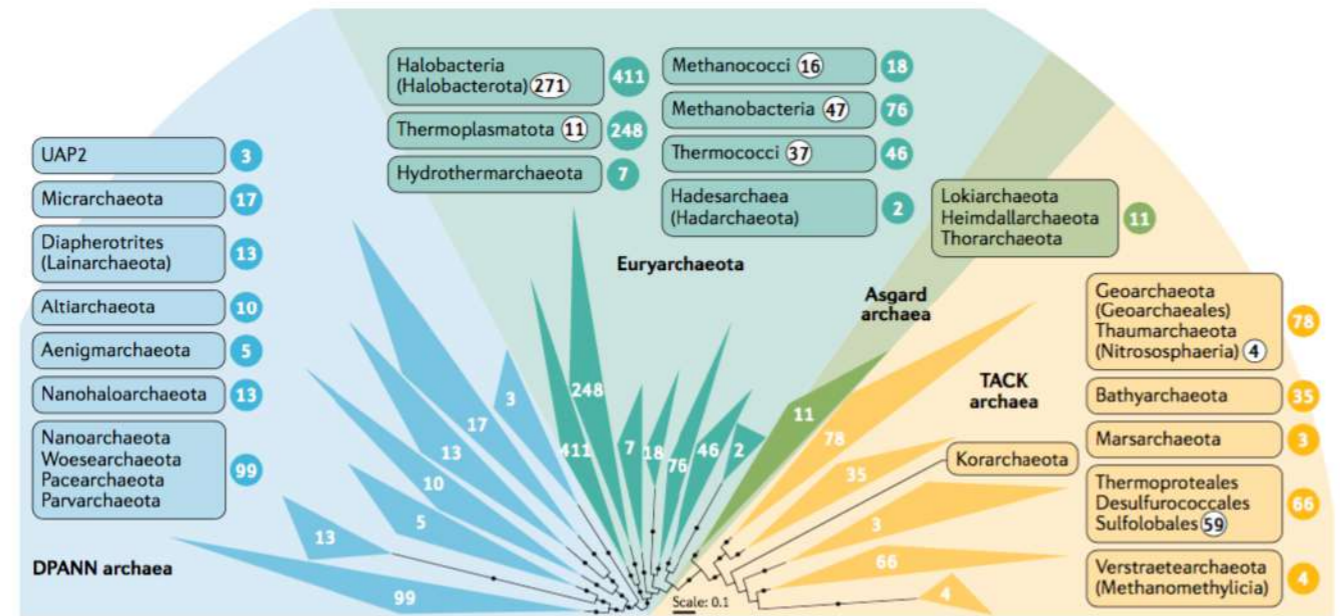
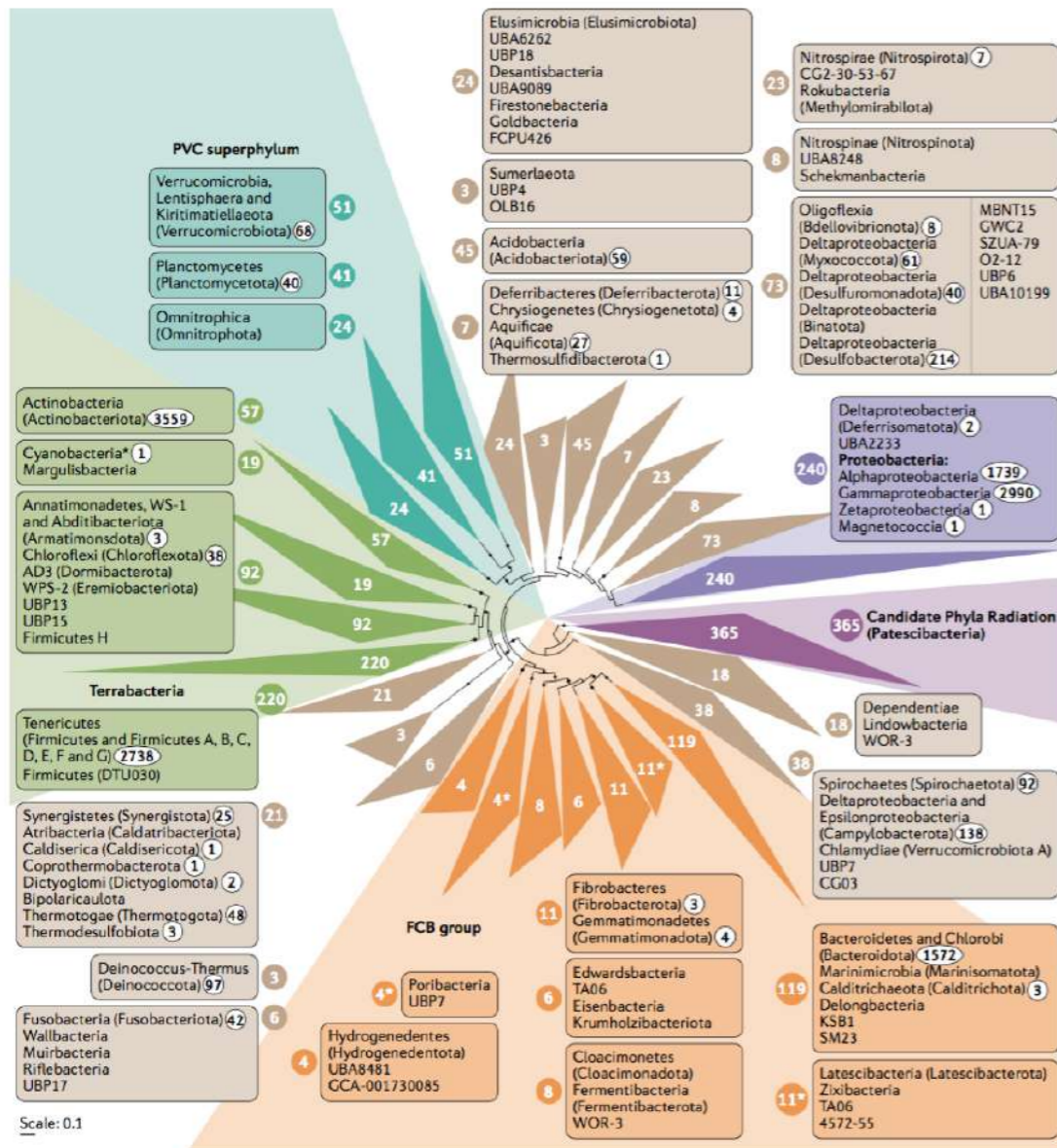
# Classical cultivation strategies and methods

Lewis et al., 2020



Method used to sterilize growth medium, the most common being autoclaving. However, besides the risk of degradation of certain components, the presence of certain components during autoclaving can lead to the formation of toxic by-products, such as hydrogen peroxide

# Census of the “cultured” ones!



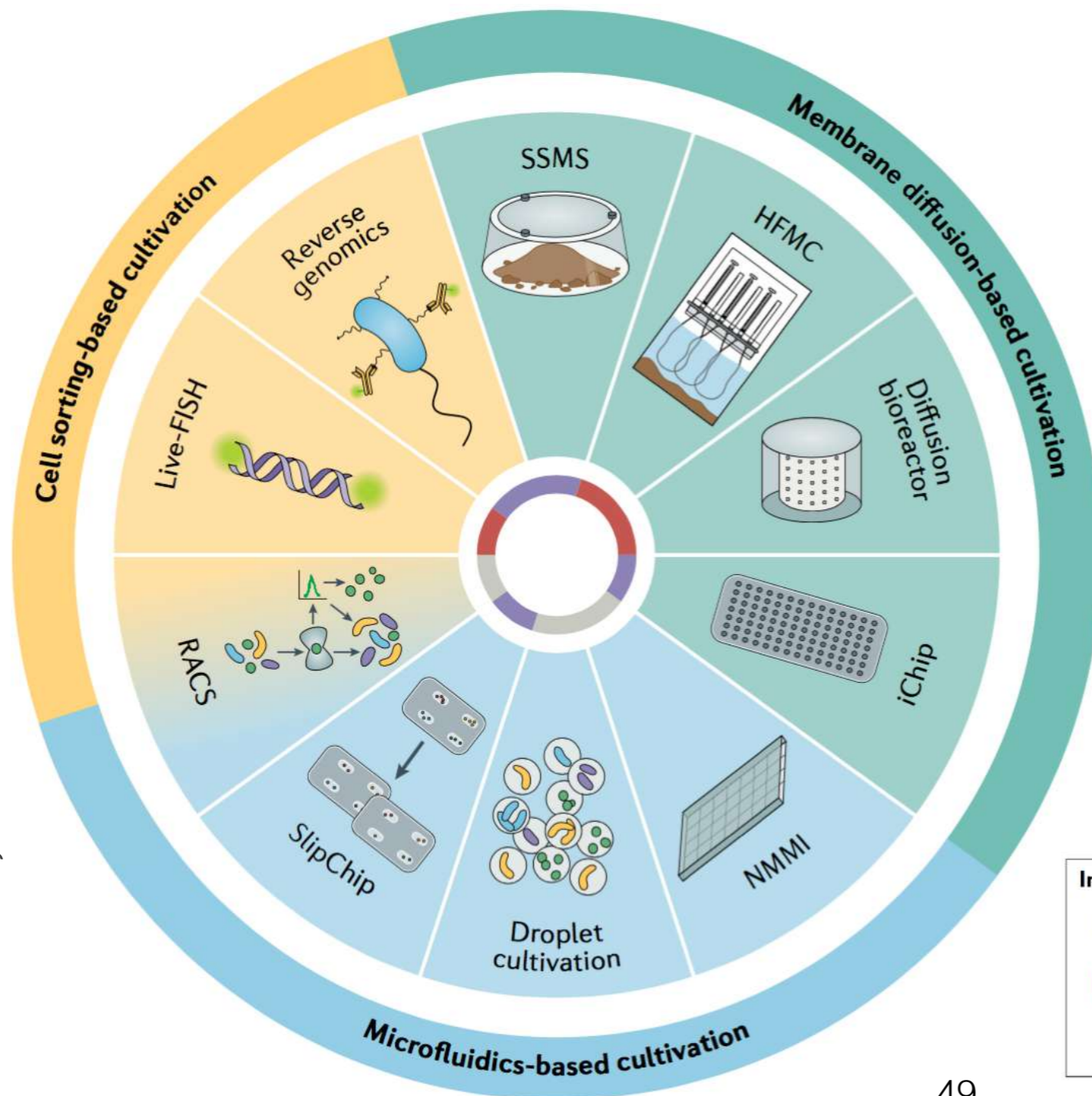
- Cultured bacteria are biased towards Bacteroidetes, Proteobacteria, Firmicutes and Actinobacteria
- Archaeal diversity is dominated by uncultured groups

A phylogenetic species tree for bacteria, inferred from concatenated alignments of a minimum of 5 out of a total 15 ribosomal proteins per species, encoded by 1,541 bacterial genomes that were obtained from the Genome Taxonomy Database

A phylogenetic species tree for archaea, inferred from concatenated alignments of a minimum of 5 out of a total 15 ribosomal proteins per species, encoded by 1,166 archaeal genomes that were obtained from the Genome Taxonomy Database



# Innovative methods for the isolation and cultivation of novel microorganisms



Membrane diffusion-based cultivation methods (**green**), such as the i(isolation)Chip, hollow-fibre membrane chambers (HFMC), diffusion bioreactors or the soil substrate membrane system (SSMS), use permeable membranes that enable nutrients and metabolites to diffuse into the cultivation medium and thereby mimic more natural conditions during cultivation.

Microfluidics-based cultivation methods (**blue**), such as nanoporous microscale microbial incubators (NMMI) or the SlipChip, are able to manipulate cells in small volumes and large numbers of replicates, and can also be combined with various droplet cultivation methods.

Cell sorting-based techniques (**yellow**), such as Raman-activated cell sorting (RACS), fluorescence in situ hybridization of live cells (live-FISH) or reverse genomics, provide a way to target a functional or taxonomic subset of cells for isolation.

Inner ring	
	Has been used to cultivate members of under-sampled or poorly characterized groups with no or few cultured representatives
	Has been used to cultivate new members of groups for which there were already cultured representatives
	Has only been applied in proof of principle experiments to cultivate or sort members of mock communities