Biological Testing of Biomaterials

Steps in Evaluation of the Biomaterials

1. Laboratory Evaluation
    - Physicochemical Characterization Tests
        - Bulk Characterization
        - Surface Characterization
    - Biological Tests
        - In vitro tests
        - In vivo tests
2. Clinical Tests

The most important property that a biomaterial should have is biocompatibility. Biocompatibility of a biomaterial is assessed using in vitro and in vivo tests.

What do we mean when we say a biomaterial is biocompatible?

- Is biocompatibility “yes” or “no,” or is there a continuum of biocompatibilities ranging from “good” to “bad?”
- How can we measure biocompatibility?
- How do we improve or enhance the biocompatibility of a biomaterial?

BIOCOMPATIBILITY TODAY ...

Definition (Williams, 1987)*:*

the ability of a material to perform with an appropriate host response in a specific application

Four factors impact that which we refer to as “biocompatibility”

- toxicology
- extrinsic organisms
- mechanical effects
- cell–biomaterial interactions.

TOXICOLOGY

- Polymeric materials often contain extractable components (unreacted monomer, oligomers, initiator fragments...).
- Metals, glasses, and ceramics can release ions and other processing components.
- The type of reaction that will be considered from a toxicology standpoint is:
  - if these substances are released and negatively impact cells (*in vitro*) or adjacent tissues (*in vivo*), or
  - if they affect an organism systemically.

THE PRODUCTS OF EXTRINSIC ORGANISMS COLONIZING THE BIOMATERIAL

- Bacteria and fungi such as *Candida* are inflammatory activators.
- In the case of implants that are contaminated with fungi, bacteria or bacterial cell-wall endotoxin, an intense and usually long-term biological reaction is seen, characterized by large numbers of white cells.
- In humans, this reaction would be described by the patient as producing pain, redness, and heat. This response to the contaminated implant can lead to exceptionally thick foreign-body capsules.
- High concentrations of extravascular white cells and thick, dense foreign-body capsules are characteristics of poor biocompatibility.

MECHANICAL EFFECTS

- “non-biocompatible”: undesirable reactions might occur if
  - rubbing, abrading or moving in contact with tissue, or
  - has sharp corners

- Mechanical mismatch between a hard biomaterial and a soft tissue:

  damage or irritation to the soft tissue

- For a better biocompatibility:
  - role of the implant designer: to ensure that the device does not excessively rub or irritate tissue
  - role of the surgeon : appropriately place and anchor the device in the implant site to minimize such rubbing and irritation

CELL–BIOMATERIALS INTERACTIONS

- Interaction of living cells with different materials may have immense influence on cell fate, including
  - attachment, spreading, proliferation, differentiation, activation, secretion, and detachment.
- Adsorbed protein film always preceding cell interaction with surfaces
- All “biocompatible” materials will heal similarly with a classic foreign-body reaction (FBR) if there are:
  (1) no leachables;
  (2) no products from extrinsic organisms; and
  (3) no mechanical irritation.
- The explanation for striking difference between *in vitro* bioreaction and *in vivo* bioreaction has yet to be identified

As a summary to biocompatibility:

- ✓Biocompatibility is negatively impacted by leachables, products of extrinsic organism surface contamination, and micromotion.
- ✓As long as leachables, extrinsic organism surface contamination, and micromotion are not impacting the reaction, all materials will give an approximately similar bioreaction *in vivo*, referred to as the normal foreign- body reaction (FBR), composed of a thin fibrous capsule and minimal ongoing inflammation.
- ✓When the foreign body capsule is thin and the reaction site is quiescent, this is an acceptable FBR and we call the implant “biocompatible (or inert).”
- ✓Inert biomaterials lead to the reaction described in point above thus are called “biocompatible.”
- ✓The favorable long-term interface between a biomaterial and the surrounding tissues is characterized by a thin, dense, collagenous capsule that isolates the biomaterial implant from the body.

NEW DEVELOPMENTS ARE CHANGING THE PARADIGM OF BIOCOMPATIBILITY

- Millions of devices made of biocompatible biomaterials are implanted in humans every year, largely with much success. But there are concerns with the way implants heal (the FBR), and new generations of materials are designed for applications in which something different than a quiescent FBR is desired.
- *Third generation* biomaterials (1990+) biospecifically orchestrate biological processes, and can have direct regeneration and restore functionality, and/or respond to the environment in a pro-active manner to favorably influence a tissue reaction. As biomaterials evolve, so the definition of biocompatibility must evolve too.
- Thus, the way we define biocompatibility may change over the next few years. Recent clarification on the diversity of macrophage phenotypes and understanding of resident tissue stem cell pools in the body may permit engineered "biocompatibility," with control of the extent, rate, and speed of integration. Precision control of biocompatibility can lead to new biomaterials-based therapies with profound advantages for the patient.

Biological Tests

Biological characterization

Biological properties of a biomaterial determine the ***short- and long-term potentials*** of the biomaterial.

- In vitro and in vivo tests (to analyze biocompatibility of the biomaterials and determine their possible time-dependent changes)
- Antibacterial tests (to evaluate antibacterial properties of the biomaterials)
- Interaction between the biomolecules and the biomaterials can be investigated by using cells, proteins, etc.

Differences…

- **In vitro:** performed or taking place in a test setup including **cells isolated from a living organism** and constructed **outside any body** in controlled laboratory conditions

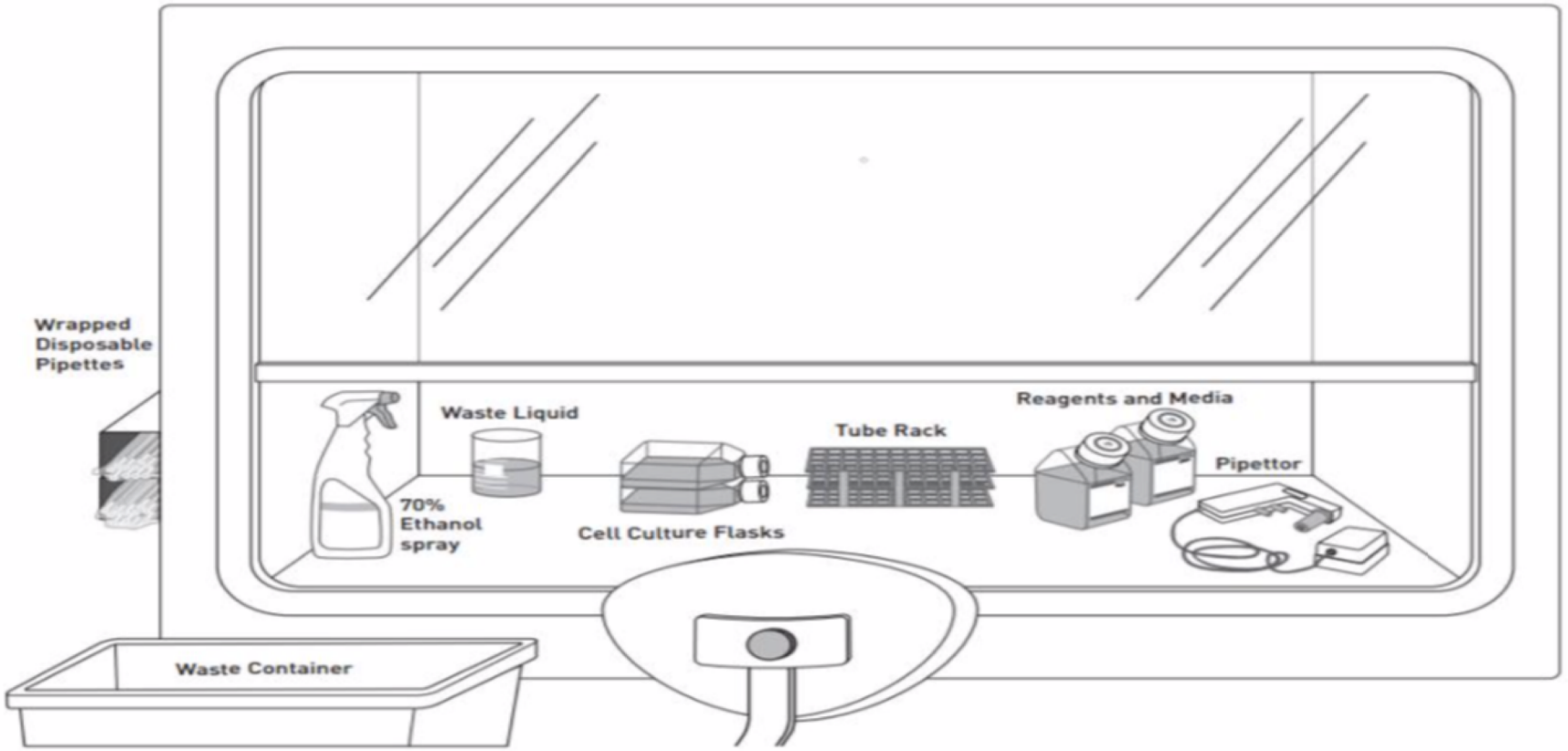
- **In vivo:** performed or taking place **inside animal bodies**

Cell Culture Studies

Cell culture is a method of multiplying cells under controlled laboratory conditions. This method is carried out to study cellular functions, structures and behaviors and in vitro tests are based on cell culture studies.

Basic Equipments of Cell Culture:

- Cell culture hood (i.e., laminar-flow hood or biosafety cabinet): Provides an aseptic work area.



The basic layout of a cell culture hood.

Other...

- Incubator (also known as CO_2 incubator)
- The purpose of the incubator is to provide the appropriate environment (37^oC, 5 % CO_2 and humidity) for cell growth.
- - Water bath
  - Centrifuge
  - Refrigerator and freezer (–20°C)
  - Cell counter
  - Inverted microscope
  - Liquid nitrogen freezer

    It is used mainly for cell storage.
  - Sterilizer (i.e., autoclave)

Important Definitions Related with Cell Culture

Culture media: Culture media is the most important component of the culture environment, because it provides the necessary nutrients, growth factors and hormones for cell growth as well as it regulates the pH and the osmotic pressure of the culture. The three basic classes of culture medium are basal medium, reduced-serum medium and serum-free medium, which differ in their requirement for supplementation with serum.

- Serum: It is medium supplement added into the basal medium. Serum is a variable mixture of growth and adhesion factors, hormones, lipids, minerals, trace elements and proteins.

(Serum is the blood fraction remaining after the coagulation of blood, followed by centrifugation to remove any remaining blood cells. In cell culture studies, serum isolated from animals like calf and bovine is generally used)

Important Definitions Related with Cell Culture

Subculturing (passaging the cells): It is the process at which the cells from a previous culture are transferred to a new culture vessel with fresh culture medium. This process is periodically required to provide cells with enough growing space as the number of cells is increased by time.

All cells used in cell culture studies are divided into two groups depending on their adhesion properties:

- Anchorage dependent cells: These cells require attachment for growth. Thus, they adhere to the surfaces of culture vessels. Anchorage dependent cells are usually derived from tissues in which the cells are immobile and embedded.
- Anchorage independent (or suspension) cells: These cells do not require attachment for growth. In other words, these cells do not adhere to the surfaces of culture vessels. All suspension cells are the cells of the blood system.

In Vitro Tests

- In vitro tests are performed in a test setup including cells isolated from an organism and constructed outside any body in controlled laboratory conditions.

Advantages	Disadvangages
✓ They are experimentally controllable and repeatable. ✓ They provide relatively fast processing of high numbers of biomaterials in comparison to in vivo tests. ✓ They avoid the ethical issues.	× In a body, nutrients and wastes are supplied and removed via a circulation system in a continuous manner. However, in in vitro tests, supplementation and removal of these substances are performed every third day since change of culture medium of the cells is conducted every third day. × Mechanical deformation is well linked with matrix formation and essential functions of the cells of the cardiovascular and musculoskeletal systems in the body. However, mechanical strains that are so prevalent in these systems are absent in in vitro tests. × In a body, different kinds of cells exist and they communicate with each other constantly. However, it is generally hard to use different kinds of cells in in vitro tests.

What can be evaluated via in-vitro tests?

- Cytotoxicity (the property of a material to kill the cells)
- Blood compatibility of a material
- Antibacterial property of a material
- Possible effects of a material on the following properties of the cells:
  - Cell morphology
  - Cell proliferation (is an increase in the number of live cells as a result of cell division.)
  - Cell differentiation (is the process by which a cell becomes specialized in order to perform a specific function)

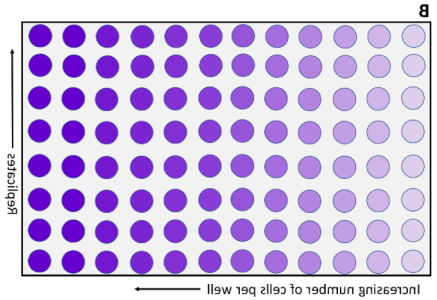
Cytotoxicity

A toxic material is defined as a material that releases a chemical in sufficient quantity to kill cells. The number of cells that are killed by the material is an indication of the cytotoxicity degree of the material.

Tests for Cytotoxicity:

a) Qualitative Tests (Optical microscopy techniques are used)
   - Direct Contact Test
   - Agar Diffusion Test
   - Extract Test (Elution Test)

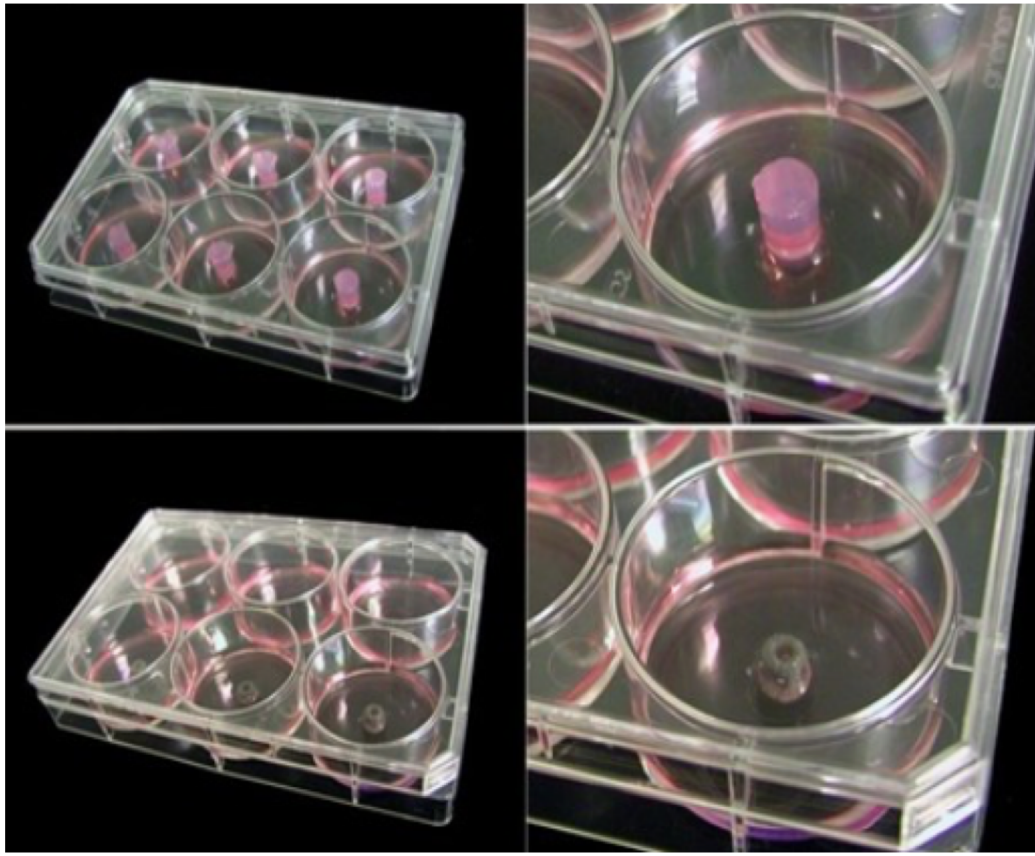
b) Quantitative Test (This test is a colorimetric method)
   - MTT Test



Cytotoxicity-Qualitative Tests

Direct Contact Test

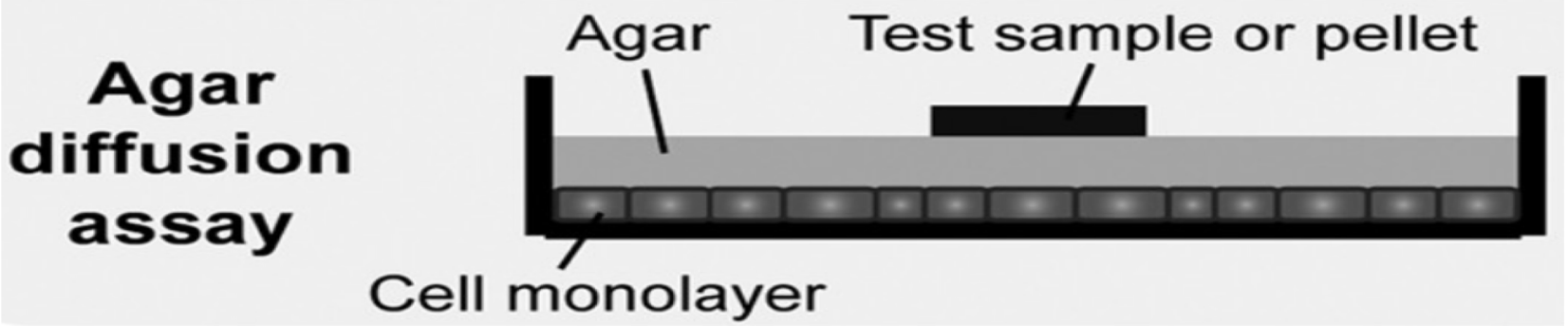
- In this qualitative method, a piece of test material is placed directly onto anchorage dependent cells growing in culture medium. At the end of a specific period of incubation at 37°C and 5 % CO_2, the culture medium and test material are removed, the cells are washed with phosphate buffered saline and stained with a cytochemical stain (dye). **Dead cells lose their adherence to the culture vessel** and are lost during the washing process. **Live cells continue to adhere to the culture vessel and are stained.**
- **Cytotoxicity degree is microscopically determined by the absence of stained cells under and around the periphery of the test material.**
- **Direct Contact Test is recommended for low density materials** such as contact lens polymers.



Cytotoxicity-Qualitative Tests

Agar Diffusion Test

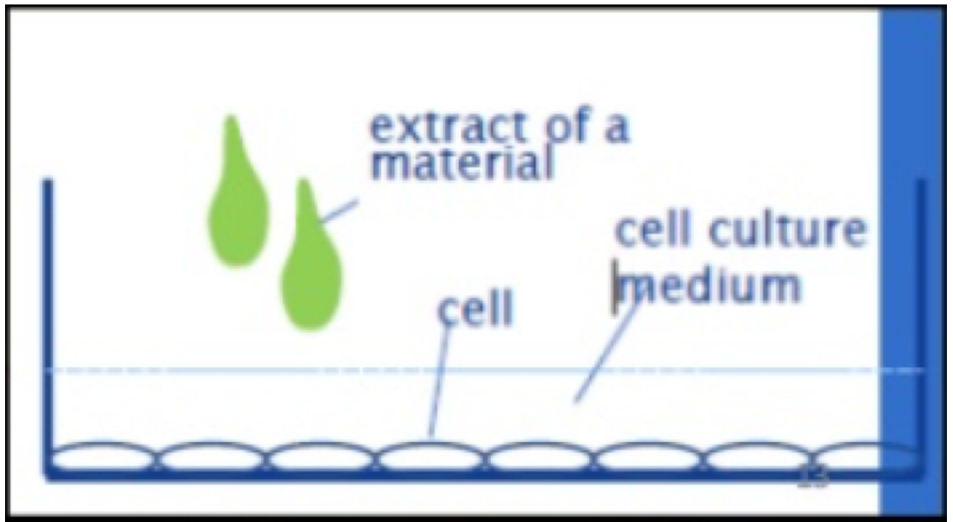
- In this qualitative method, a thin layer of nutrient-supplemented agar is placed over the anchorage dependent cultured cells. The test material is placed on top of the agar layer and the cells are incubated with the test material for a specific period of time at 37°C and 5 % CO_2. This method often includes a neutral red vital stain which allows ready visualization of live cells. After corresponding incubation, the test material is removed and the stain is incorporated into the agar. **The stain passes through the agar, reaches the cells, and is taken up and retained by live cells. Dead cells do not retain neutral red and remain colorless.**
- Similar to Direct Contact Test, **cytotoxicity degree is microscopically determined by the lack of the vital stain under and around the periphery of the test material**.
- This method **can be used for high density materials**.



Cytotoxicity-Qualitative Tests

Extract Test (Elution Test)

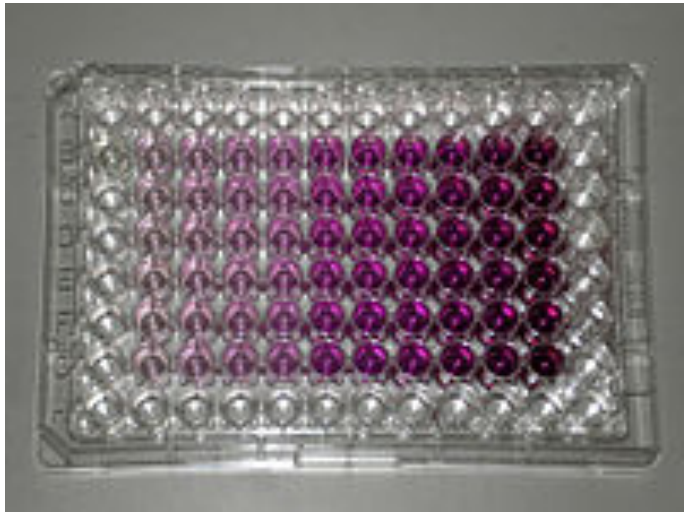
- In this qualitative test, **an extract** (a solution containing the extracted compounds) of a material **is prepared**. The cells are treated with the extract for a specific period of time at 37°C and 5 % CO_2.
- Similar to Direct Contact and Agar Diffusion Tests, **cytotoxicity degree is determined by distinguishing live and dead cells with the use of special kinds of stains. This method is also based on microscopic evaluation.**



Cytotoxicity-Quantitative Test

MTT Test

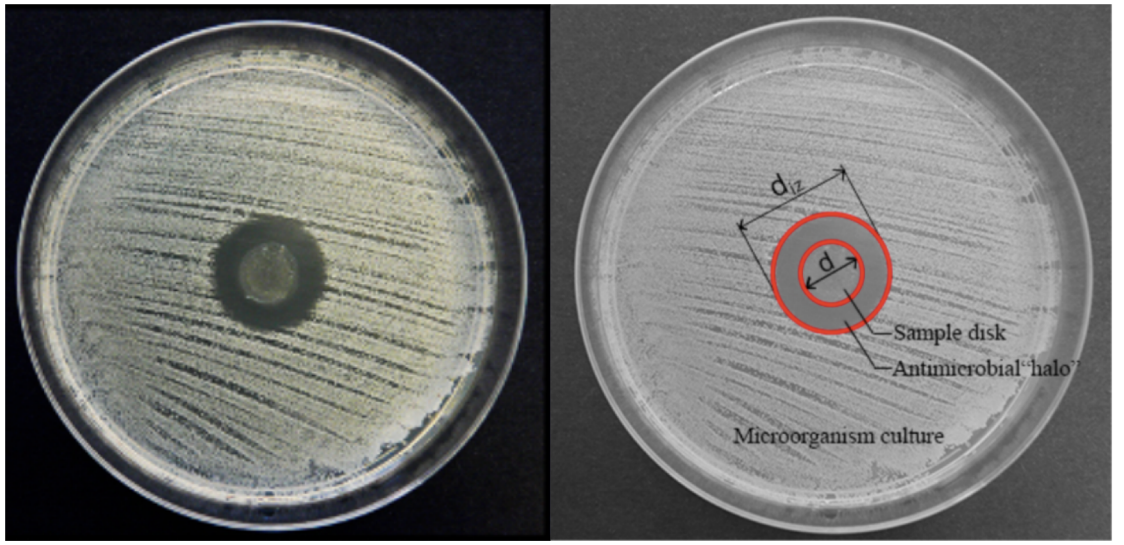
In this quantitative test, after the cells are either directly interacted with the material as in Direct Contact Test or treated with an extract of a material as in Extract Test (Elution Test), the solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is added into the culture medium of the cells. After specific period of incubation at 37°C and 5 % CO_2, it is seen that the color of the culture medium changes to purple. Because **live cells produce formazan which has a purple color. Thus, the intensity of the purple color is correlated with number of live cells.** The intensity of the color is determined by measuring the optical density of the culture medium at the specific wavelength with a microplate spectrophotometer. **By comparing with the control** (cells which are not interacted with the material), **the amount of decrease in the optical density of the culture medium is found and it refers to cytotoxicity degree of the material.**



Antibacterial Testing

- Implant failure is often a consequence of microbial infections.
- Testing of antibacterial behaviour is generally carried out by 2 ways:
  - **Agar Disk Diffusion Test (Diffusion Method)**
  - **Measurement of Antimicrobial Activity on Material Surfaces (Contact Method)**

Antibacterial Testing



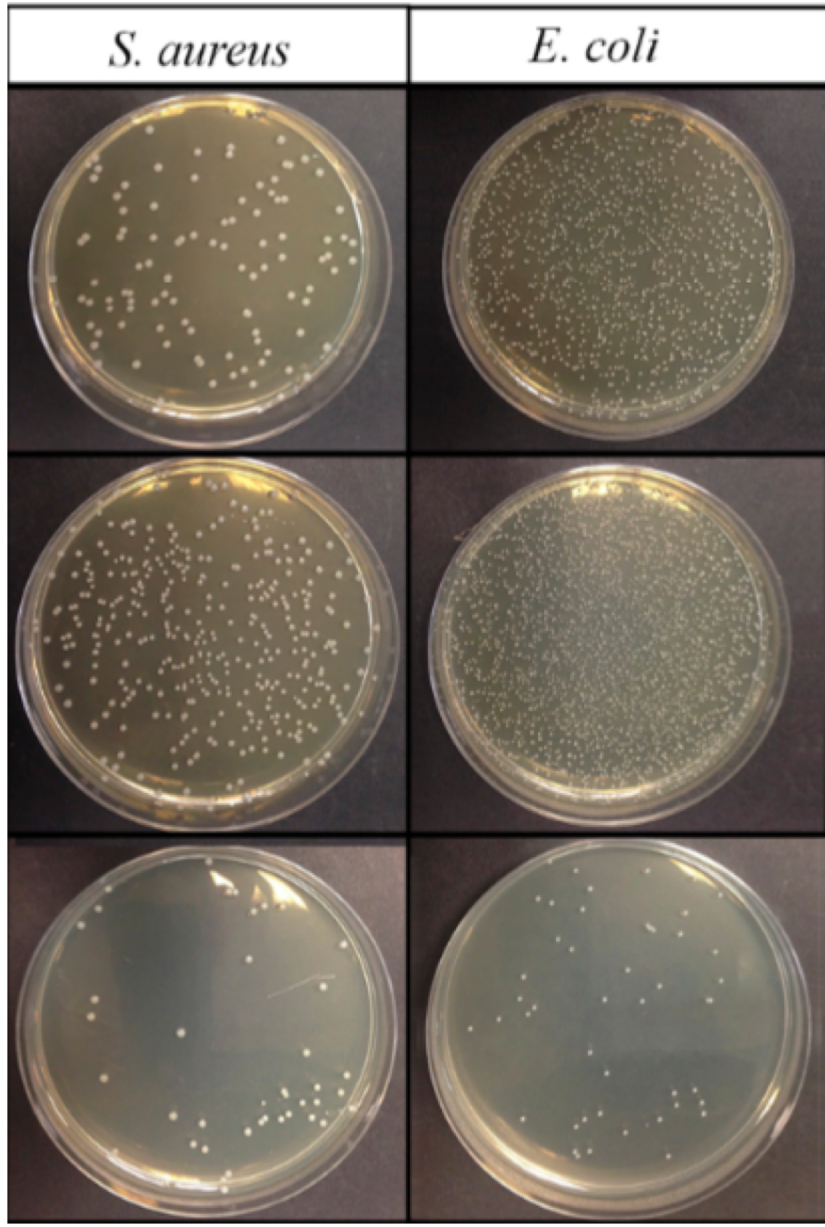
Disk Diffusion Test

- Agar plate is prepared.
- Bacteria is put onto the agar plate.
- The biomaterial in the shape of a disc is placed onto the surface of the agar plate.
- Plate is incubated at 37°C for a specific period of time.
- If present, the diameter of inhibition zone is measured. Inhibition zone is the area where the bacterial growth is inhibited due to existence of the biomaterial. Therefore, **the diameter of inhibition zone corresponds to the antibacterial capability of the biomaterial. High diameter of inhibition zone refers to high antibacterial property of the biomaterial.**

Antibacterial Testing

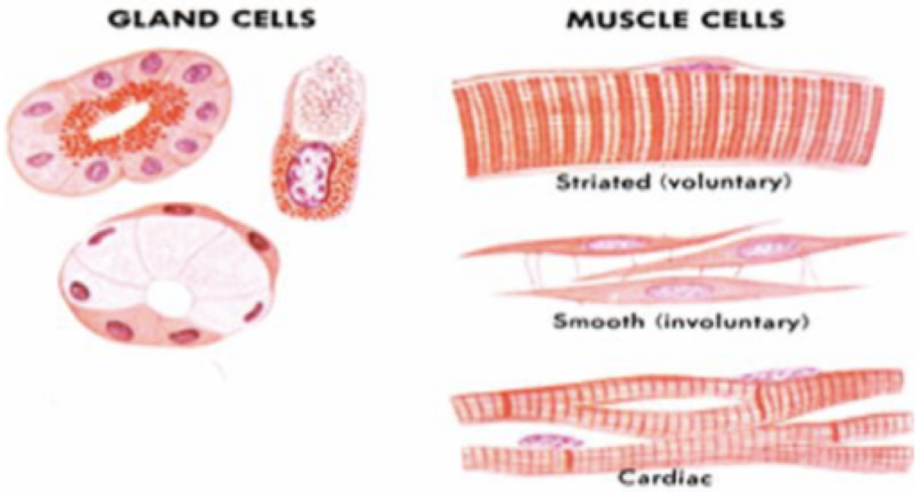
Elution Method

- One or two bacteria colonies were introduced into the bacteria growth media (Trypsin Soy Broth-TSB) and mixed
- Putting it in the shaker incubator for 12 to 24 hours.
- Taking it out of the incubator and read the optical absorbance to prepare the initial concentration of bacteria for your experiments.
- Putting the samples on a well plate and cover them with the previously prepared bacteria solution.
- Incubating the well plate for the amount of time required (usually between 4 and 24 hours).
- Samples are washed with Phosphate Buffer Solution (PBS) and introduced in proper tubes tubes with proper amount of PBS added
- Samples are sonicated your for 10min and vortexed for 10sec. Make 3 to 4 dilutions (x100, x1000, x10000, ...) of each sample, and add 3 droplets of each dilution in an agar plate.
- Incubating for 12 hours until the colonies are formed and they are big enough to count them (put the plates upside down inside the incubator).
- The colonies are counted after the 12hours incubation.



Cell Morphology

- **The shape and size (morphology) of the cells can be analyzed in order to qualitatively evaluate the health of the cells.** The morphology of cells can be influenced by a variety of factors including their interaction with the biomaterial, signals from other cells and cell density, etc.
- Cell morphology is evaluated using optical and confocal microscopy techniques. Electron microscopy provides a high level of detail but is a destructive test as it entails the fixing and coating of cells.



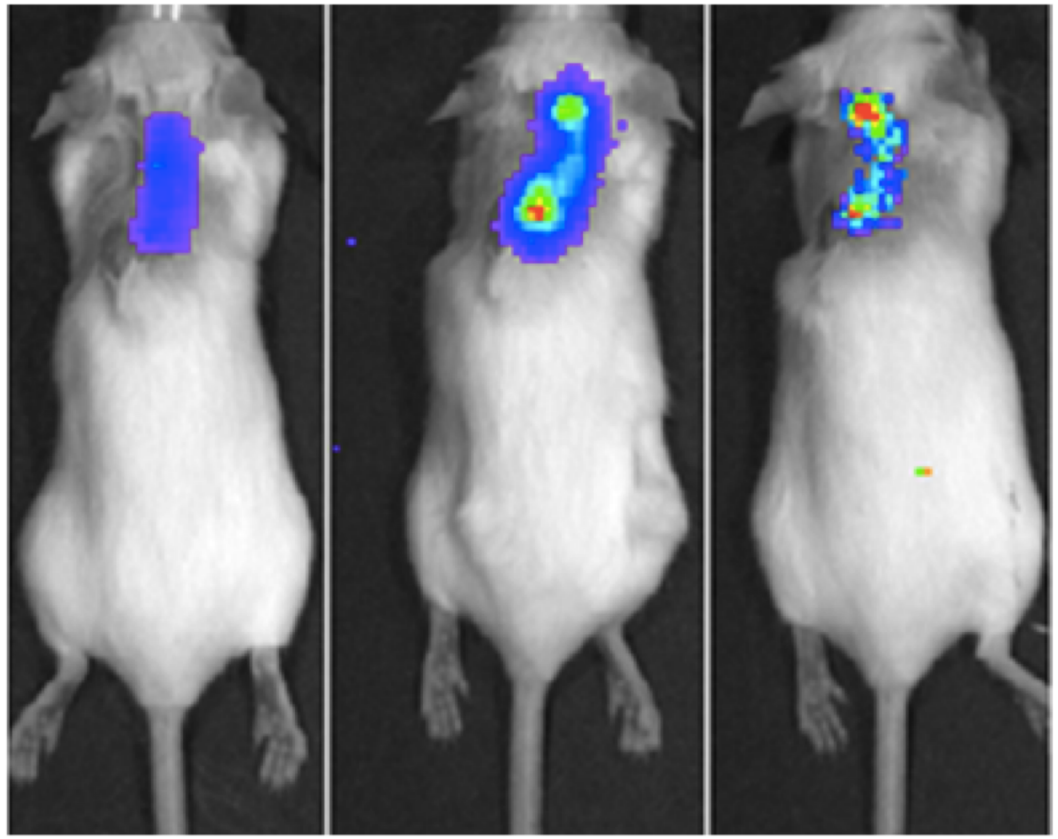
Different types of cells exhibit different morphologies.

Cell Proliferation

The cells interacted with a biomaterial (e.g. cells seeded on a biomaterial) **should ideally proliferate. Proliferation of the cells interacting with a biomaterial for a particular period of time is determined by conducting quantitative tests like MTT at different time points of the period.** By these tests, at each point, the number of live cells is calculated and the curve of the number of live cells versus time is plotted to obtain proliferation profile of the cells. However, such tests require a reference standard correlating the parameter measured by the tests (e.g. optical density measured by MTT) to the number of live cells.

In Vivo Tests

In vivo tests are performed **inside animal bodies**. The **main goal** of in vivo tests is to **evaluate the biocompatibility of a biomaterial in a biological environment.**



In vivo optical imaging of implant associated infection at consecutive days in the same mouse.

Types of In Vivo Tests

Sensitization tests: These tests help to determine whether a material contains chemicals that cause adverse local or systemic effects after repeated or prolonged exposure. These allergic or hypersensitivity reactions involve immunologic mechanisms. Studies to determine sensitization potential may be performed using either specific chemicals from the test material, the test material itself, or most often, extracts of the test material. Symptoms of sensitization are often seen on skin and these tests are generally carried out topically.

Intracutaneous (intradermal) reactivity tests: These tests provide to determine the localized reaction of the skin of an animal to an extract of a material after the extract is injected within the skin and extract-skin interaction is maintained for a specific period of time.

Blood compatibility tests: These tests provide to evaluate the effects of a blood-contacting material on blood coagulation and/or components of the blood of an animal.

Types of In Vivo Tests

Systemic toxicity tests: These tests provide to estimate the potential harmful effects of a material or its extract on tissues and organs away from the point of contact (i.e. implantation/injection site of the material or its extract). These tests are carried out after either single or multiple exposures of the tissues and organs to a material or its extract for a specific period of time. In these tests, materials that release constituents into the body or their extracts are analyzed. These tests are divided into four groups:

i) Acute toxicity tests: These tests provide to evaluate the adverse effects occurring after administration of a single dose or multiple doses of a test material within a period of 24 hours.

ii) Subacute toxicity tests: These tests provide to evaluate the adverse effects occurring after administration of a single dose or multiple doses of a test material per day within a period of 14 to 28 days.

iii) Subchronic toxicity tests: These tests provide to evaluate the adverse effects occurring after administration of a single dose or multiple doses of a test material per day within a period not exceeding 10 % of the life span of the animal (e.g. 10 % of the life span of rats corresponds to about 90 days).

iv) Chronic toxicity tests: These tests provide to evaluate the adverse effects occurring after administration of a single dose or multiple doses of a test material per day within a period of at least 10 % of the life span of the animal.

Types of In Vivo Tests

Genotoxicity tests: These tests are carried out if in vitro test results indicate potential genotoxicity of a material.

> Genotoxicity is the property of a material to negatively effect DNA of the cells of an organism and, in turn, to cause changes in cell proliferation, differentiation and/or function, etc.

Implantation tests: These tests provide to assess the local effects of a material on the structure and functions of a tissue at the site where the material is surgically implanted. In the scope of these tests, immunohistochemical staining of histological sections of the tissue can be performed to determine the types of cells present and collagen formation or destruction in the tissue.

Carcinogenicity tests: These tests provide to determine the tumorigenic potential of a material or its extract after the body of an animal is exposed to the material or its extract for a period of the major portion of the life span of the animal.