MICRO-561

Biomicroscopy I

Syllabus (tentative)

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Live Cell Imaging

Live cell imaging has become a major research focus in cell biology.

➔ Biomicroscopy offers ability to monitor a variety of dynamic intracellular events with high spatial and temporal resolution over a wide range of time scales.

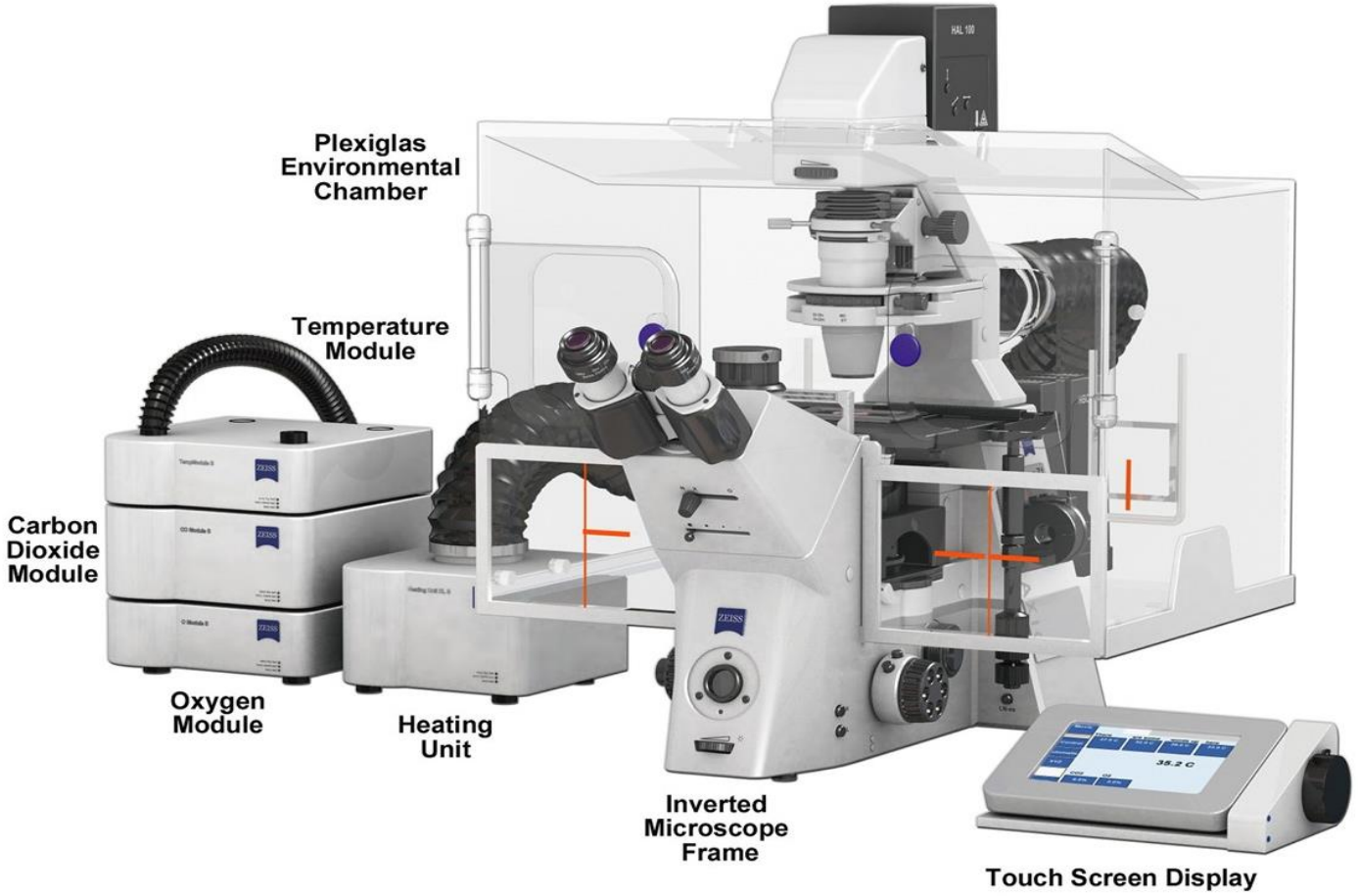
Some of the main concerns are:

- Maintaining cell viability on the microscope
- Minimizing photo-toxicity from exposure to light
- Reducing the rate of photobleaching of dyes.

New developments in the instrumentations are helping to overcome these changes, such as:

- Climate-controlled environmental incubator systems
- High-performance digital cameras
- Advanced filter technology
- Narrowband illumination sources
- Advances in fluorescent dyes (bright, photo-stable, genetic coupling) for single & multi-color imaging

Live-cell imaging microscope configuration



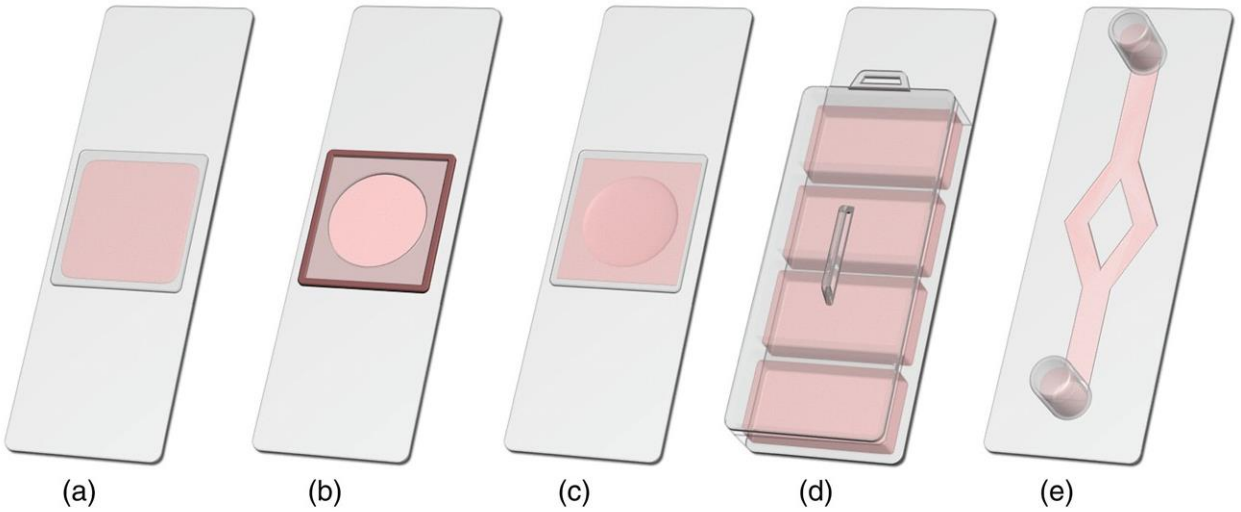
- The upper portion of an inverted microscope frame is enclosed in a Plexiglas environmental chamber to maintain temperature.
- Warm and humidified air mixture with 5% Co2 is injected into the chamber.
- Several hinged and sliding doors provide access to the stage and specimen.
- The microscope control panel is accessible to the operator

Control of Environmental Conditions

- Control of environmental factors is critical for successful live-cell imaging.
- While it may be easier to maintain cells in a tissue culture incubator, the task of growing cells on a microscope stage for long term imaging experiments is more demanding.
- Table below gives some typical environmental parameters:

Variable	Optimum Range	Comments
Humidity	97–100%	Closed chamber or humidified environmental chamber
pH	7.0–7.7	Use HEPES-buffered media or change medium regularly or use perfusion
Osmolarity	260–320 mOsm	Avoid evaporation, seal chamber or use humidified environmental chamber
Oxygen	Variable	Perfuse or change media regularly or use large chamber volume
Temperature	28–37°C	Control with chamber heaters, objective heaters, inline heaters, or environmental control boxes

Example: Live-cell imaging chambers with microscope slides



(a) Coverslip attached to the microscope slide with rubber cement.

(b) Coverslip with adhesive - backed silicone gasket to seal the chamber.

(c) Coverslip attached to a concave microscope slide for imaging thicker specimens, such as embryos of small intact organisms.

(d) Multi-chamber slide (called “lab-on-a-slide ”) for holding several individual cultures. These slides are available from several manufacturers in a number of configurations, ranging from a single chamber to ~ten separate chambers.

(e) Flow chamber for introducing external factors (e.g. fresh medium, stimulants, analytes/drugs …). These slides have Luer-style adapters for perfusion couplers.

Time-Lapse

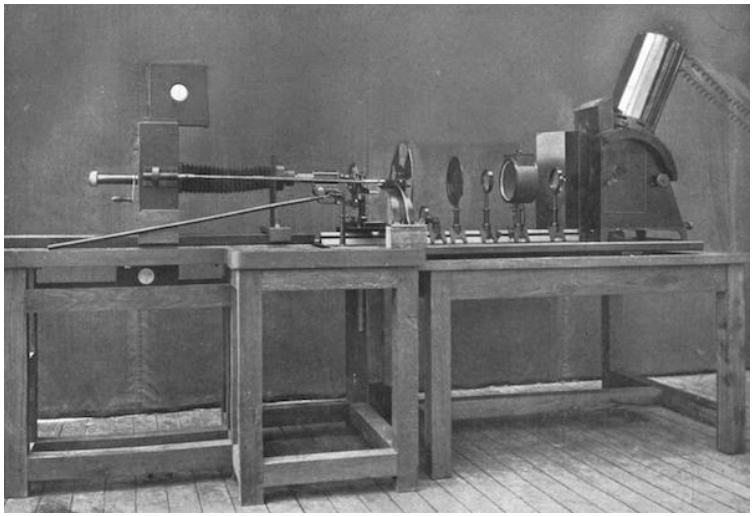
Time-lapse microscopy is the method that extends live cell imaging from a single observation in **time** to the observation of cellular dynamics over long periods of **time**.

- Dynamic imaging of biological activity first was introduced in 1909 by the French doctoral student Jean Comandon, who presented the time-lapse films of living spirochaetes, 5 years before Charlie Chaplin made his first movie.

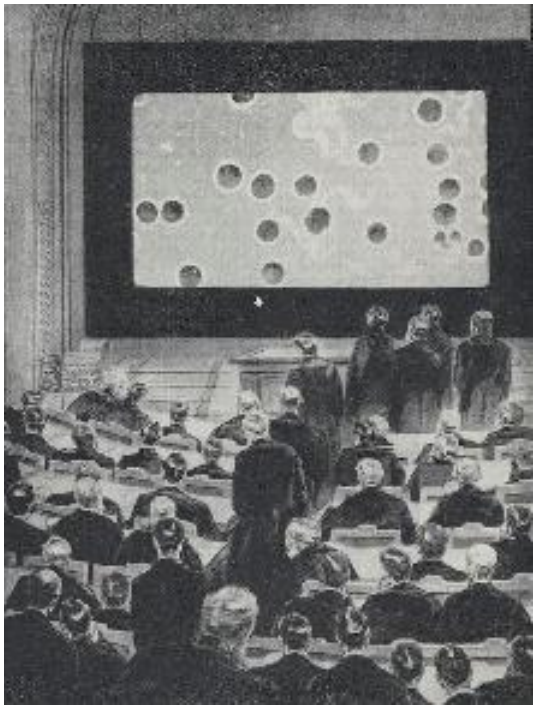
➔ The technique, which he called *micro-cinematography*, enabled movies by recoding events at microscopic scales with a cinema-style camera bolted onto a darkfield microscope.

➔ These films were useful in teaching physicians how to distinguish disease-causing spirochaetes from those that are harmless.

Jean Comandon in the laboratory of the Hôpital Saint-Louis, 1914



Jean Comandon's apparatus for taking motion pictures of microbes, circa 1914



An image of one of Jean Comandon's presentations at the Académie de Médécine, originally printed in the magazine "Je sais tout" in January 1914. Image credit: BnF

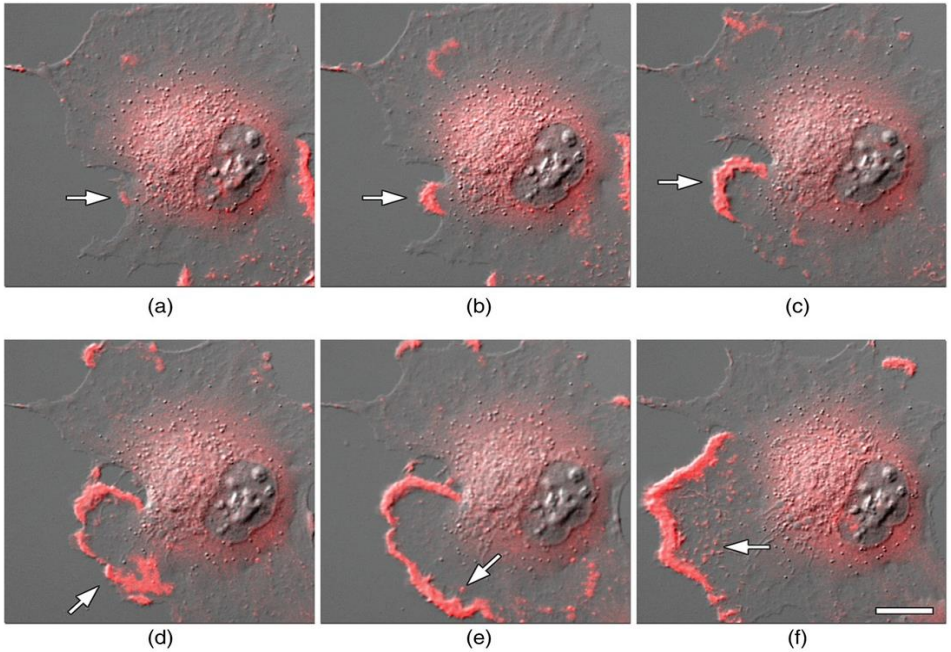
Time-Lapse

Time-lapse microscopy is the method that extends live cell imaging from a single observation in **time** to the observation of cellular dynamics over long periods of **time**.

- With the state-of-the-art digital camera systems, time-lapse microscopy is being applied to capturing events that occur in living cells over periods ranging from a few seconds to several weeks (or even months).

- When time-lapse investigations are coupled to labeling cells with synthetic fluorophores and genetically encoded fluorescent proteins, dynamic events and interactions at the subcellular and molecular levels can be investigated.

Time-lapse microscopy – an example



Selected images from a 24-hour time-lapse sequence of adherent rabbit kidney epithelial cells.

The cells contain a chimeric vector composed of mCherry fluorescent protein and human β-actin to reveal the distribution of actin in lamellipodia.

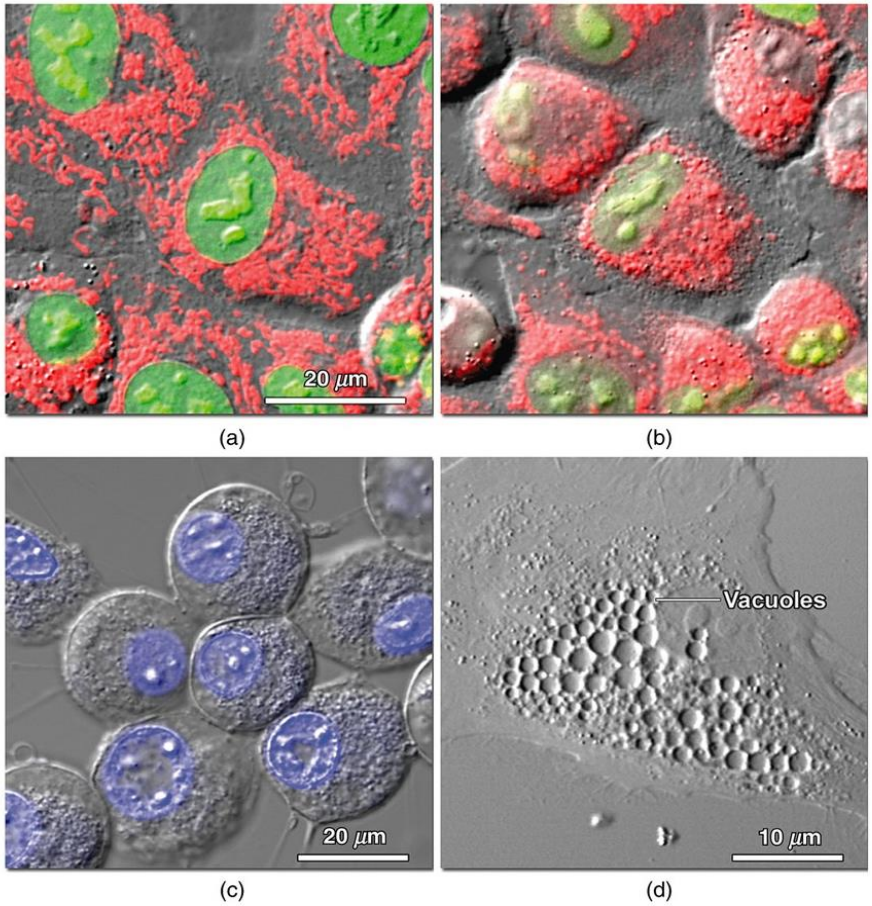
Scale bar = 20 μ m.

(a) Elapsed time (ET) = 1 minute; the white arrow points to the initiation of a cytoplasmic ruffle near the large pool of actin in the central region of the cell.
(b) ET = 5 minutes; arrow points to growing ruffle.
(c) ET = 10 minutes; ruffle continues to grow.
(d) ET = 15 minutes; the small ruffle begins to swell and extrude toward the upper left-hand side of the image, in a wave-like motion, concentrating the brightly labeled actin fusion protein into the leading edge as it grows.
(e) ET = 20 minutes; as the lamellipodium spreads to cover a large area, small clusters of labeled actin form behind the leading edge to form structural elements that may be podosomes (white arrows in panels e and f) that play a role in cell adhesion to the glass substrate.
(f) ET = 25 minutes.

Phototoxicity → Control of Illumination

- In time-lapse microscopy, in addition to the photo-bleaching issue of fluorescent labels, the **detrimental effects of light on living cells must also be considered**.

- There is a trade-off between limiting the amount of light exposure to prevent toxicity while still being able to acquire images with sufficient detector signal –to noise ratio.



Examples for phototoxicity during live-cell imaging:

(a) Rabbit kidney cells expressing EYFP fused to a nuclear localization signal (green nucleus) were treated with synthetic dye MitoTracker Red.
(b) Same view-field as in panel **a** after time-lapse imaging for 2 hours at 15 seconds intervals. Note the fragmentation of mitochondria and rounding of cells due to photo-toxicity.

(c) Labelled HeLa cells are imaged for 30 min at 1-sec intervals with 405-nm laser. Cells have detached from the coverslip and are rounded.

(d) Vacuole formation in a fiberblast cell after imaging for 8 hours at 30-sec intervals using tungsten halogen illumination

Photo-toxicity and Photo-damage

- Aside from **cellular toxicity** due to high concentration of dyes and overexpression of fluorescent proteins, living cells are subject to light-induced damage, called **photo-toxicity**.

- Light, especially at short wavelengths, reacts with molecules to create free radicals.
- The presence of molecular oxygen (O_2) enhances this process.
- Mammalian cells are sensitive to UV, blue and infrared (IR), and least sensitive to green, yellow and red light.

- Additionally, some constituents of cell culture media (i.e. vitamin riboflavin and tryptophan) may also contribute to adverse light-induced effects in cultured cells.

- Fluorescent proteins are generally less toxic (at low and optimized concentrations), but the synthetic dyes (i.e. MitoTracker) can be toxic when they are illuminated.
- Some fluorescent molecules at their excited states can react with O_2 such that their fluorescent properties get destroyed by producing free radicals and these in turn can damage cells.

Dealing with Photo-toxicity and Photo-damage

➔ **Best practice is to minimize exposure to light:** for example use fluorophores with longer excitation wavelengths, and avoid exposing cells to short wavelengths of illumination.

➔ **Photo toxicity can be reduced by:**

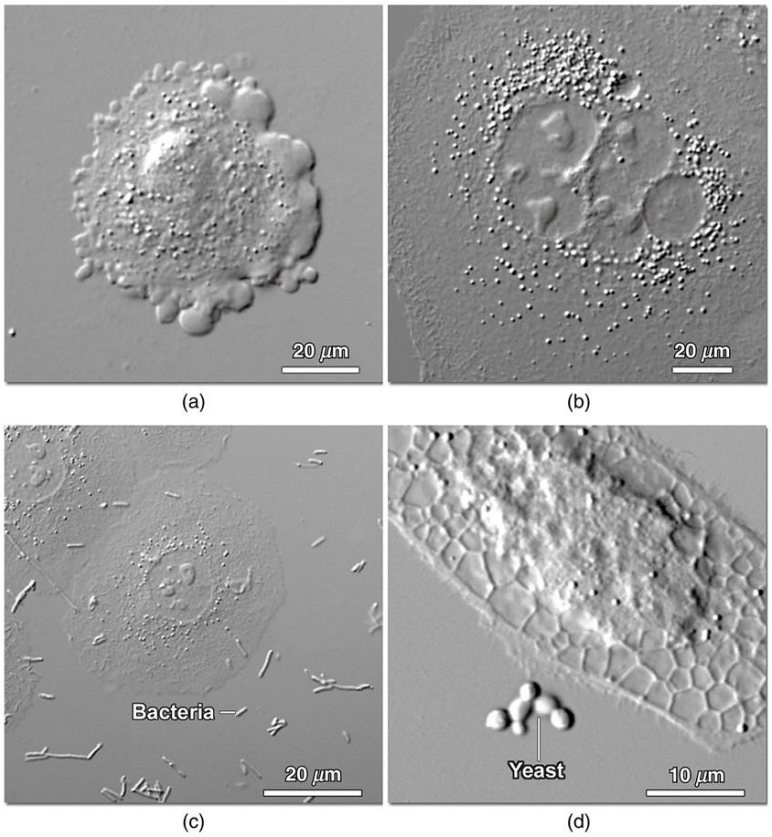
- choosing efficient bandpass filters (filter out traces of UV & IR),
- using sensitive cameras and detectors to reduce light exposure.
- By introducing electronic shutters to limit exposure of the cells to light during the periods when the images are not being captured.

➔ Choosing the exact level of light attenuation and the correct exposure time is mostly an empirical exercise.

- For a new cell line with unknown parameters, the best strategy is to attenuate the light as much as possible and apply very short exposure times so that subcellular structures are just barely visible in the acquired image.
- If the cells are able to tolerate this light level through a long-term experiment, then the intensity & exposure time can be slowly increased until a workable compromise is achieved between SNR & cell viability.

Other mechanisms affecting cell viability

- Aside from the problems associated with photo-toxicity and maintaining cells on the microscope, one must be alert to the possibility of **microbial contamination** during an experiment.
- The most common infections are due to bacteria, fungi, mycoplasma, yeast, and in rare cases, protozoa.



Examples of some common health problems and infections of cultured mammalian cells.

(a) Membrane blebbing

(b) Multiple nuclei

(c) Bacterial contamination

(d) Yeast contamination and vacuole formation.

- It is important to visualize the cells to establish their overall condition and morphology.
- If cells show slight deviation from a normal & health appearance they should not be considerate for data collection.