SIBO
Società Italiana Banche degli Occhi

Evaluation atlas of corneas

Edition 2018

"I spent my life looking in people's eyes,

It's the only place in the body where perhaps a soul still exists."

José Saramago

(Azinhaga, 16 November 1922 – Tías, 18 June 2010)

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1 Evaluation of the cornea with the slit lamp

1.1 The slit lamp

The slit lamp (biomicroscope) is a binocular microscope designed to be used in a horizontal position (**Figure 1**).

Figure 1: slit lamp.

In vivo it provides a magnified, stereoscopic (three-dimensional) view of the anterior ocular structures and, with the use of additional lenses, also of the posterior ones (**Figures 2** and **3**).

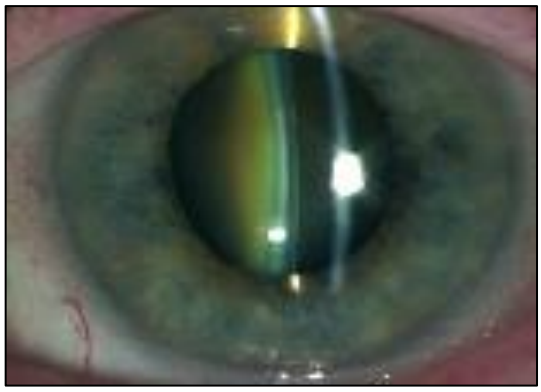


Figure 2: *in vivo* evaluation of the anterior segment at the slit lamp.

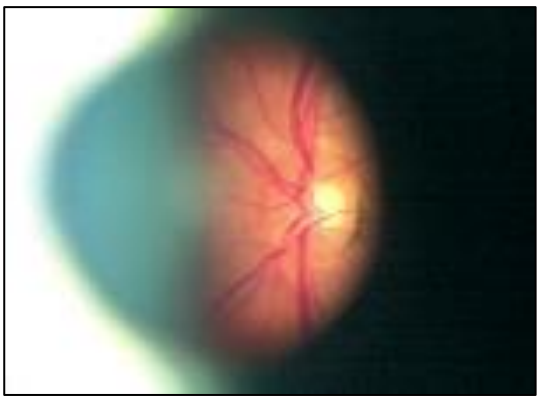


Figure 3: *in vivo* evaluation of the retina at the slit lamp.

The slit lamp is a useful method to evaluate the cornea both *in vivo* and in isolated tissue, it is able to provide way the equivalent of a histological section, in a non-invasive: epithelium, stroma and endothelium (**Figure 4**).

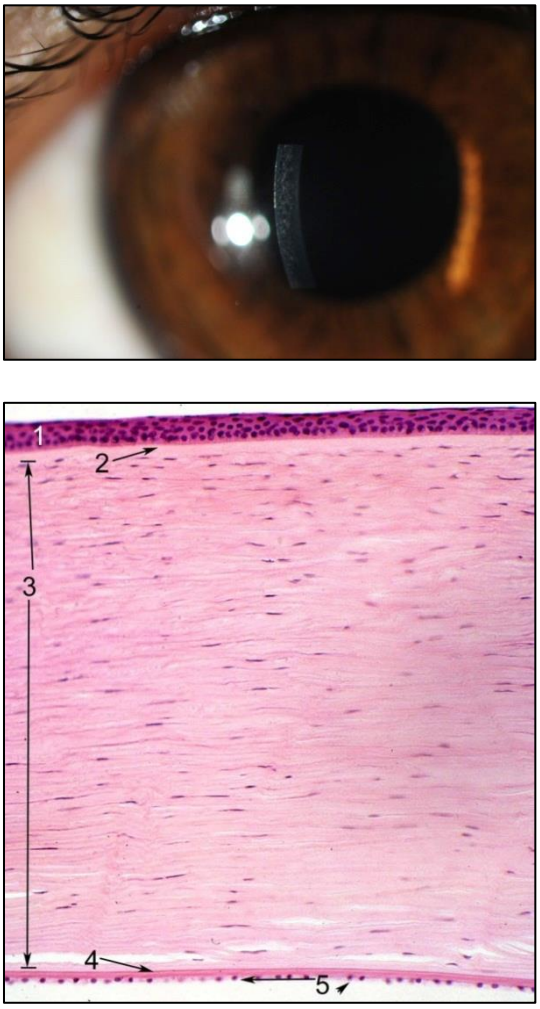


Figure 4: evaluation of the cornea at the slit lamp ("*in vivo* histological section") [top], corneal section: epithelium (1), Bowman's membrane (2), stroma (3), Descemet's membrane (4) and endothelium (5) [bottom].

This device allows a preliminary selection of the tissues, according to the EEBA Guidelines ("Technical Guidelines for Ocular Tissue"): "*[…] slit lamp examination, performed when whole eyes are enucleated or when corneoscleral buttons are excised, is recommended because it provides additional information […]*".

The slit lamp consists of:

- microscope;
- lighting system;
- moveable stand;
- additional photographic tools.

The microscope consists of:

- stereomicroscope with variable magnification from 6× to 40× (**Figure 5**);
- parallel eyepieces, where the eyes focused at infinity allows a fatigue-free vision when the instrument is used over a long period of time, or convergent, where the eyes focused at near allows the best vision when the instrument is used for short periods of time (**Figure 6**).

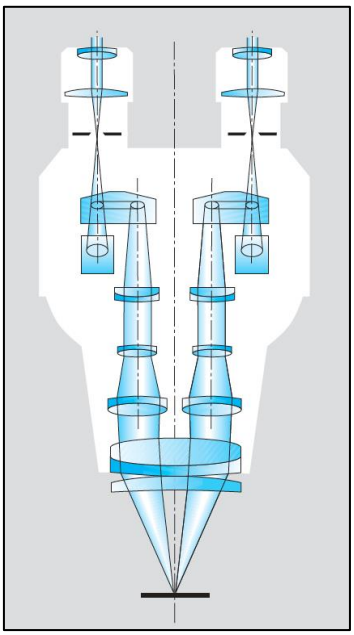


Figure 5: general scheme of a stereomicroscope.

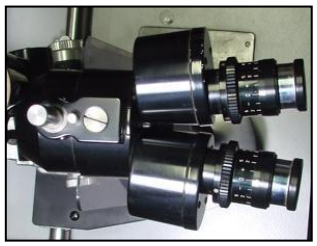


Figure 6: parallel [top] and convergent [bottom] eyepieces.

The lighting system consists of a halogen light source and a lens condenser which are able to produce a slit light beam at a defined distance from the instrument.

The slit can vary in length, width and position, with the possibility of crossing specific filters (blue, green, etc.) for specific applications (**Figure 7**).

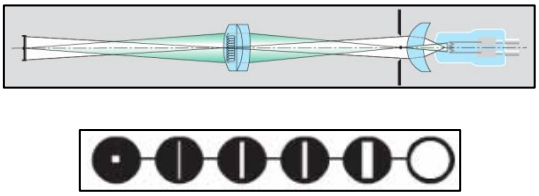


Figure 7: general scheme of a lighting system [top] and of the slit produced [bottom].

The moveable stand is the mechanical system that allows the microscope and the lighting system to be hinged on the same axis around which they rotate independently.

This allows both to focus on the same plane, at the same point (for particular observations – see scleral diffusion – it is possible to dissociate them), but with different inclinations on the horizontal axis (**Figure 8**).

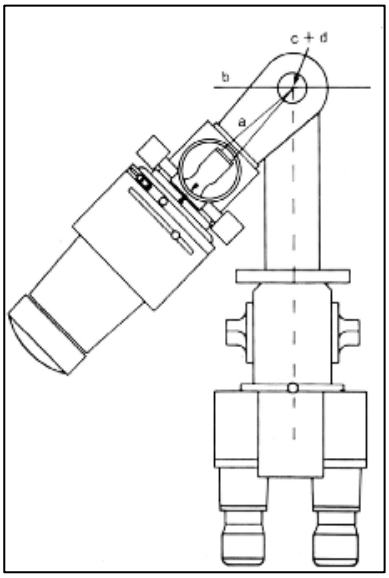


Figure 8: general scheme of mobile support.

A joystick control allows to move the instrument in the three directions of the space: left/right, forward/backward (focusing) and up/down.

Additional photographic tools can include:

- digital camera;
- beam splitter (to provide a coaxial view);
- electronic flash (to reproduce the effect of lighting);
- fill light (an accessory source of diffused lighting to obtain general information on the picture highlighted by the slit).

1.2 How to hold the cornea

The donor cornea can be placed in front of the slit lamp for the correct focusing process using different devices (**Figures 9** and **10**):

- cornea transport vial supported by laboratory clamp;
- corneal observation chamber supported by laboratory clamp;
- cornea transport vial supported by a perforated support equipped with a 45° inclined mirror;
- moist transport chamber for eyeball to examine the whole eye.

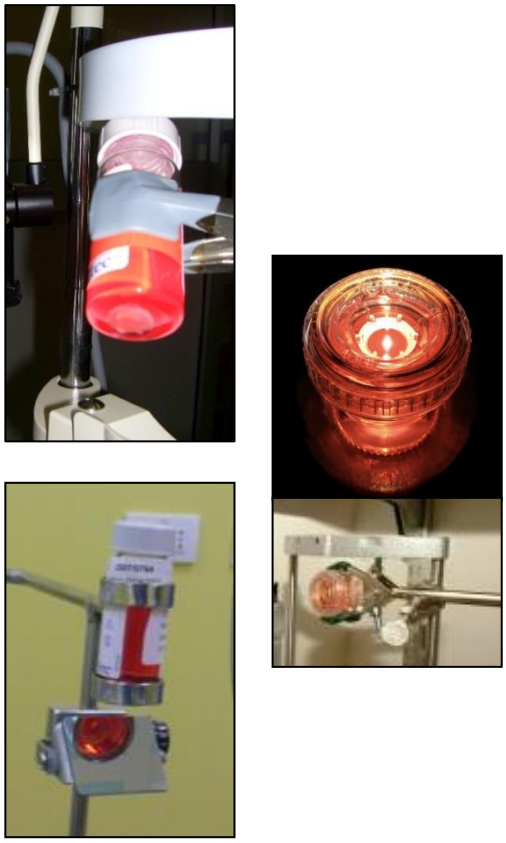


Figure 9: cornea transport vial on laboratory clamp [top], corneal visualization chamber on laboratory clamp [middle], cornea transport vial on mirror equipped holder [bottom].

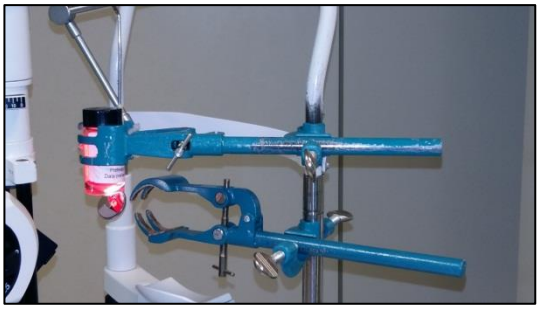


Figure 10: laboratory clamp for crimping and mirror support system.

1.3 Principles of use of the slit lamp

Depending on the type of lighting, particular details of the tissue under examination can be viewed and studied:

- with direct lighting, the light beam is directly pointed at the focused object (coupled mode);
- with indirect lighting, the beam of light is off-center to illuminate behind the focused object (uncoupled mode).

The types of direct lighting are:

- widespread;
- focal (section or parallelepiped);
- specular reflection.

The types of indirect lighting are:

- backlighting;
- sclerotic scatter.

In the evaluation of a cornea, both lighting techniques can be applied on the epithelial or endothelial side (**Figure 11**).

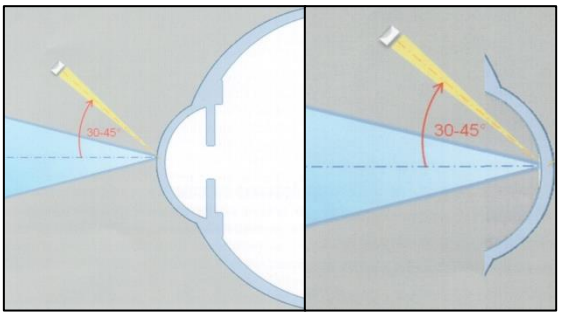


Figure 11: lighting techniques applied to the evaluation of the cornea.

In the direct diffuse lighting of the cornea, the magnification is low and the slit beam is completely open, in order to perform a panoramic evaluation of the tissue to examine the surface, size, shape, transparency and any foreign bodies or opacities present in it (**Figures** from **12** to **17**).

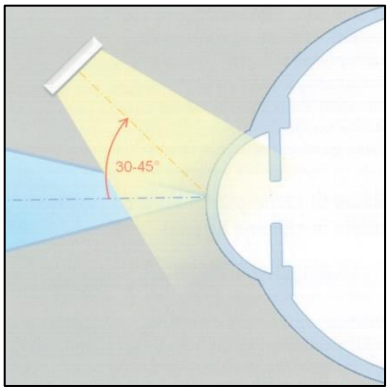


Figure 12: diffuse direct illumination of the cornea.

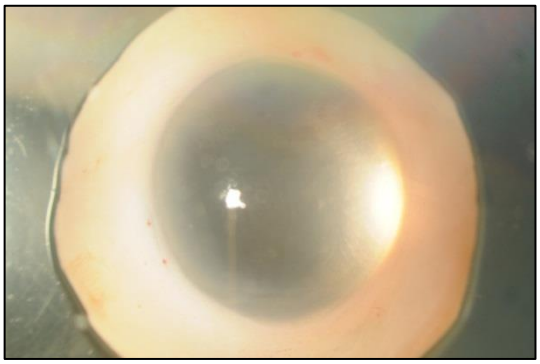


Figure 13: anterior view – normal corneal surface, size, shape and transparency; scleral ring of regular shape and correct size (≥2mm).

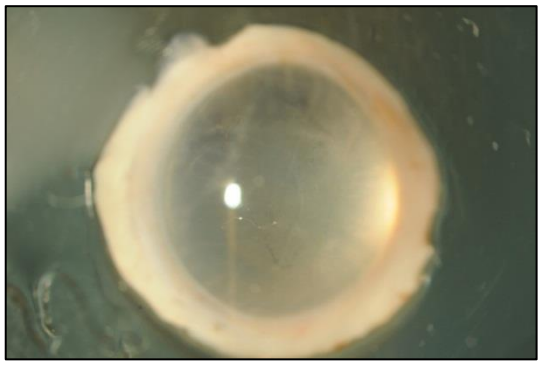


Figure 14: anterior view – normal corneal surface, size and shape, with some folds; scleral ring of fairly regular shape, but incorrect size (≤2mm).

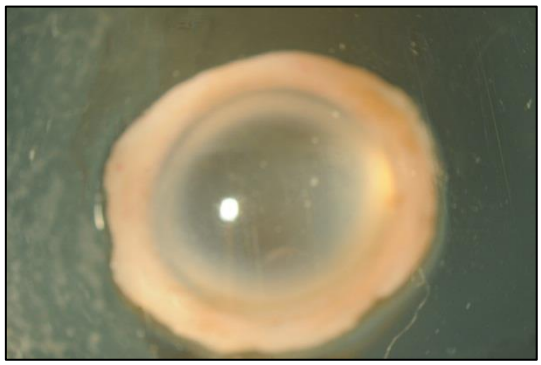


Figure 15: anterior view – normal corneal surface, size and shape, diameter of transparency <8mm (gerontoxon); scleral ring of fairly regular shape, but incorrect size (≤2mm).

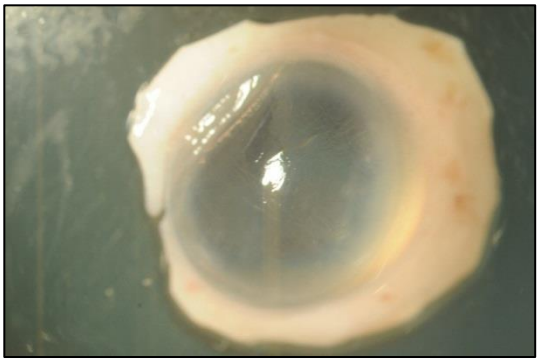
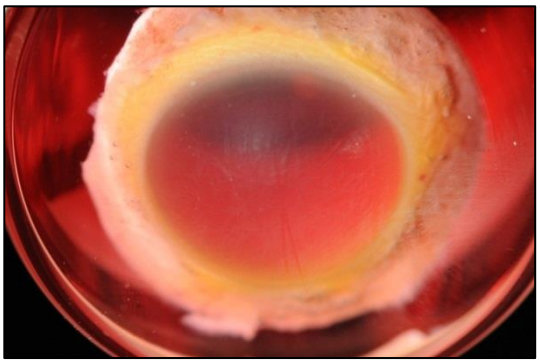
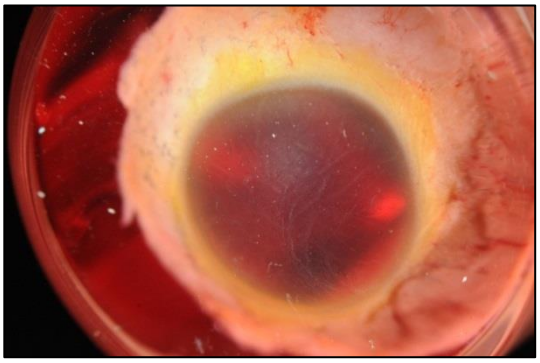


Figure 16: anterior view – normal corneal surface, size and shape, with some folds; scleral ring of irregular shape and incorrect size (≤2mm); incision in light cornea due to the excision procedure.





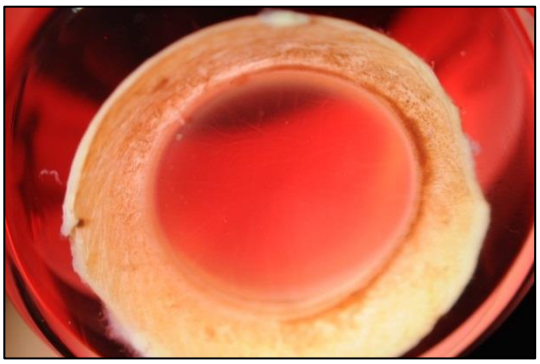


Figure 17: front view at the top and in the middle, rear view in the bottom – normal surface, with some folds; scleral ring quite regular, but indented cornea (minimum distance from scleral edge ≤2mm); yellowish color due to the presence of povidone-iodine residues.

In the direct focal lighting of the cornea, the magnification is medium or high and the slit beam is narrow (<0,5mm for the section) or medium (between 0,5mm and 2mm for the parallelepiped): the section consists of a "slice of light" useful for determining the depth of the lesions, the parallelepiped consists of a "curved cube" useful for evaluating the epithelium, the stroma, the Descemet's membrane and the possible presence of edema (**Figures** from **18** to **22**).

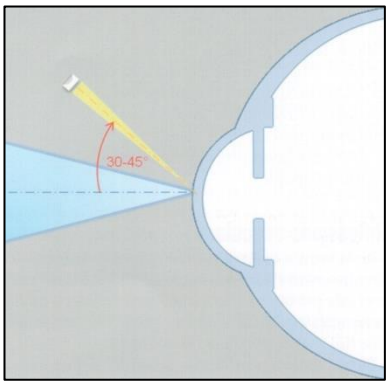


Figure 18: focal direct illumination of the cornea.

Figure 19: the section and the parallelepiped show the size and depth of leucomas, deposits and debris.

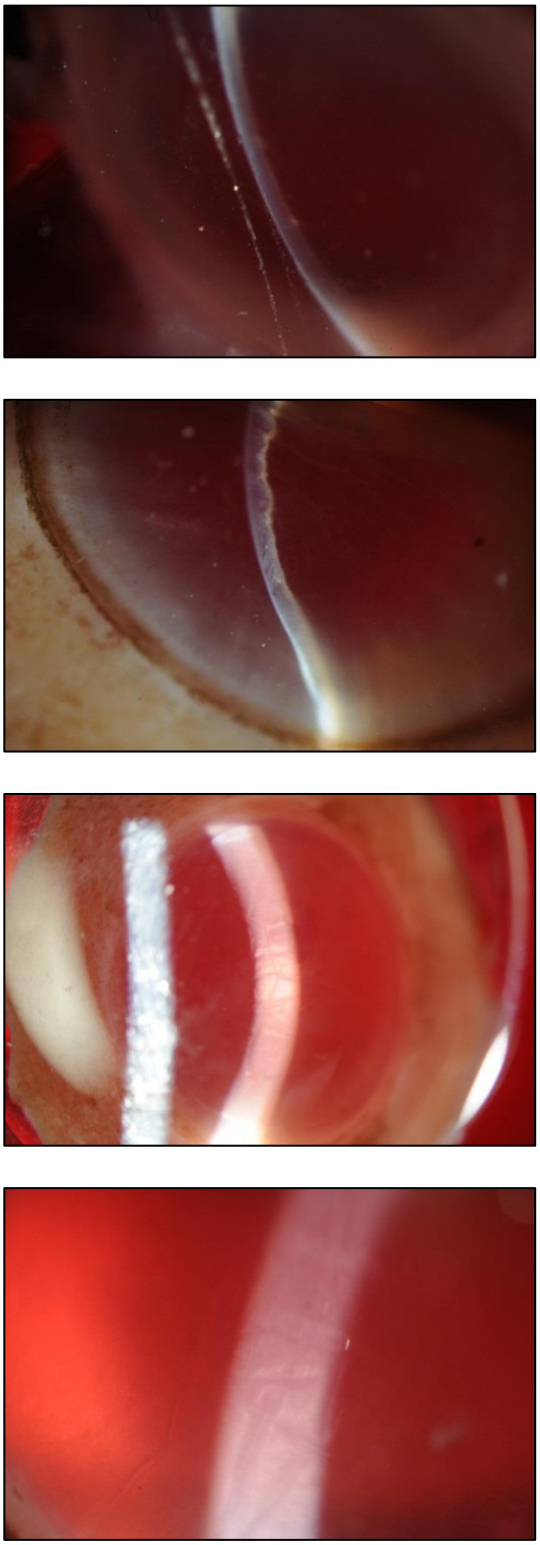
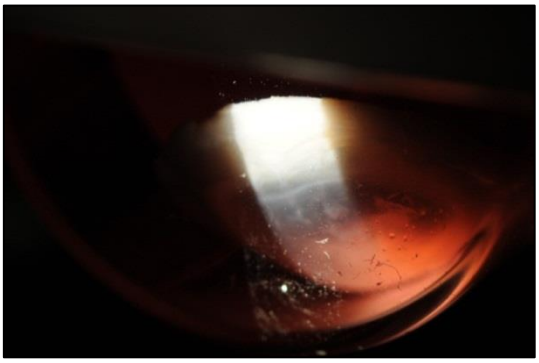
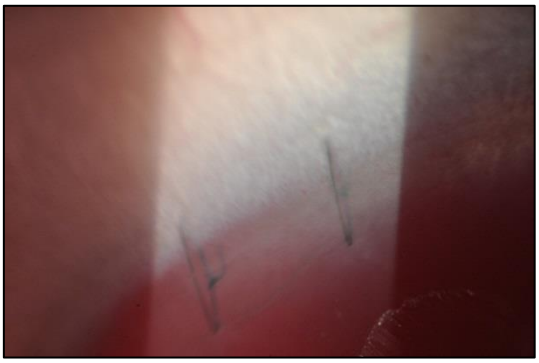


Figure 20: the section and the parallelepiped allow the classification of the folds of the Descemet's membrane due to hypotonia and traction (slight, medium and coarse).

Figure 21: the torsion of the cornea during excision procedure causes deep radial folds and mortality of the endothelium.





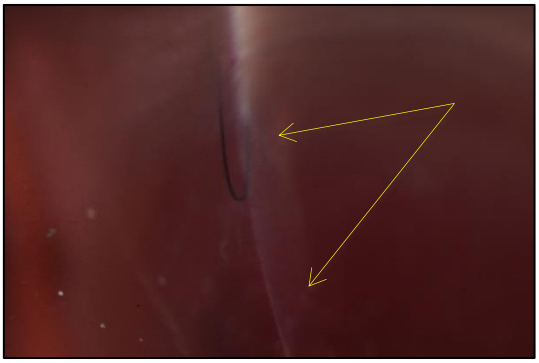


Figure 22: evidence of cataract surgery: incision scar [top], single sutures [middle], corneal tunnel [bottom].

In the direct lighting with specular reflection of the cornea, the magnification is high and the slit beam is a small and short parallelepiped: the angle of

incidence of the beam of light on the cornea is equal to the angle of reflection of light into the biomicroscope and allows to evaluate the endothelium, highlighting the cell margins and the possible presence of guttae (**Figures 23** and **24**).

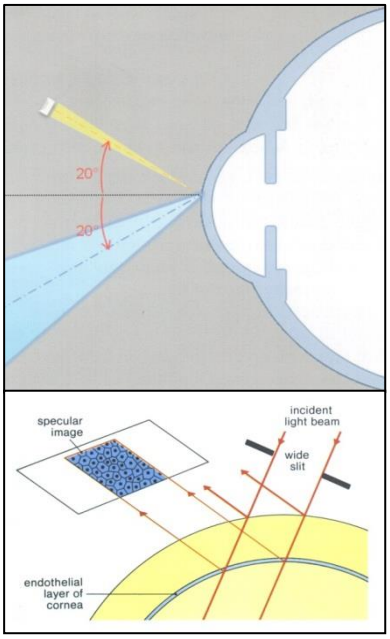


Figure 23: direct illumination with specular reflection of the cornea.

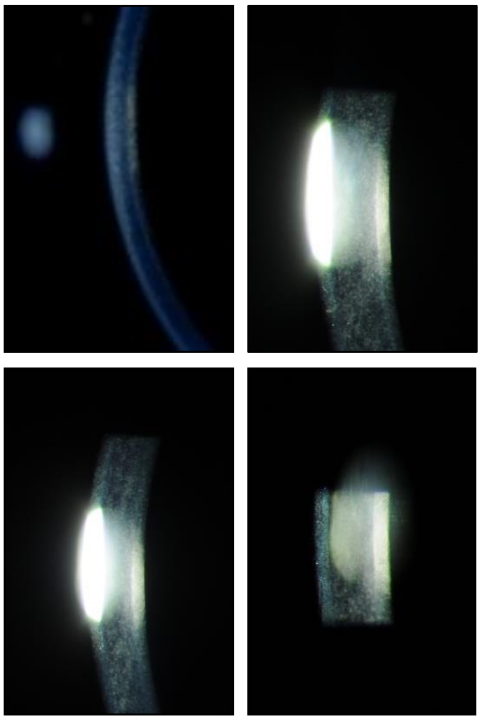


Figure 24: corneal endothelium with evidence of dystrophy.

In indirect backlighting of the cornea, the magnification is low or medium and the slit beam is a small parallelepiped: the area to be examined is lighted by the diffuse reflection of the light beam in the middle (*in vivo* also by direct reflection on areas such as iris, crystalline lens or fundus), highlighting the possible presence of scars, epithelial edema, pigmentation and corneal precipitates, blood, vacuoles and ghost vessels (**Figures 25** and **26**).

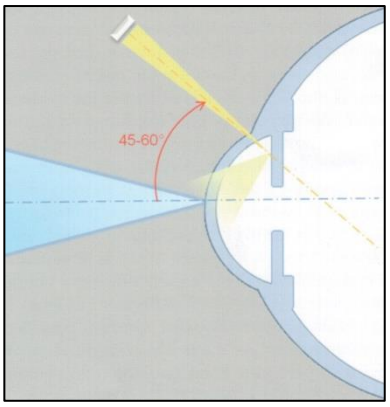


Figure 25: indirect corneal backlight illumination.

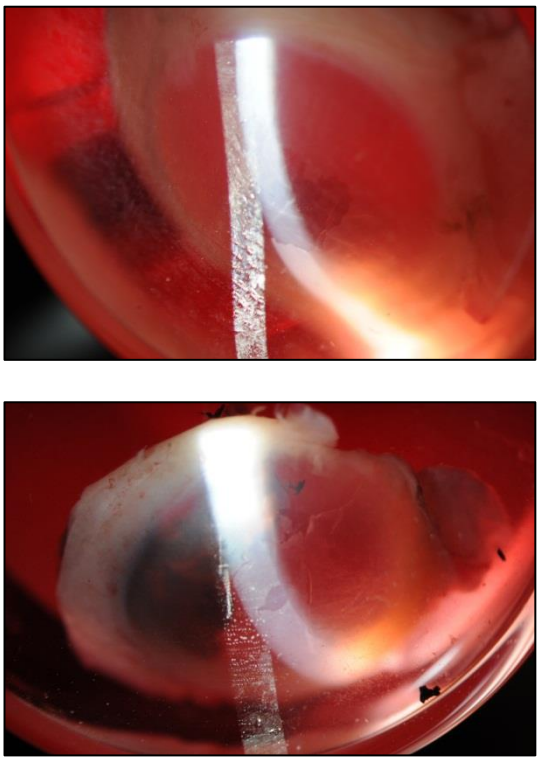


Figure 26: epithelial defects and edema.

In indirect scattered sclerotic lighting of the cornea, the magnification is low and the slit beam is a small parallelepiped: the decentralized light beam is projected at the limbus level and internally reflected through the corneal tissue (internal reflection similar to the propagation of light through an optical fiber), highlighting low-density alterations such as dystrophy, epithelial edema and rupture of Descemet's membrane (**Figures** from **27** to **29**).

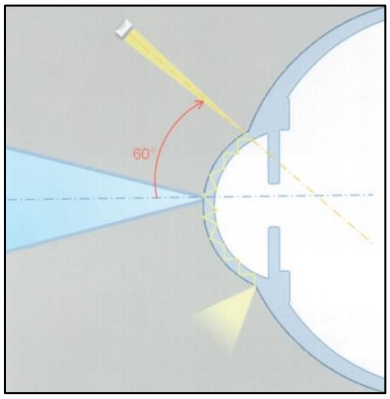
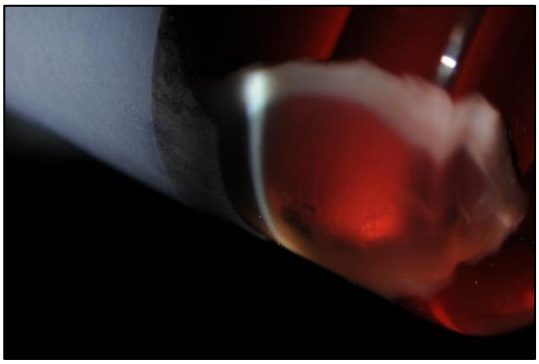


Figure 27: indirect sclerotic scattering of the cornea.



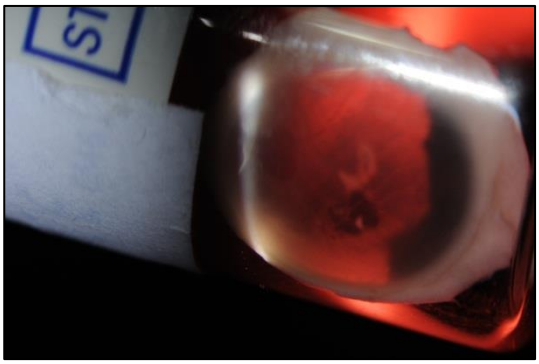


Figure 28: exposure keratopathy: de-epithelialization, swelling and opacification of the epithelium.

Figure 29: Descemet's membrane breakage.

The slit lamp is a useful instrument for assessing the suitability of corneal tissues at transplantation.

The quantity and quality of information derived from a slit lamp examination are related to the level of practice acquired in the application of the various techniques.

1.4 Miscellaneous

The following is a series of representative figures of tissue abnormalities that can be found at a slit lamp cornea analysis (**Figures** from **30** to **35**).

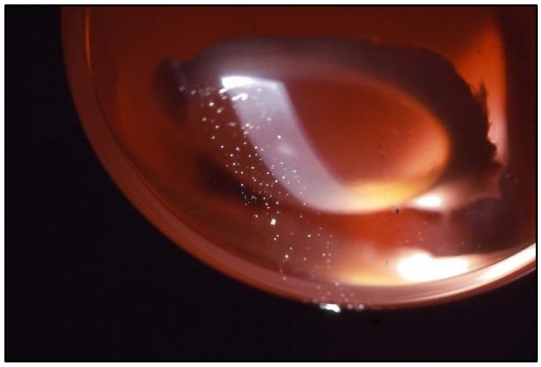


Figure 30: leucoma.

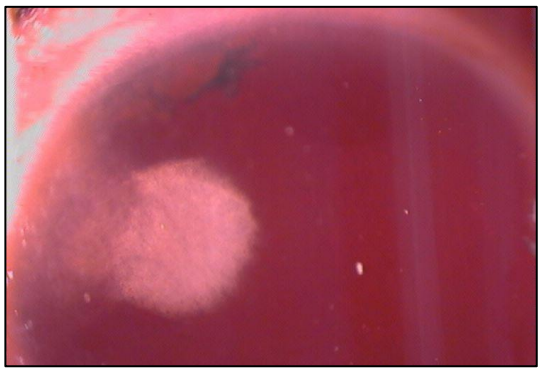


Figure 31: leucoma.

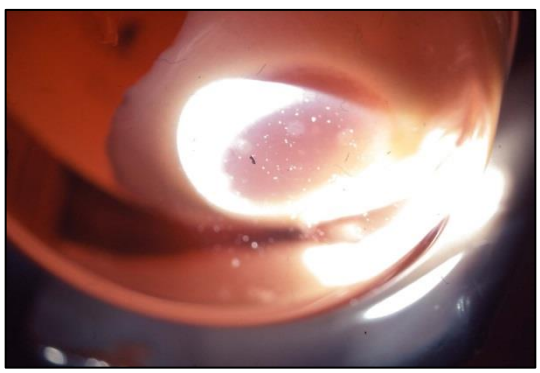


Figure 32: thermocheratoplasty (refractive surgery).

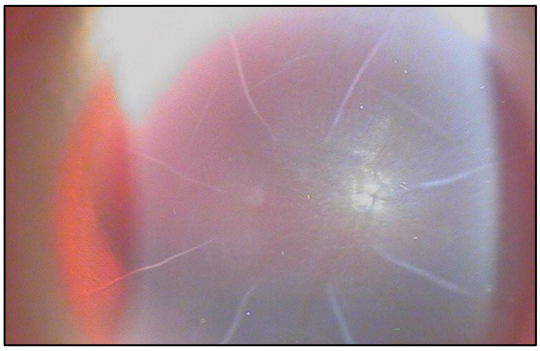
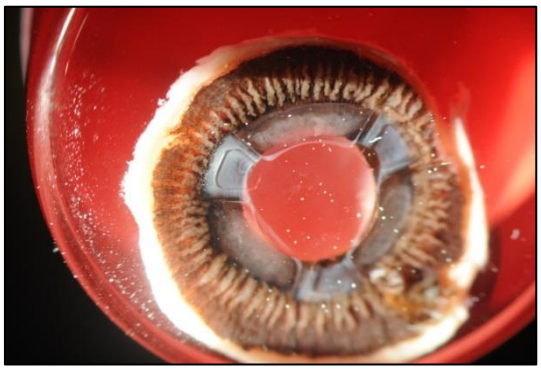
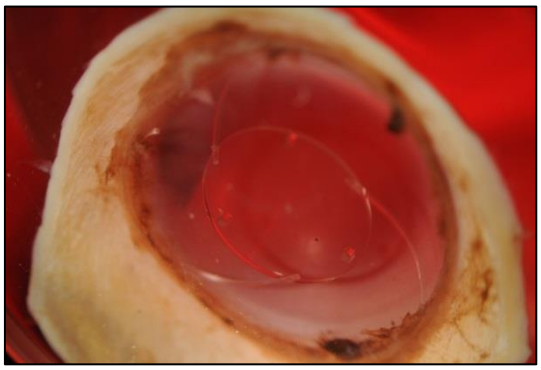


Figure 33: radial keratotomy (refractive surgery).

Figure 34: pterygium.

Figure 35: presence of eyelash.





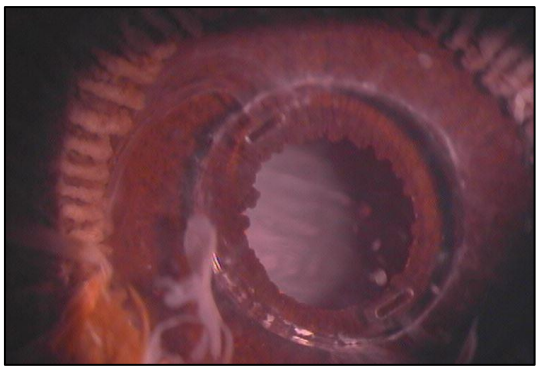


Figure 36: IOL, iris and ciliary body.

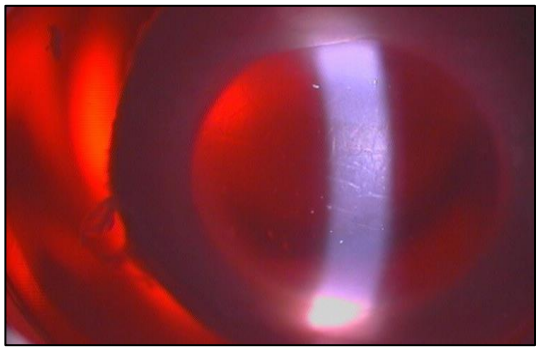


Figure 37: hypotonus folds.

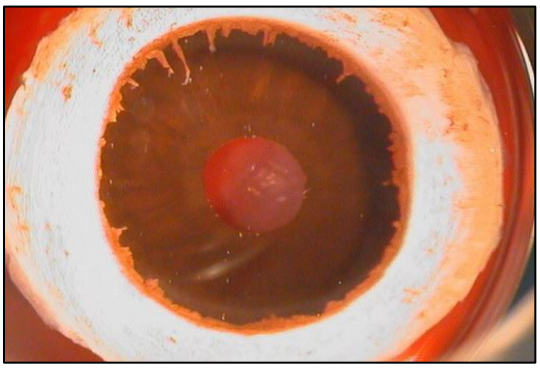
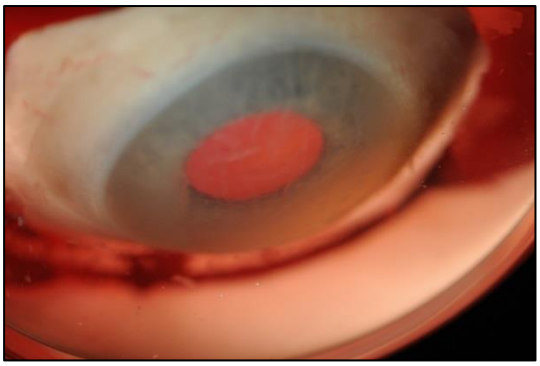


Figure 38: presence of iris in place.



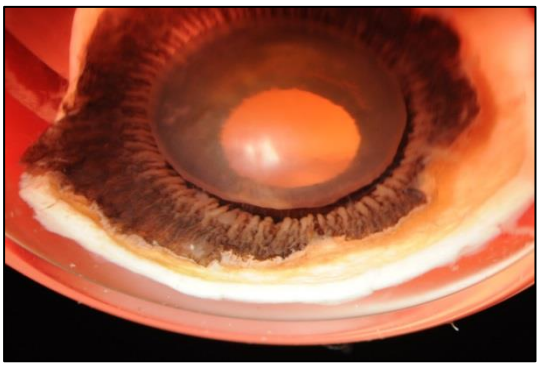


Figure 39: presence of iris and crystalline.

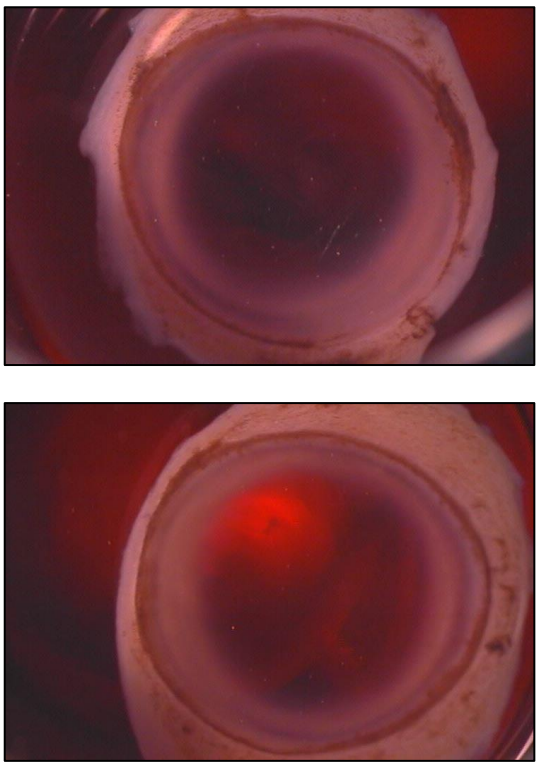


Figure 40: insufficient scleral ring.

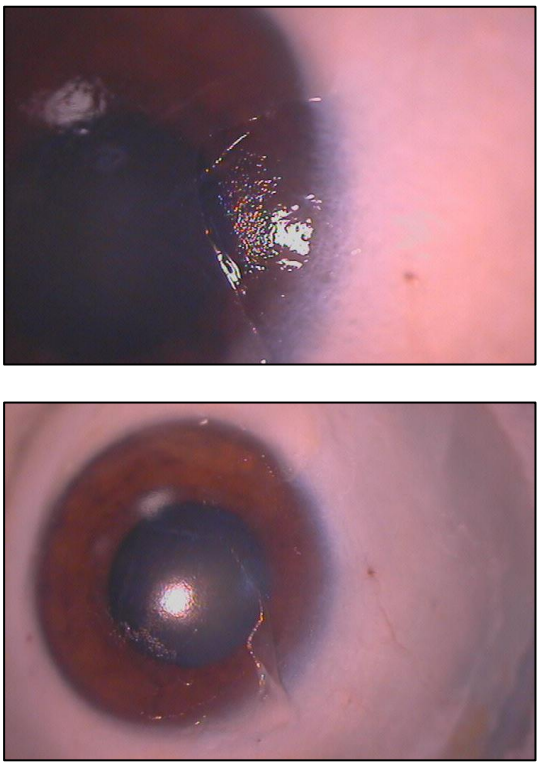


Figure 42: bulb with corneal damage due to excision procedure.

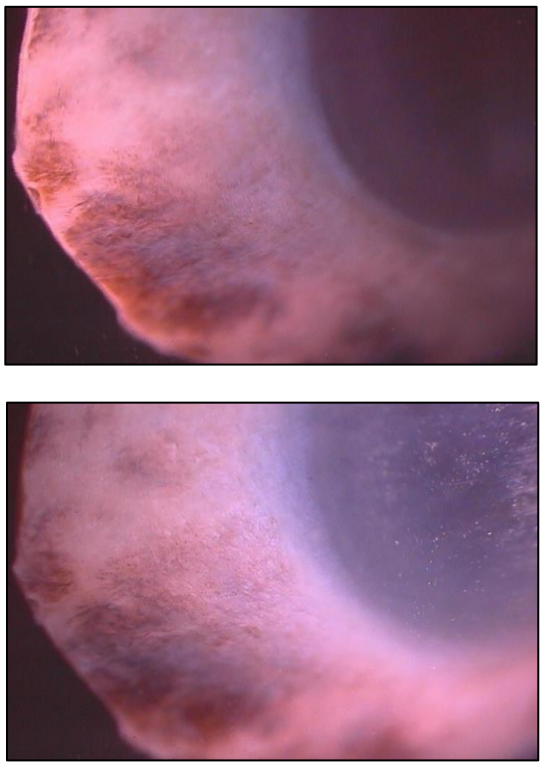


Figure 41: suspected melanoma.

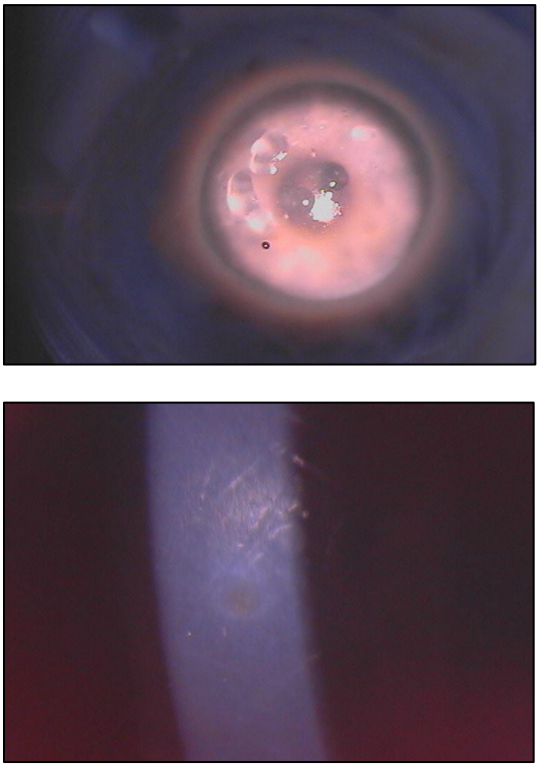


Figure 43: foreign body [top], outcomes of foreign body with opacity [bottom].

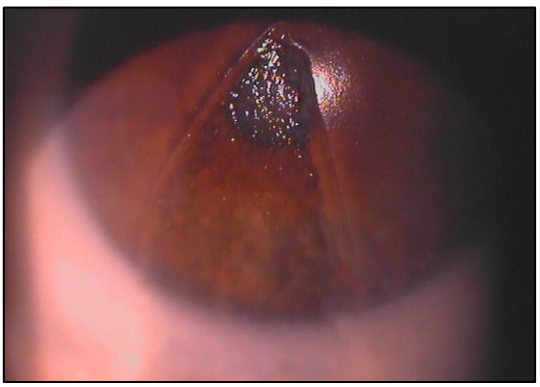


Figure 44: stromal damage due to excision procedure.

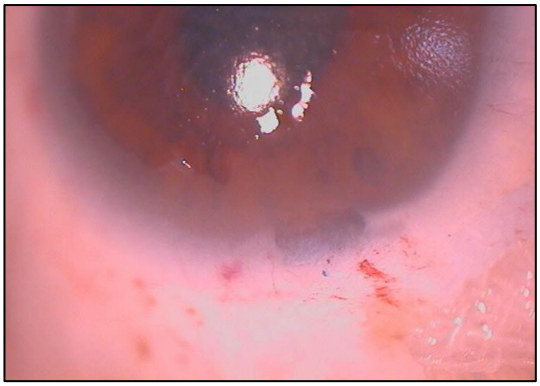


Figure 45: stromal erosion at the limbus.

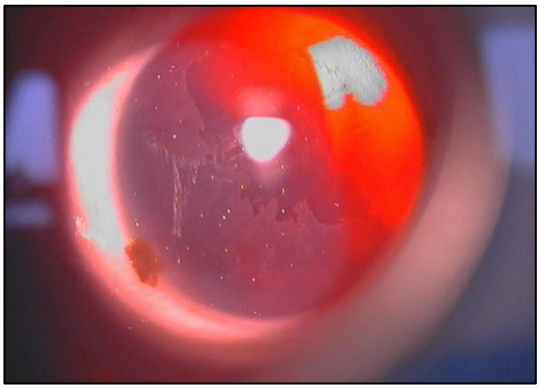


Figure 46: : suspected pigmentation.

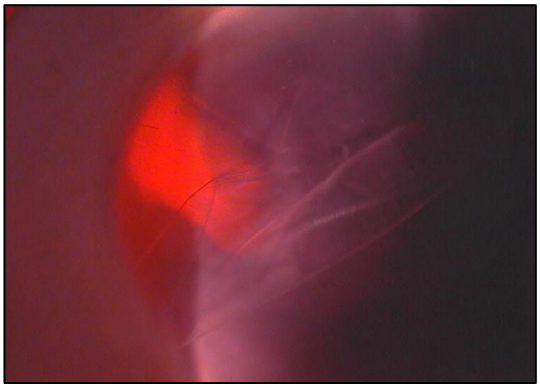


Figure 47: Descemet's membrane tear.

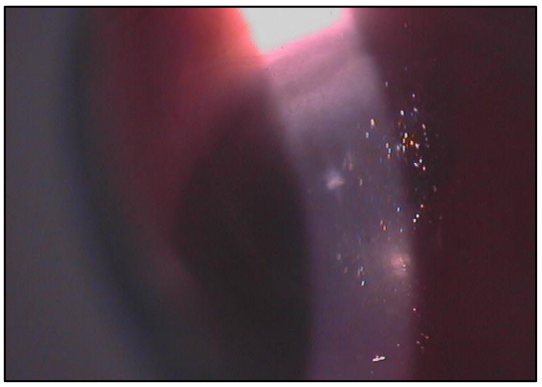


Figure 48: outcome of corneal perforation.

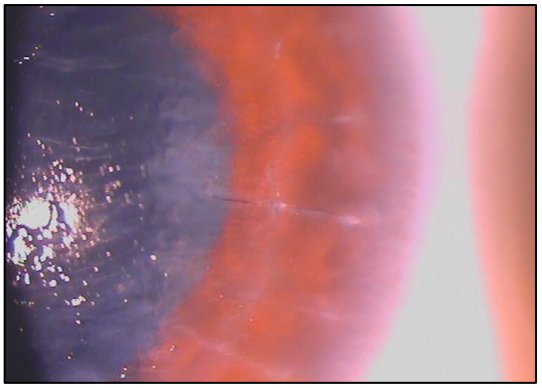


Figure 49: outcome of surgery with point in place.

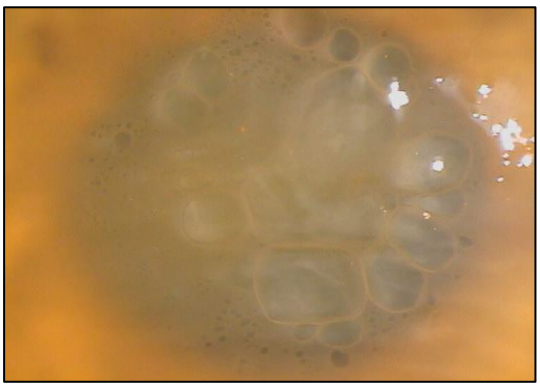


Figure 50: bubble edema.

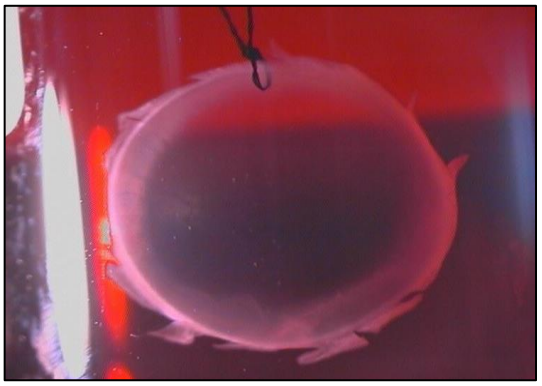


Figure 51: excision without sclera with suture in place.

2 Evaluation of the cornea with the specular microscope

2.1 Optical principles and instrumentation

The light striking a surface can be reflected, as well as absorbed and refracted.

A small portion of light is reflected specularly: the angle of reflection is equal to the angle of incidence (**Figure 52**).

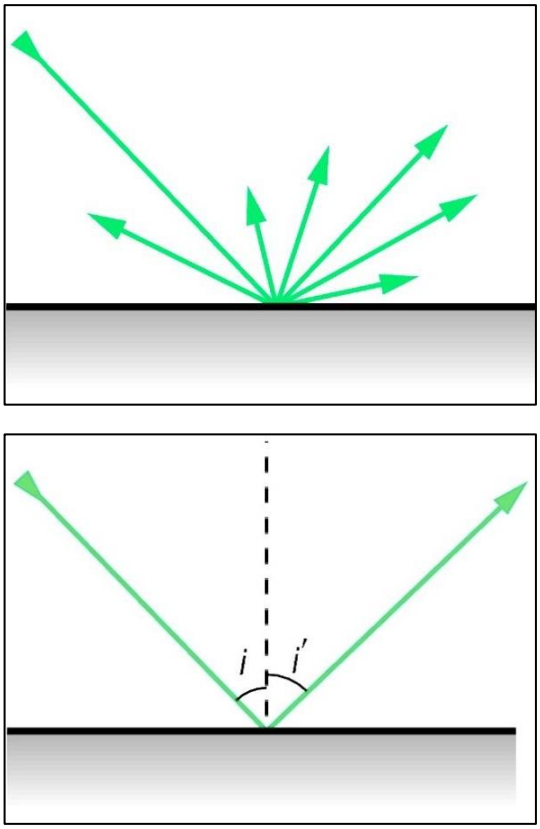


Figure 52: incident light and specular reflection.

When a ray of light passes through a non-homogeneous medium, part of the light is reflected at each interface.

The light reflected specularly from the posterior corneal surface is collected through a focused system.

To process signals of this type, it is used a specific microscope (**Figure 53**) equipped with:

- integrated camera;
- analysis software;
- optical pachymetry device.

Figure 53: specular microscope.

The transport vial with preservation liquid or the viewing chamber is positioned in the appropriate adaptor (**Figure 54**).

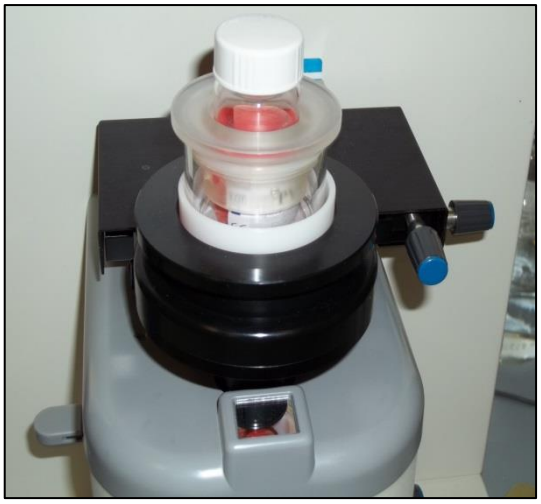


Figure 54: positioning of the transport vial.

The cornea should be placed on the bottom of the transport vial with the endothelial side down (**Figure 55**).

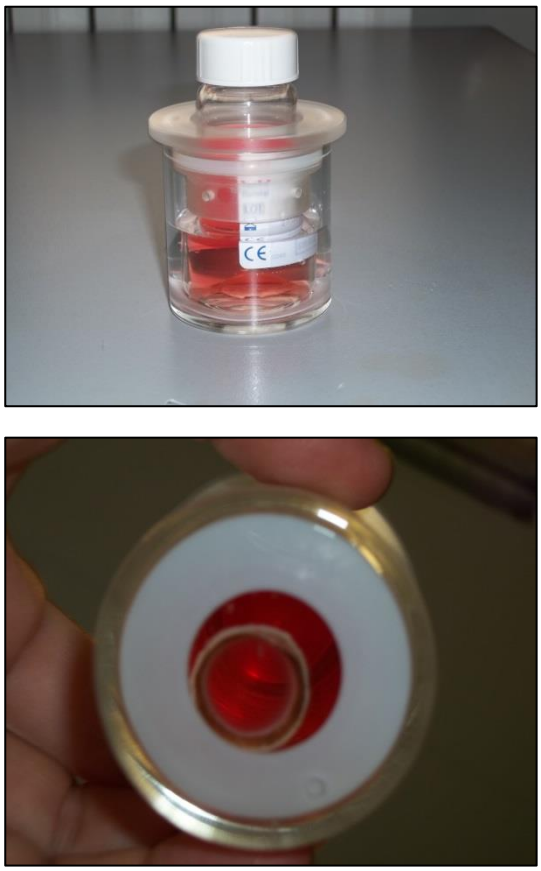


Figure 55: correct positioning of the cornea inside the transport vial.

The joints allow a movement on the x, y and z axes, where the rocking platform mechanism allows to tilt the tissue respect to the microscope slit (**Figure 56**).

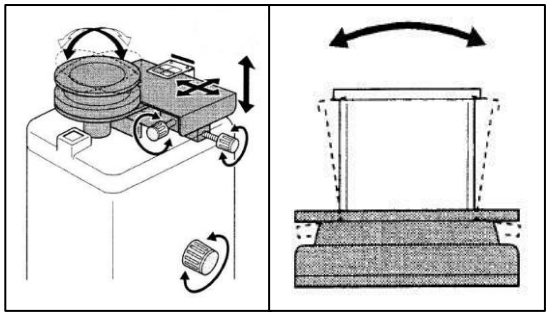


Figure 56: adjusting and centering system of the sample to be analyzed.

The software allows to carry out the endothelial count with the "Center Method" procedure.

By identifying the center of each cell, the software determines its margins and calculates the area using the corresponding pixels.

Peripheral cells are excluded, because they are not entirely surrounded by other contiguous cells (**Figure 57**).

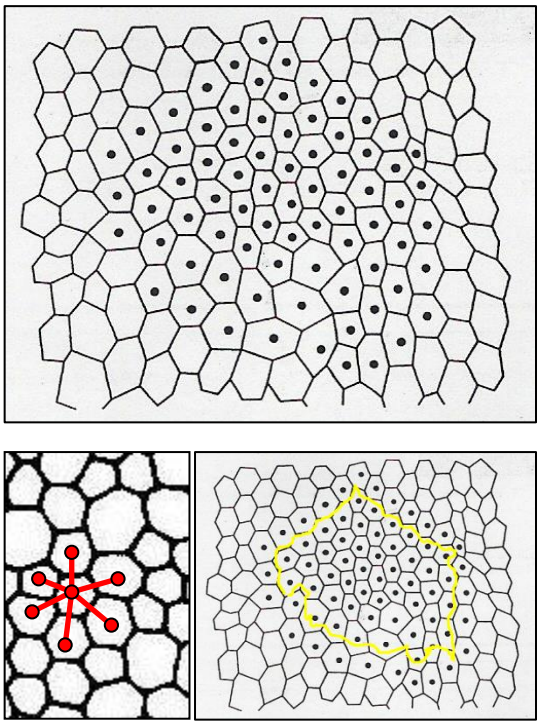


Figure 57: "Center Method".

2.2 Quantitative analysis

The most important parameters that characterize the result of the analysis are (**Figure 58**):

- cell density (CD);
- coefficient of variation (CV);
- hexagonality (6A);
- pachymetry (µm).

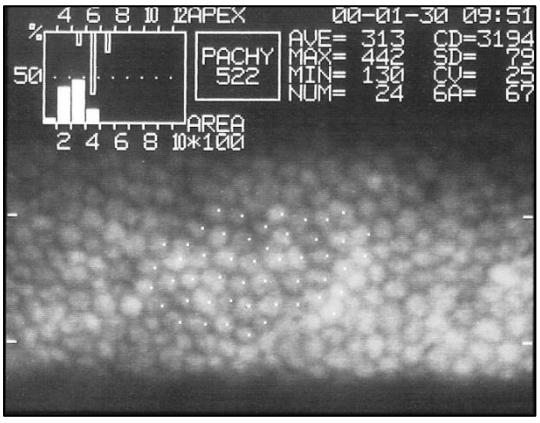


Figure 58: result of the analysis.

Cell density (CD) is defined as:

$$CD\ [cell/mm^2] = \frac{106}{average\ cell\ area}$$

The coefficient of variation (CV) is defined as:

$$CV = \frac{SD}{average\ cell\ area}$$

The coefficient of variation should take values in the range 0,25-0,30.

High values mean a considerable variability of the cellular dimensions that is called polymegatism (**Figure 59**).

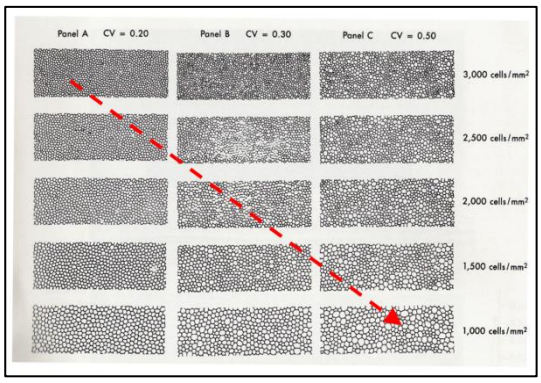


Figure 59: correlation between CD and CV (the red arrow indicates the "direction of instability").

Corneas with the same CD can have different CV.

The CD alone does not show corneal stability.

The hexagonality (6A) is defined as:

$$6A = \%\ cells\ with\ 6\ sides$$

The hexagonality should assume values greater than 50%.

A high number of cells with more or less than six sides indicates cellular instability and is called polymorphism.

It is important to remember that the specular microscope analyzes a small central area (<1mm^2) even with multiple measurements.

Some authors have reported that:

- the axial CD is a good indicator of the total CD;
- a peripheral cell loss can be suspected in presence of marked pleomorphism and polymegatism, by demonstrating a significantly higher CD in the contralateral eye.

Specular microscope data must always be interpreted within the context of the slit lamp tissue examination.

To calculate the optical pachymetry with "manual" mode it is necessary (**Figure 60**):

- setting to zero the micrometer scale by focusing the epithelium;
- reading the value focusing on the endothelium (distance ep-end).

The pachymetry should assume values greater than 500µm (only reliable for "extreme values").

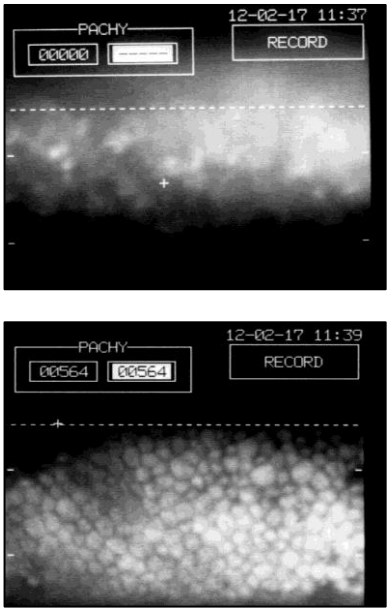


Figure 60: calculation of optical pachymetry with manual mode.

2.3 Qualitative analysis

Before starting the examination it is essential warm the tissue back to room temperature (about 25°C) to avoid artifacts.

The cold cornea does not allow proper visualization of the endothelium (**Figure 61**).

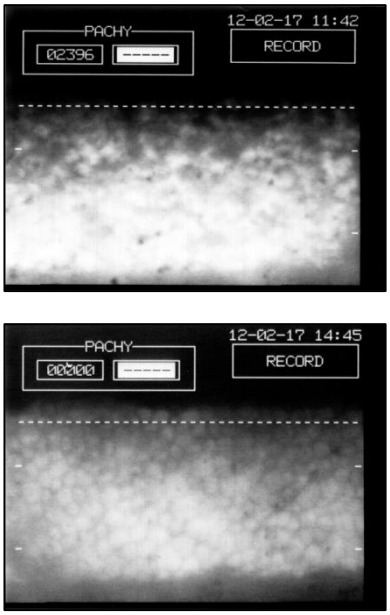


Figure 61: comparison between cornea in hypothermia [top] and at room temperature [bottom].

Some Author correlated the morphological changes observed in specular microscopy with:

- histological preparations in optical microscopy;
- histological preparations in scanning electron microscopy.

It is essential to recognize the normal and pathological structures, that means "to interpret the light and the dark".

The images depend on the regularity of the endothelial surface (**Figure 62**):

- a smooth area is represented by a lighter area;
- a rough or wavy surface is represented by not uniform light and dark areas;
- a posterior excrescence is represented by a dark area with a light apex.

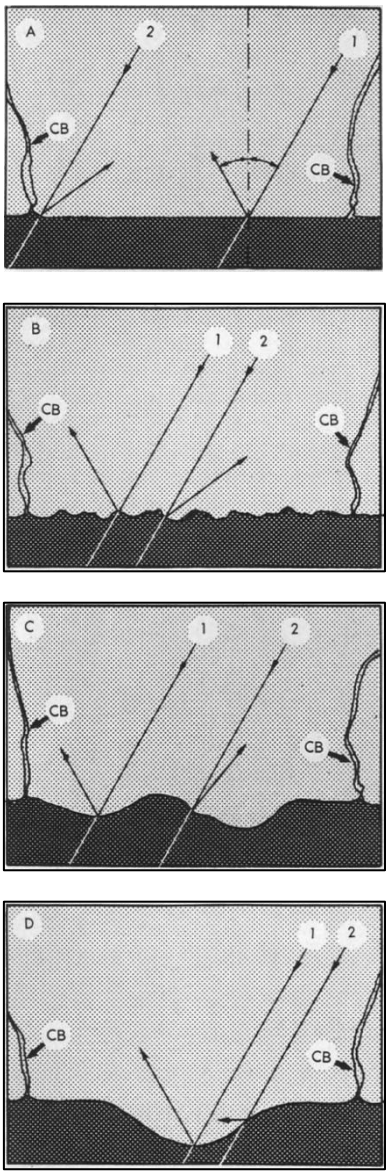


Figure 62: variation of the specular reflection angle in case of an irregular surface.

Cell margins appear as thin dark lines (**Figure 63**).

Figure 63: cell margins.

The difference in height between adjacent cells simulates doubling of the borders (**Figure 64**).

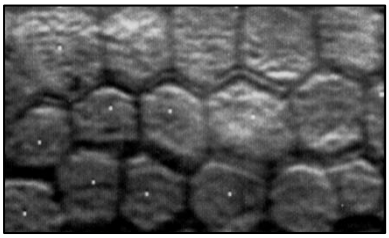


Figure 64: double cell margins between contiguous cells.

The prevalent form of cells is hexagonal.

In the case of polymorphism, which results from cellular suffering, bizarre cellular frameworks are observed: giant, elongated, compressed, indented and daisy cells (**Figures 65** and **66**).

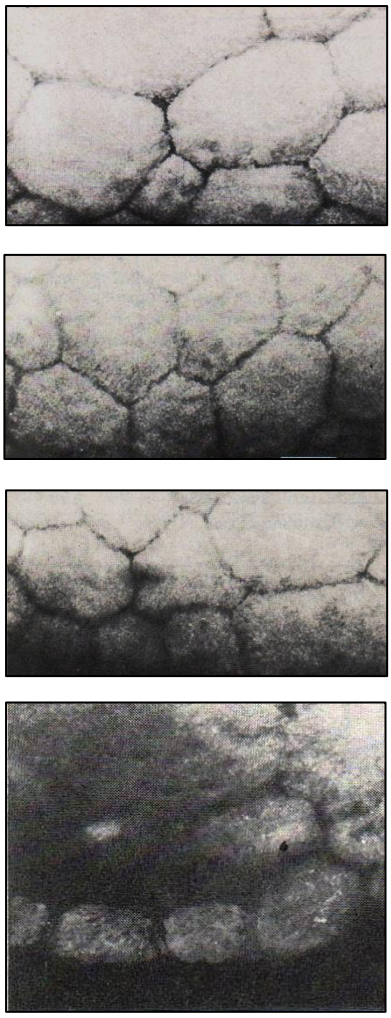


Figure 65: variation of cell shape.

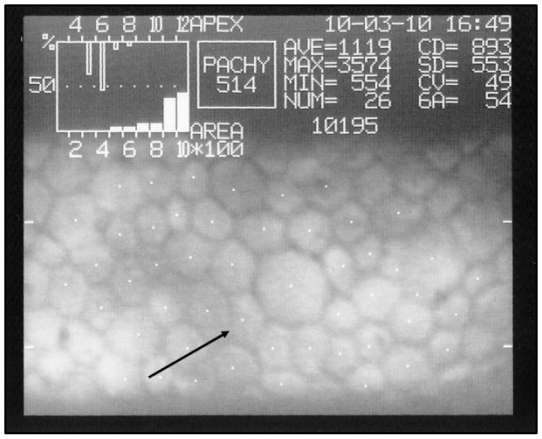


Figure 66: daisy cells.

The dark areas represent: cilia, vacuoles or blebs, red blood cells, pigment deposits (**Figure 67**).

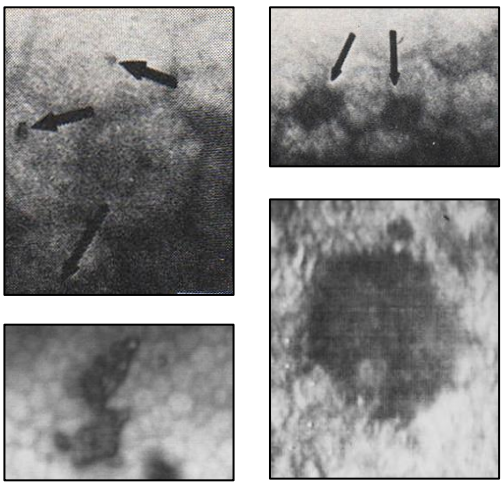


Figure 67: examples of dark areas.

The light areas represent: nuclei, sticking leukocytes, hyaline bodies (**Figure 68**).

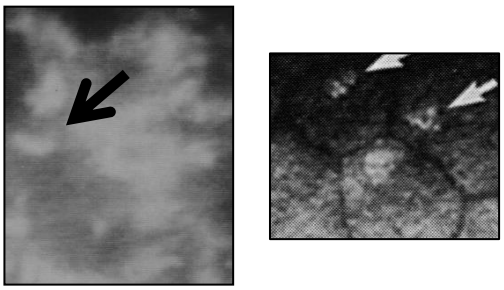
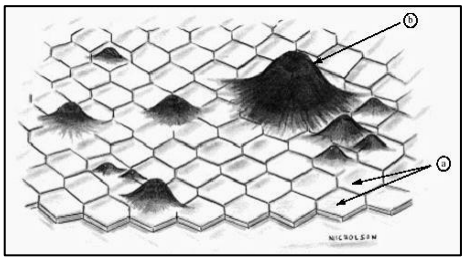
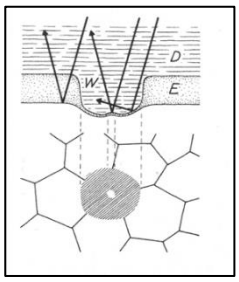


Figure 68: white areas.

In Fuchs' dystrophy, endothelial cells show wart like excrescences: guttae.

In the gutta (**Figure 69**) there is a scattering of light (dark area) and a reflection of light (light area).





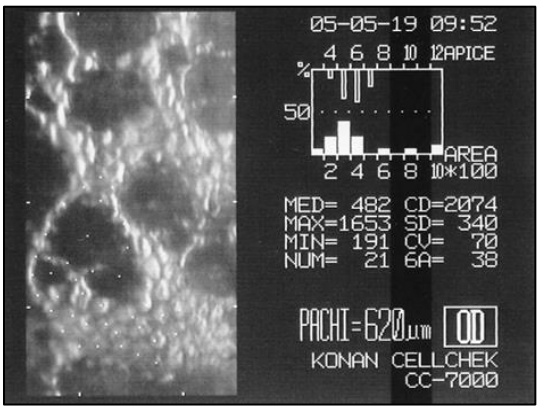


Figure 69: appearance of guttae.

The folds are the physical manifestation of corneal swelling: they can be mild, moderate or severe and populated with normal or suffering – necrotic cells (**Figure 70**).

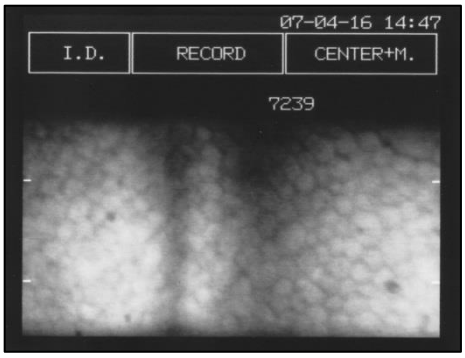


Figure 70: appearance of the folds.

In necrosis three morphological stages can be identified (**Figure 71** and **72**):

- initially the cell has a bulging appearance and soft edges;
- subsequently the cell necrosis is present (cellular debris remains);
- finally the surrounding cells migrate and change to cover the denudated surface (rosetta).

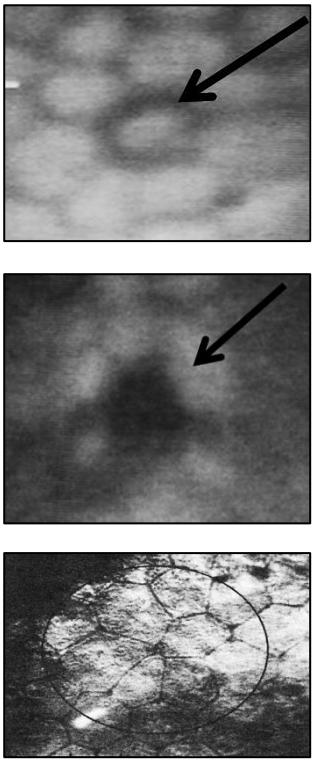


Figure 71: evolution of a necrotic cell.

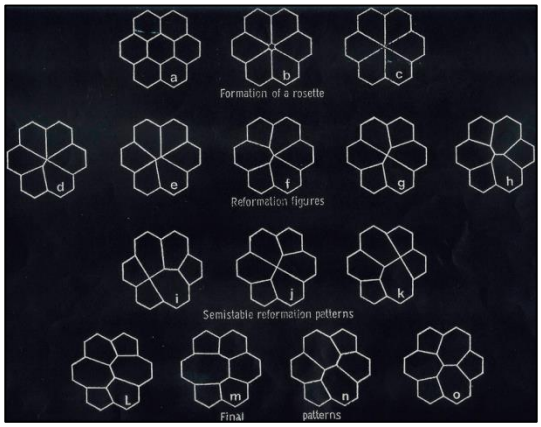


Figure 72: evolution of cells surrounding a necrotic cell (by Steffen Sperling – courtesy of Birte Olesen – Danish Cornea Bank).

Large dark plaques represent areas of massive cellular necrosis (**Figure 73**).

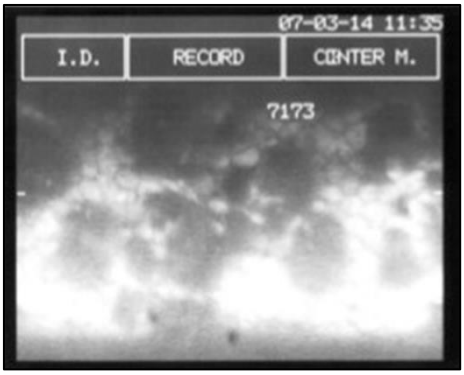


Figure 73: necrotic areas.

3 Evaluation of cornea with the light microscope

3.1 Physics in pills

When using the light microscope, light can interact with matter in the following ways:

- reflection;
- refraction;
- diffraction;
- absorption.

When light arrives on a smooth surface and is reflected with the same angle, there is specular reflection. If the surface is rough, however, the reflection occurs at all possible angles and there is diffuse reflection.

When the light passes from a medium into another with different index of refraction is deviated from a straight line, because of the different speed of light waves in the different media. The index of refraction of a medium is the ratio between the speed of light in the vacuum and that in the medium itself.

Snell's law, also known as the Descartes law or Snell-Descartes law, describes the refraction mode of a light ray in the transition between two different refractive index media (**Figure 74**):

$$\frac{\sin\theta_1}{\sin\theta_2} = \frac{n_1}{n_2}$$

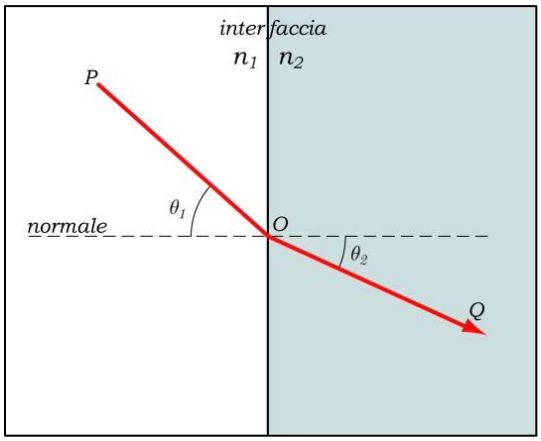


Figure 74: Snell's law.

The light that passes through an edge or a small part of an object is diffused by diffraction, according to the equation:

$$d = \frac{\lambda}{n \sin\alpha}$$

where

d = linear dimension of an object;

λ = wavelength of light;

n = index of refraction of the medium;

α = diffraction angle.

When the light wave passes through a transparent object its amplitude (intensity) is reduced compared to the light that passes around it. The difference in light intensity is perceived by the eye as a contrast. Most biological samples observed in the light field are transparent. In this case the contrast is created by staining or microscopy techniques.

More specifically in our field: light is diverted when it passes through media with different indices of refraction; fine structures produce strongly deflected rays; biological samples are transparent (they do not absorb light) and their poor contrast can be increased optically.

3.2 The light microscope

An object closer to the eye appears larger because the viewing angle and its projection on the retina are increased (**Figure 75**).

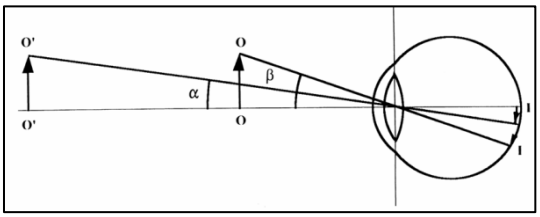


Figure 75: α and β angles are examples of viewing angles of the same object placed at two different distances from the eye.

The main limits for the perception of small details are:

- the normal human eye does not focus on an object placed less than 25cm (nearest distance of distinct vision);
- if the viewing angle becomes extremely small (less than one minute of arc) two points do not appear to be separate, because their images on the retina do not stimulate distinct retinal cells.

If an object is placed near the focus of a convex lens, the viewing angle is increased and the object appears bigger. In this way it is possible to resolve the small details of the magnified image (**Figure 76**).

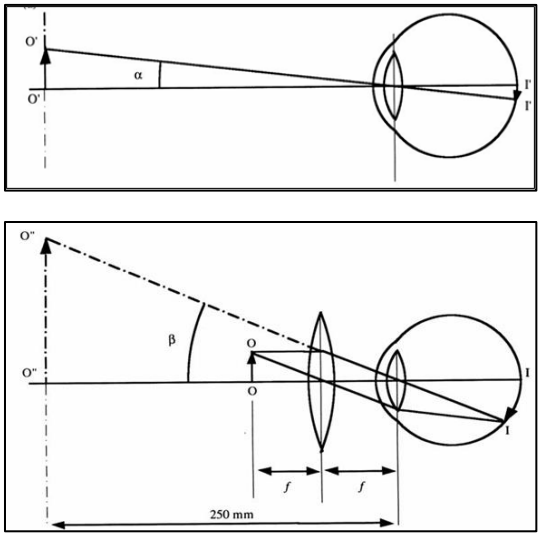


Figure 76: vision with a magnifying glass.

The magnification of a lens is the ratio between the tangent of the angles α and β (tan α : tan β). In practice, the magnification can be expressed as the ratio between the nearest distance of distinct vision and the focal distance of the lens:

$$Magnification = 25cm : focal\ distance\ [cm]$$

With a single convex lens it is not possible to obtain a magnification greater than di 8-10×.

If a single lens is not sufficient, several lenses can be placed one after the other to get the compound microscope.

A typical compound microscope magnifies in two steps (**Figure 77**):

- the objective (2) produces a magnified image of the specimen (1) in the intermediate image plane (4);
- the eyepiece (5) magnifies the intermediate image like a magnifying glass.

In modern microscopes, the infinitely corrected lens (ICS) projects parallel rays at an infinite distance and the intermediate image is formed by an additional tube lens (3).

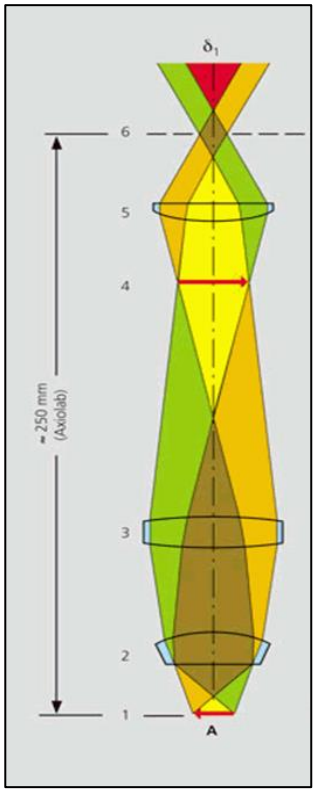


Figure 77: optical system of a compound microscope.

ICS microscopes have two main advantages:

- the combination of objective and tube lens allows to eliminate most of the aberrations;

- focusing is done by moving only the objective, because the distance between objective and tube lens can be varied without problems.

The overall magnification of the microscope is given by the formula:

$$M_{Microscope} = M_{Objective} \times M_{Eyepiece}$$

Resolution is the minimum distance at which two points are distinguished as separate.

$$d_0 = \frac{\lambda}{N.A._{Objective} + N.A._{Condenser}} = \frac{\lambda}{2N.A.}$$

where

d_0 = resolution limit;

λ = wavelength of light;

N.A. = numerical aperture.

A high magnification that is not accompanied by a corresponding resolution is not effective. The resolution limit of the light microscope is 0,2µm.

To achieve the maximum resolution:

- the objective should have high N.A. to collect more of diffracted light;
- it is necessary to use the shortest possible wavelength of light (the light green-blue or green is the best compromise between visibility and resolution).

3.3 Anatomy of the light microscope

The main components of the light microscope are (**Figure 78**):

- light source (1);
- condenser (2);
- objective (3);
- eyepiece (4).

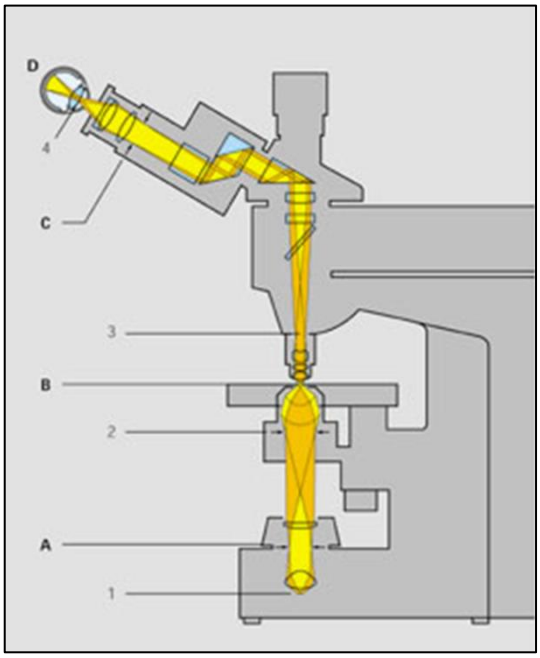


Figure 78: scheme of an optical microscope.

The light source can consist of:

- a tungsten filament, which allows a continuous light spectrum from 300 to 1500nm;
- a halogen bulb, characterized by intense brightness (no blackening of the envelope occurs).

The condenser concentrates light on the specimen, with uniform intensity over the whole field, and provides specific lightings for phase contrast, darkfield or other.

The objective collects the light from the specimen and with the tube lens forms the image on the intermediate plane. Provides most of the magnification and resolution of the microscope.

The objective is characterized by the numerical aperture value (N.A.). This value represents the measurement of the angle of light covered and constitutes an indirect index of the resolving power of the microscope (**Figure 79**).

$$N.A. = n \sin \alpha$$

where

n = index of refraction of the medium (n_{air} = 1);

α = half of the lens opening angle.

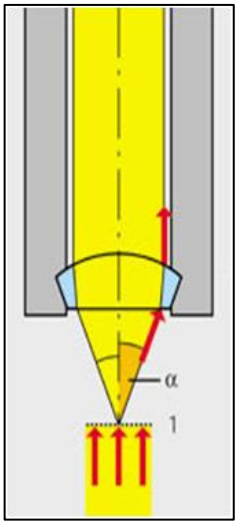


Figure 79: numerical objective and aperture.

The value of the half-angle of acceptance increases if you use immersion liquids of the same refractive index of the glass, placed between the object and the cover slip.

The maximum resolution is obtained when all the diffracted light is collected by the objective, namely when the condenser diaphragm has the same N.A. of the objective (**Figure 80**).

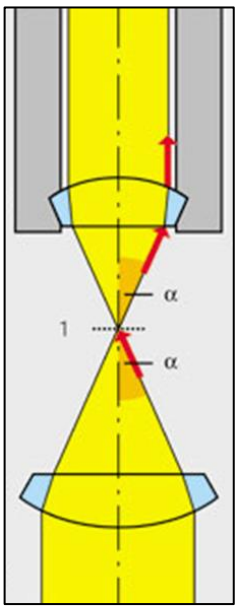


Figure 80: maximum resolution.

The lenses show a color code for the magnification and the type of immersion fluid that can be used (**Figure 81**).

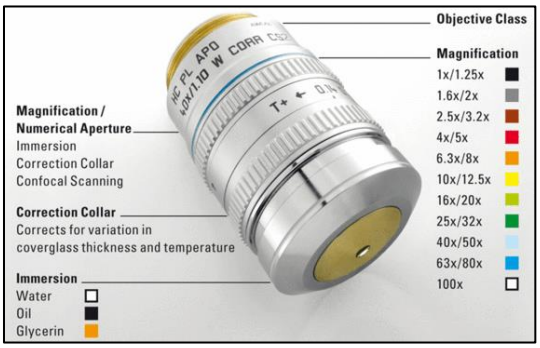


Figure 81: color code represented on the objectives.

The eyepiece magnifies the intermediate image formed by the objective and the intermediate lens, completes the correction of the residual aberrations of the intermediate image and introduces reticles or pointers in the conjugated plane of the intermediate image.

3.4 Physiology of the light microscope

The image of an object placed in an optical plane is projected into each successive plane of the same series:

- aperture series → lighting;
- field series → image.

The two series are completely separate.

Understanding these plans is important for using appropriate lighting and for inserting reticules or filters in the right position:

SERIES OF OPENINGS	SERIES OF FIELDS
Filament of the lamp	
	Diaphragm of the lamp
Diaphragm of the condenser	
	Plan of the specimen
Back focal plan of the objective	
	Plan of the intermediate image
Pupil of the eye	
	Retina of the eye

A homogeneous light source is projected directly from the condenser onto the plane of the specimen and then onto the retina of the eye (according to the conjugate planes).

The light source must be large and without structure.

In 1893, August Köhler devised a technique according to which a collecting lens is placed in front of the lamp with its filament located near the focal point and projects an image of the filament on the diaphragm plan of the condenser. Then the image of the lamp filament will not be on the retina of the observer and the lamp collector lens will appear as a homogeneous secondary source, projected on the plan of the specimen.

3.5 Pathology of the light microscope

There are two orders of aberration of the lenses:

- 1st order → chromatic and spherical;
- 2nd order → coma, astigmatism and field curvature.

In the chromatic aberration, the component wavelengths of the white light are refracted at different angles and are not focused (**Figure 82**).

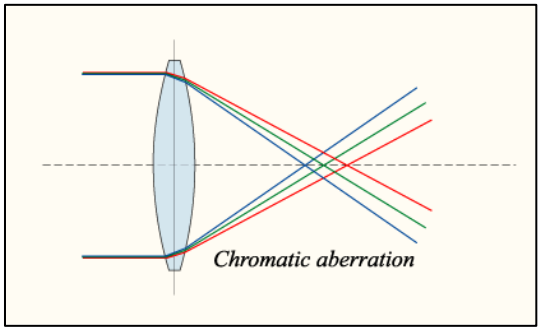


Figure 82: chromatic aberration.

The image appears surrounded by fringes that vary in color depending on their focus. This aberration is corrected by the combination of lenses.

In spherical aberration the light waves passing to the periphery of the lens are refracted more than those passing in the center: the beams of light are not concentrated in the same point and there is a extensive area of confusion (**Figure 83**).

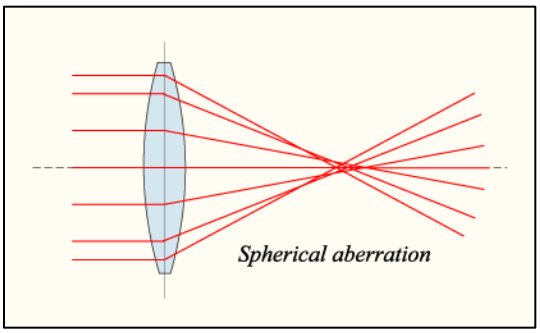


Figure 83: spherical aberration.

Coma derives its name from the appearance similar to a comet of the image that undergoes aberration. In general, points that lie outside the axis are projected as a conical or comet-shaped blur (**Figure 84**).

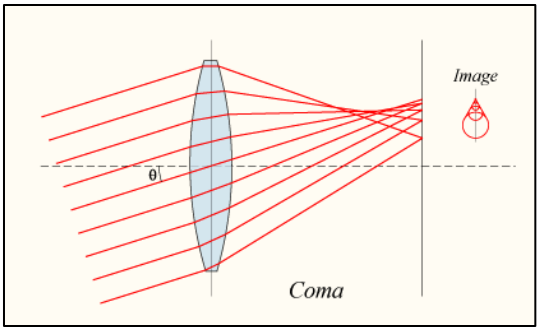


Figure 84: coma.

Astigmatism is similar to chromatic aberration, but depends more strongly on the obliquity of the light beam.

In field curvature, the image plan is curved and appears sharp in the center or at the edges of the field of view, but not both. Field curvature can be a serious problem for microphotography.

The objectives can be constructed in such a way as to correct the aberrations described. There are therefore various types of objectives:

- achromatic, which have a correction for chromatic aberration according to two wavelengths (red and blue);
- semi-apochromatic, which have a better color correction than achromatic, allow a greater numerical opening of achromatics of equal magnification, higher resolution and better contrast;
- apochromatic, which have a correction for chromatic aberration according to three wavelengths (red, blue and green).

3.6 Miscellaneous

The following is a series of representative figures of tissue anomalies that can be found at an analysis of the cornea under an optical microscope (**Figures** from **85** to **92**).

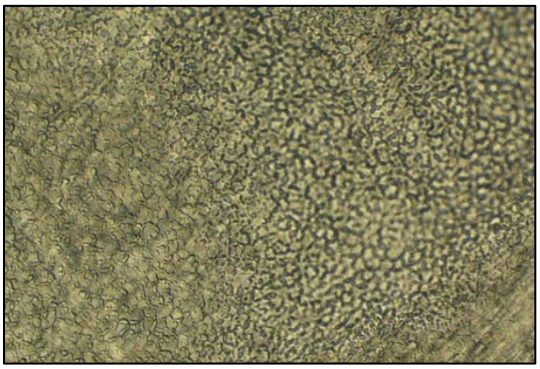


Figure 85: normal endothelium.

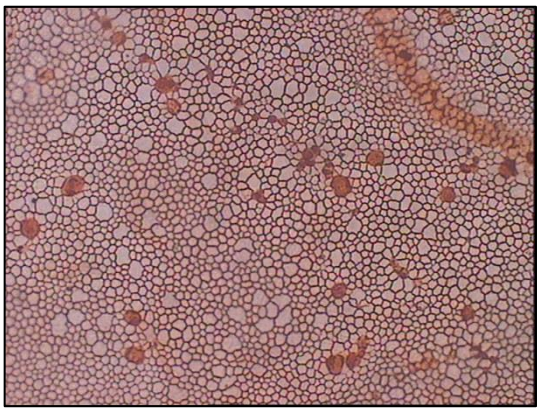
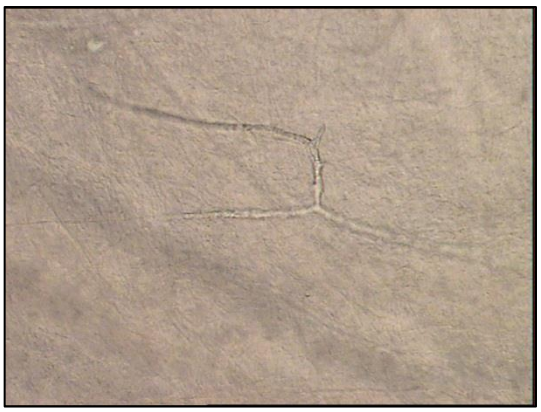


Figure 86: polymorphic endothelium (alizarin red).



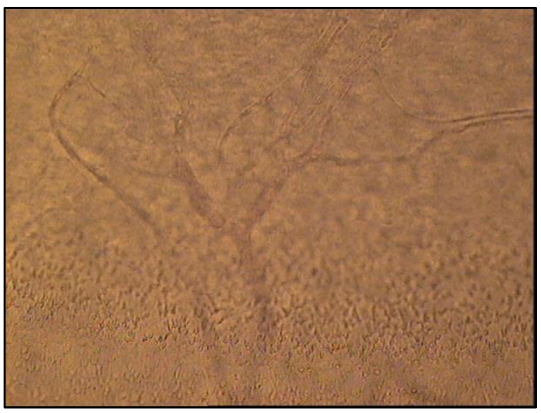


Figure 87: corneal vascularization.

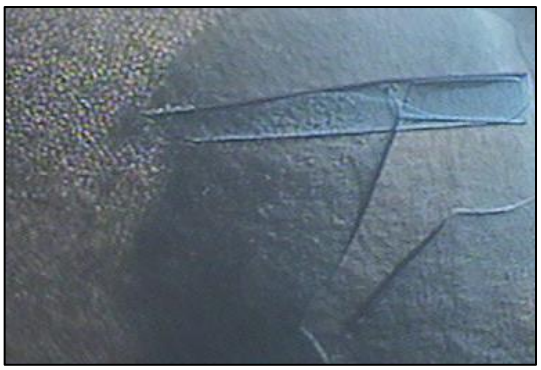


Figure 88: endothelium excised (trypan blue).

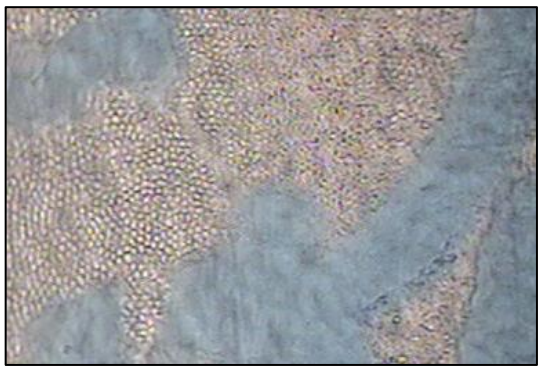
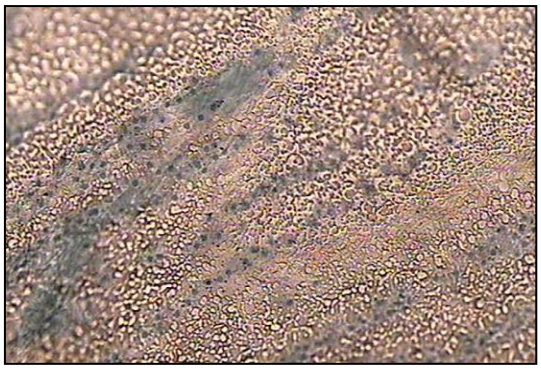


Figure 89: iatrogenic excision damage (trypan blue).



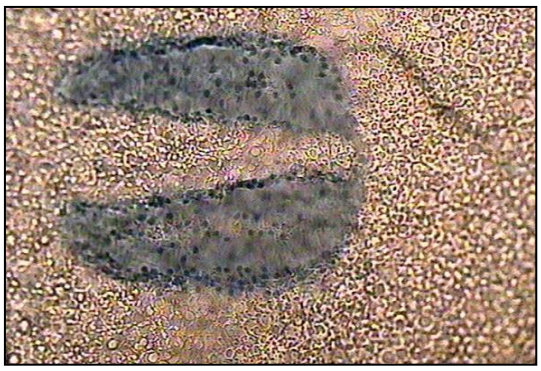


Figure 90: iatrogenic excision damage (trypan blue).

Figure 91: outcome of a foreign body in an optical zone.

Figure 92: post-surgical corneal scar.

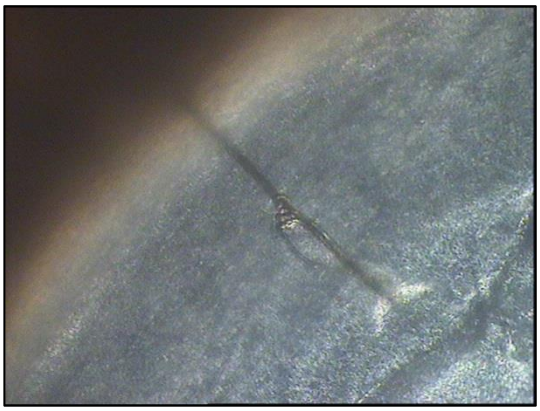


Figure 93: post-surgical residual corneal suture.

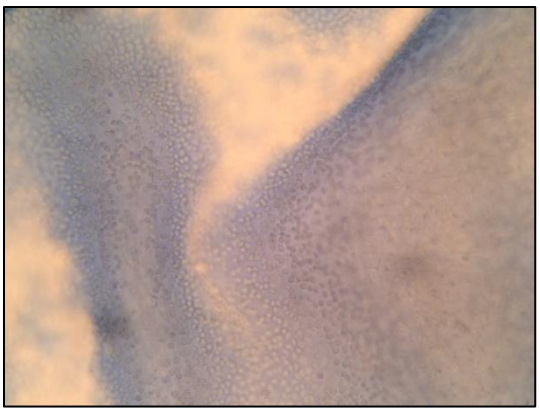
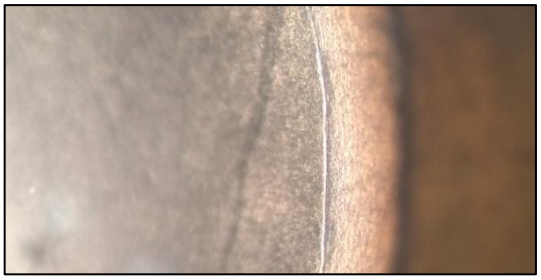


Figure 95: diffuse and uniform coloration with trypan blue of the posterior cornea after culture at 31°C (40×) [top]. In detail (100×) [below] we can appreciate the total absence of the physiological endothelial mosaic with the cells that appear degenerating in spheroidal form.



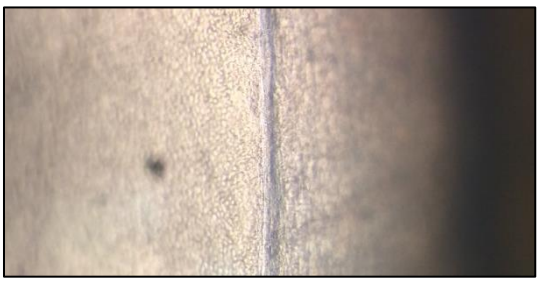
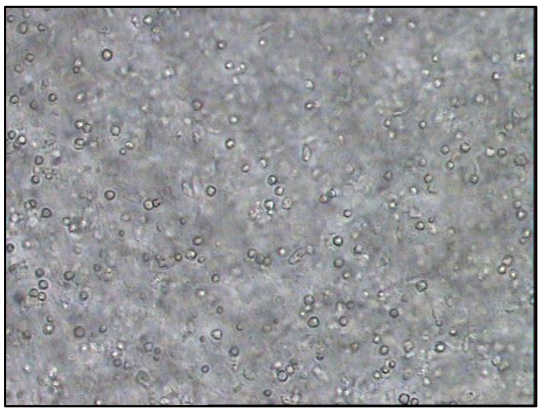


Figure 94: prominent and centralized Schwalbe's line within the 360° transparent cornea (40×) [top]. In detail (100×) [below] we can recognize the Schwalbe's line because it limits the corneal endothelium (left side of the photo).



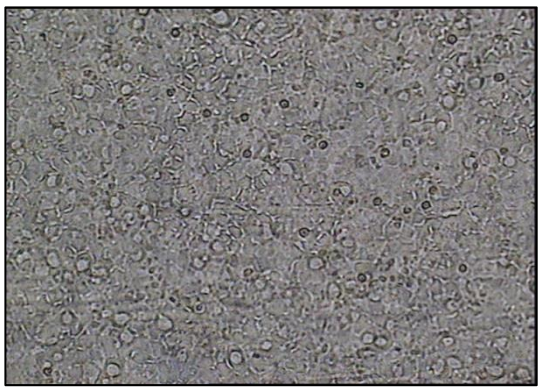


Figure 96: Acanthamoeba corneal contamination.

Figures index