La membrana amniotica in dermatologia: stato dell’arte e prospettive.

Dott. Elisa Pianigiani

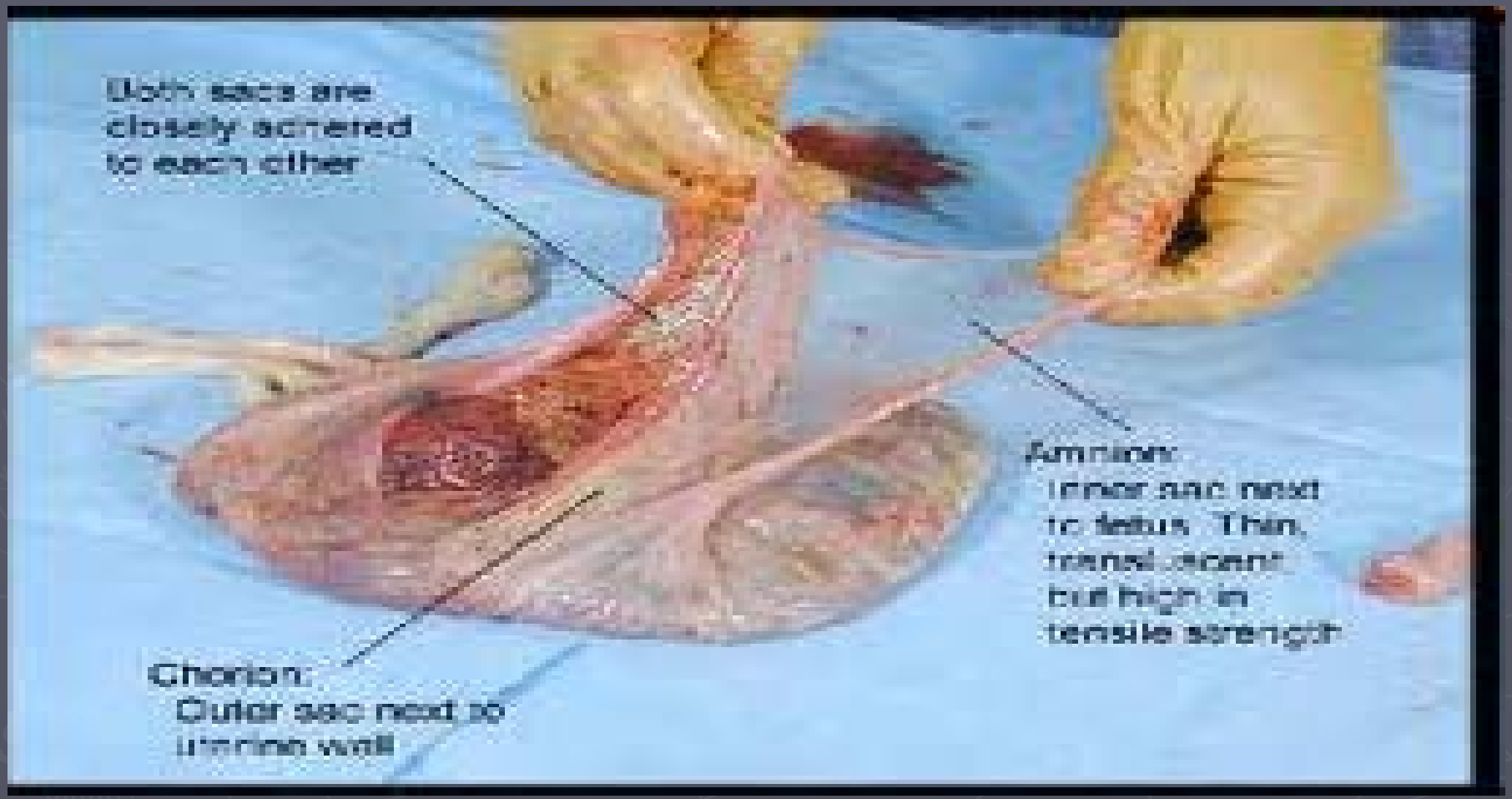
Resp. Centro Conservazione Cute

Amnion in dermatologia – la storia

- 1910 Davis - amnion come graft cutaneo
- 1920 Sabella - amnion come copertura permanente in pazienti ustionati
- 1952 Douglas – amnion come sostituto biologico temporaneo
- 1979 Trelford – infezioni
- 2003 Sawamura – Impiego in EB

Venetia LO et al. Int J Dermatol 2009

Separazione dell’amnion dal corion



La membrana amniotica deriva dal trofoblasto.

Le sue cellule ricoprono l'embrione in fase di sviluppo e presentano soltanto antigeni di classe II del sistema maggiore di istocompatibilità (MHC)

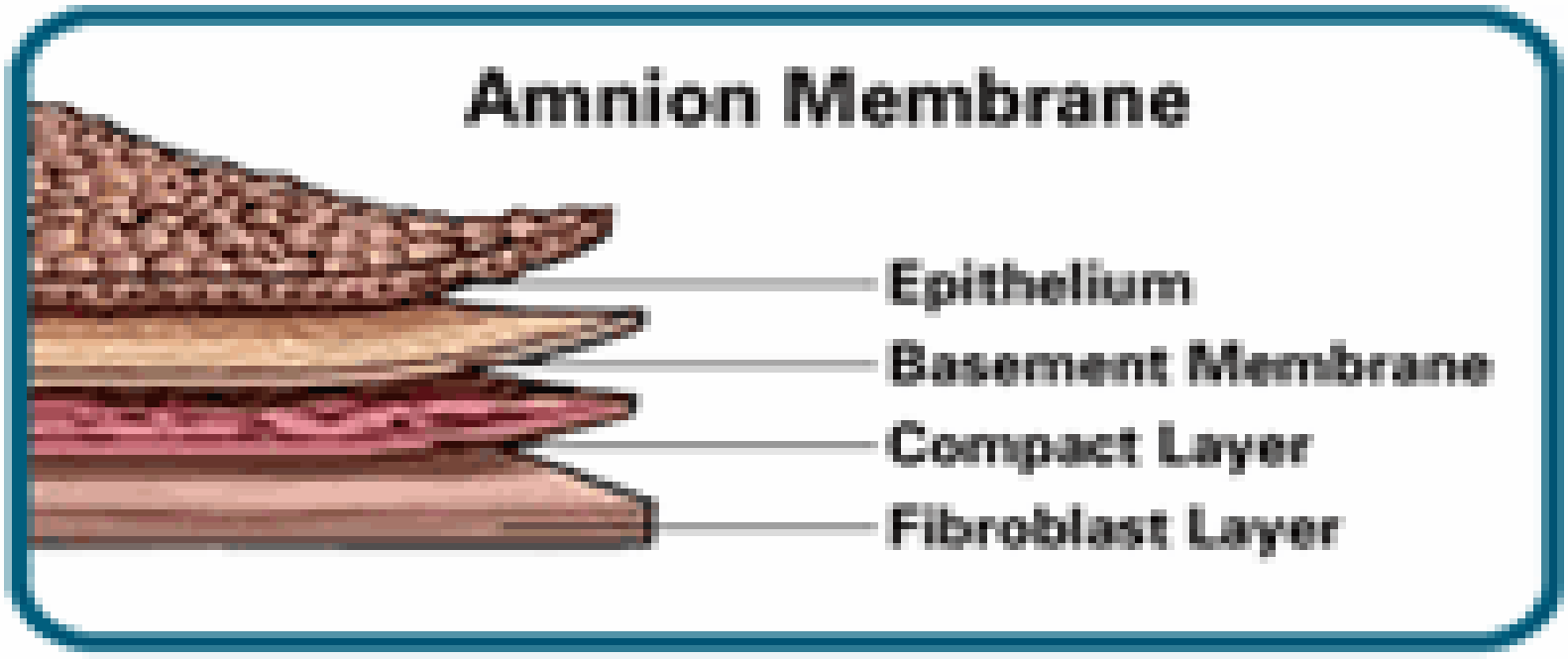


Illustration courtesy of Surgical Biologics, LLC.

Composizione istologica

- La membrana amniotica è parte della placenta e si compone di amnion e chorion. L'Amnion è lo strato interno ed è costituito da un singolo strato di cellule epiteliali, una membrana basale (MB), uno spesso strato compatto, e uno strato di fibroblasti. La MB contiene collagene tipo III, IV, V, fibronectina e laminina.

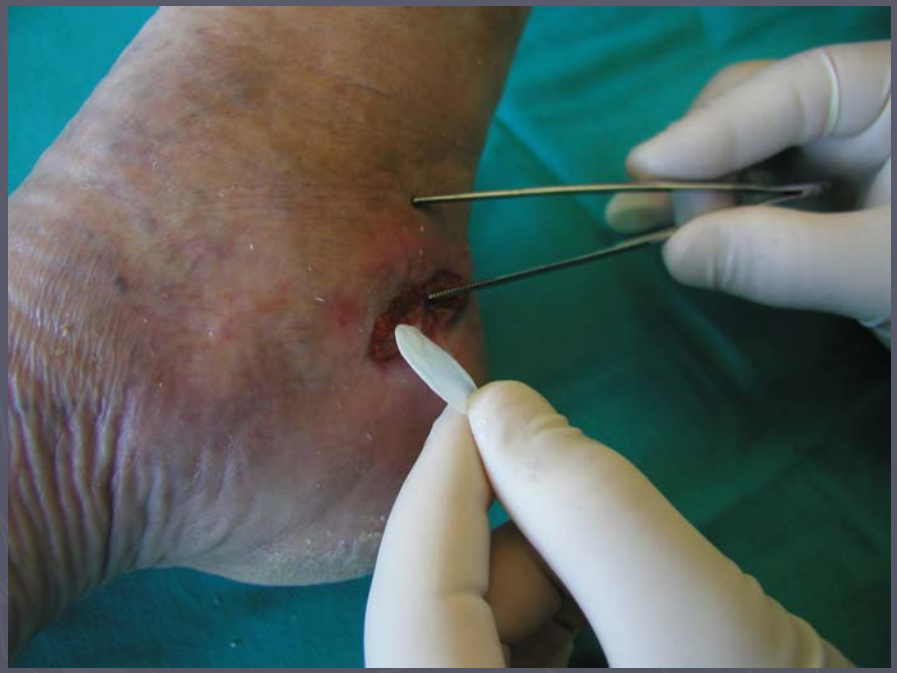
Proprietà della membrana amniotica:

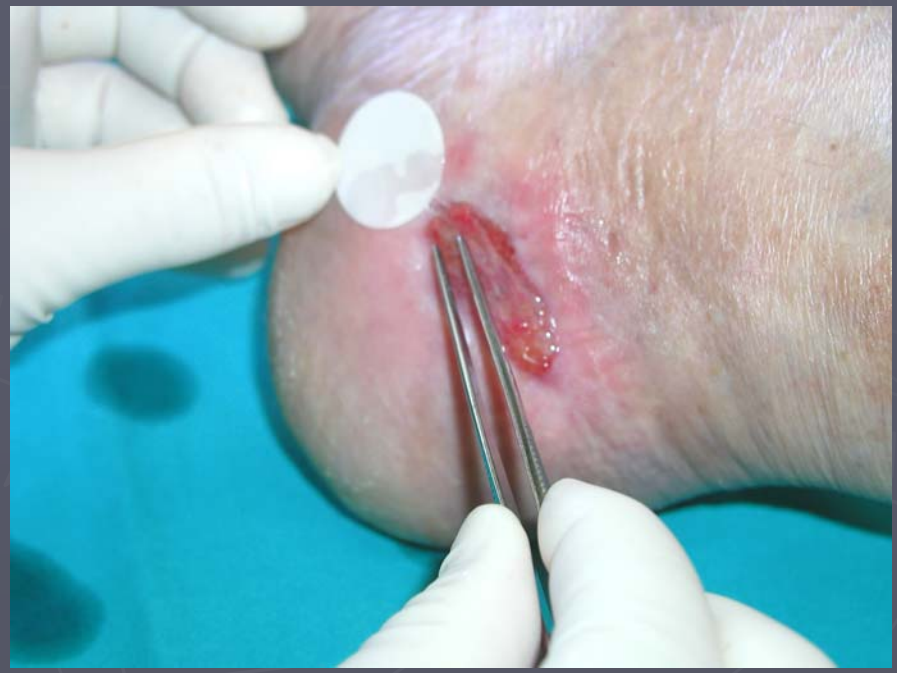
- Anti-infiammatorie
- Antiangiogeniche
- Antifibrotiche
- Anticicatriziali
- Pro-rigenerative
- Antibatteriche

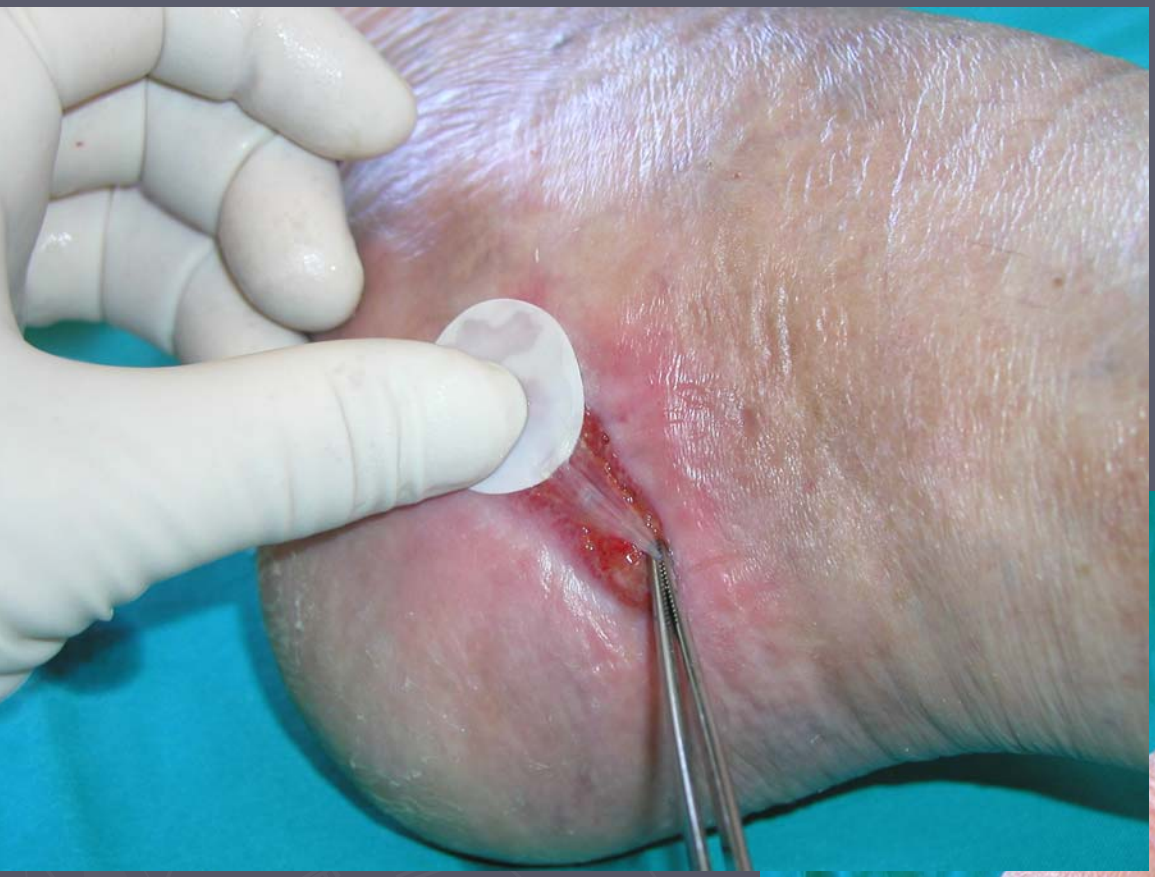
Uso in dermatologia

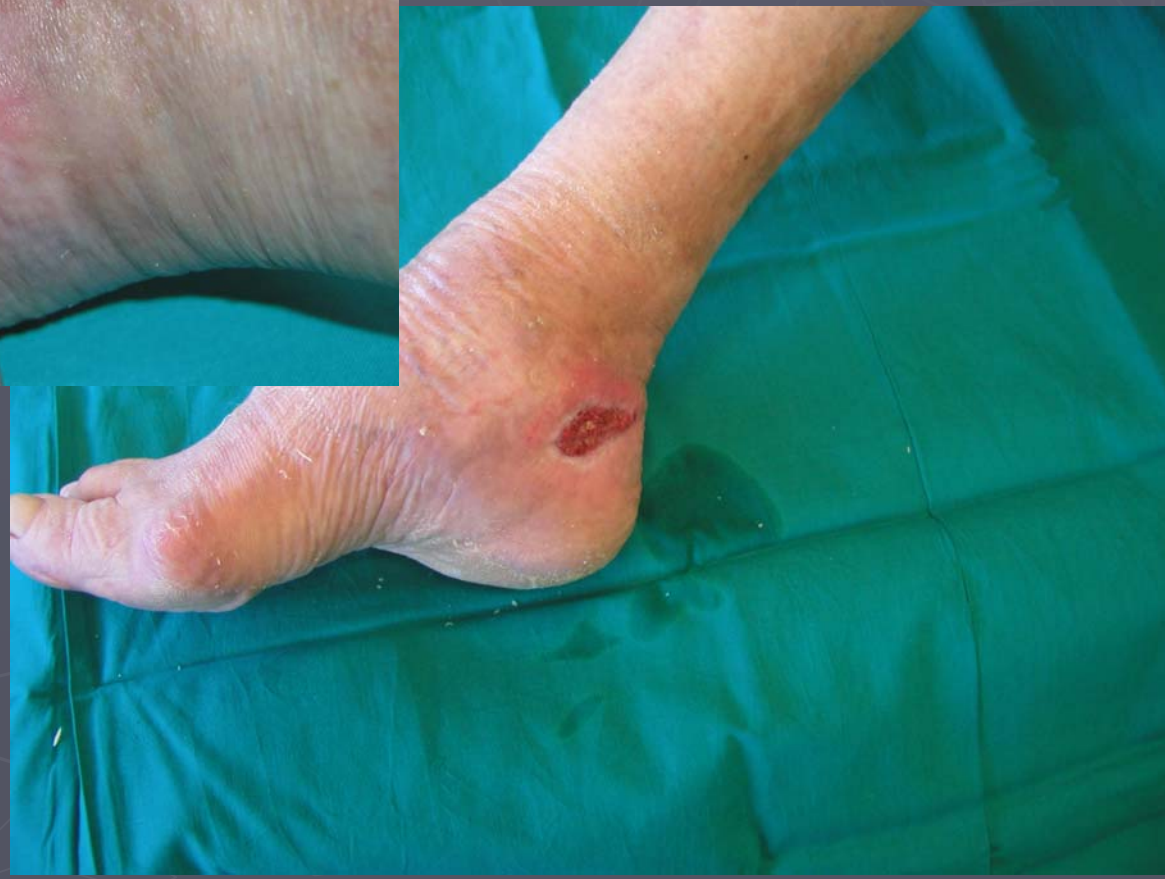
- **Ustioni** (Gajiwala 2004): copertura temporanea in attesa/sostituzione di graft cutaneo
- **EB** (Matinez Pardo 1999): riduzione tempi di cicatrizzazione EB distrofiche
- **Ulcere croniche** (Singh 2004): azione prorigenerativa in ulcere “non-healing”
- **Lyell syndrome** (Prasad 1986): guarigione in 3 gg. pazienti pediatrici con coinvolgimento cutaneo del 90%
- **Pemfigo** (Annamma 2010): copertura temporanea, effetto antalgico, prorigenerativo

Un caso dermatologico







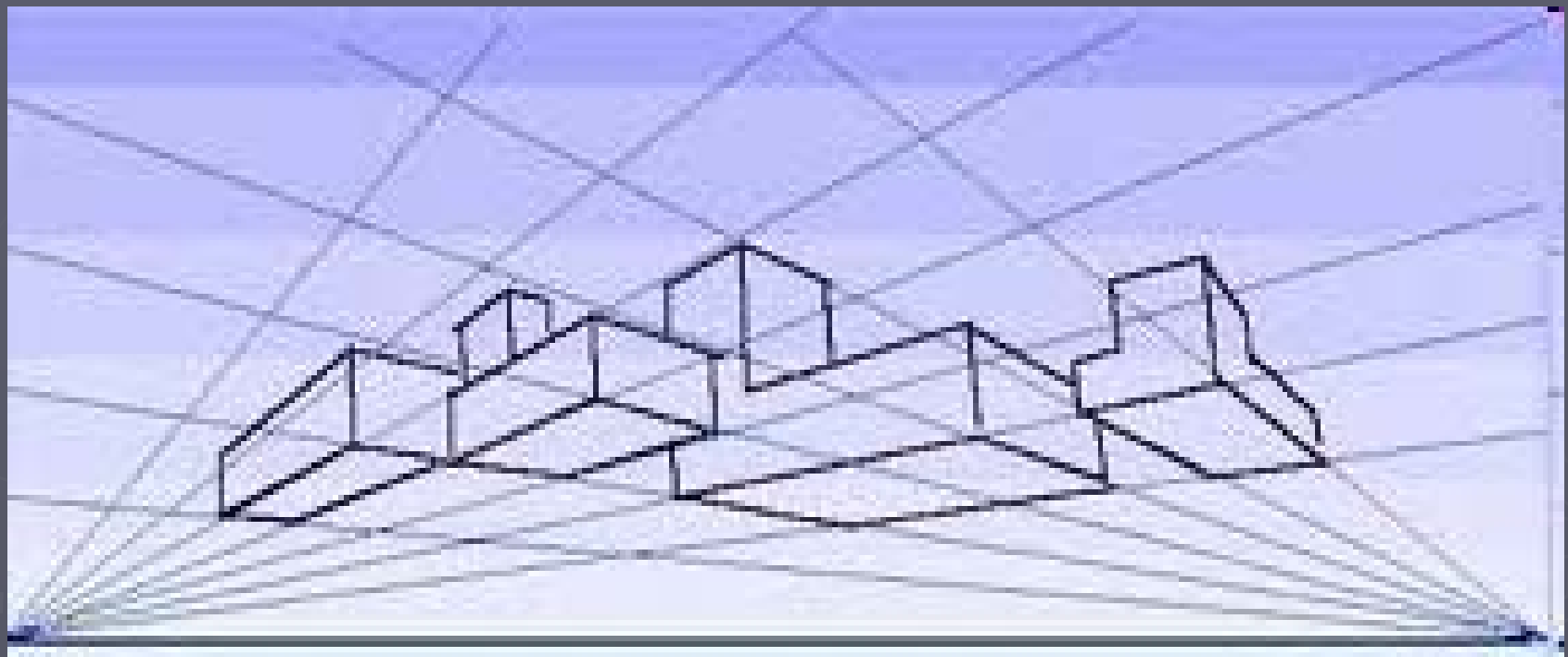


TREATMENT OF CHRONIC ULCERATION OF THE LEGS WITH HUMAN AMNION
JohnP. Bennett et al. Lancet 1980

- “The appearance of healthy granulation tissue after amnion application was associated with successful autografting”.

HUMAN AMNION AS AN ADJUNCT IN WOUND HEALING
W. Page Faulk et al. Lancet 1980

- "After amnion application there was considerable granulation tissue in the ulcer bed and microscopical evidence of thinned connective tissues, vessel development, more compact resolution of vascular basement membranes.."



Prospettive

L’amnion come cell carrier

- **la membrana basale della membrana amniotica facilita la migrazione delle cellule epiteliali, facilita l’ adesione delle cellule epiteliali basali, promuove la differenziazione epiteliale .**
- **Costituisce il substrato ideale per l’adesione e la crescita delle cellule staminali.**

Guo M. J Invest Dermatol 1989

L’amnion nel “tissue engineering”

Cultured limbal epithelial cells

ocular surface reconstruction → cultured human corneal endothelial cells

Cultured lacrimal gland acinar cells (animal model)

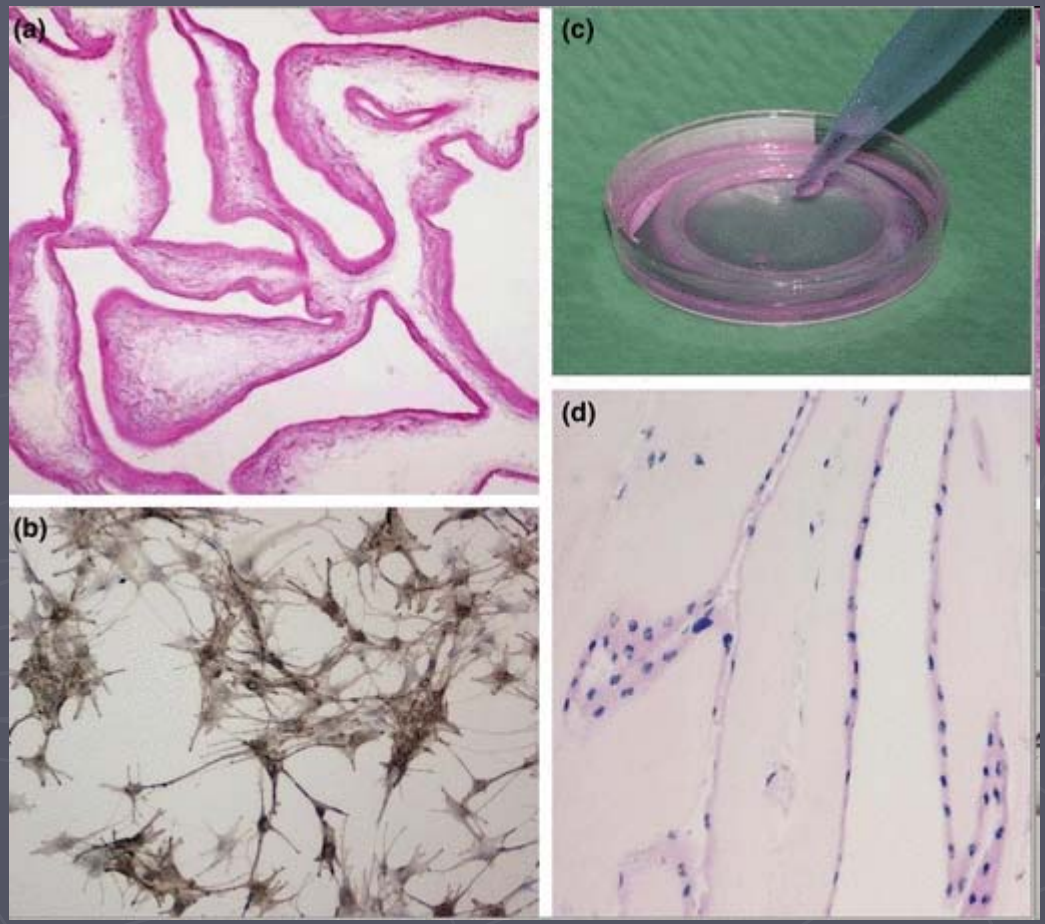
tissue engineering of the urinary tract system

chondrocyte carrier

Autologous melanocyte-keratinocyte cell culture

Cultivated autologous oral epithelial cells

Autologous melanocyte- keratinocyte cell culture – Redondo P. 2008



La nostra esperienza nella ricostruzione dell'epitelio corneale

UNIVERSITA' DEGLI STUDI DI SIENA
DIPARTIMENTO DI SCIENZE OFTALMOLOGICHE E NEUROCHIRURGICHE
DIPARTIMENTO DI DERMATOLOGIA*
ISTITUTO DI ANATOMIA PATOLOGICA**

CORNEAL EPITHELIAL CELLS CULTURE ON HYALURONAT BASED CARRIER : HISTOLOGICAL STUDY AND CLINICAL USE

TRAVERSI C. , PIANIGIANI E.*,TOTI P.**, ANDREASSI L.*, CAPOROSSI A.

RIVA DEL SOLE 5-7 OTTOBRE 2000

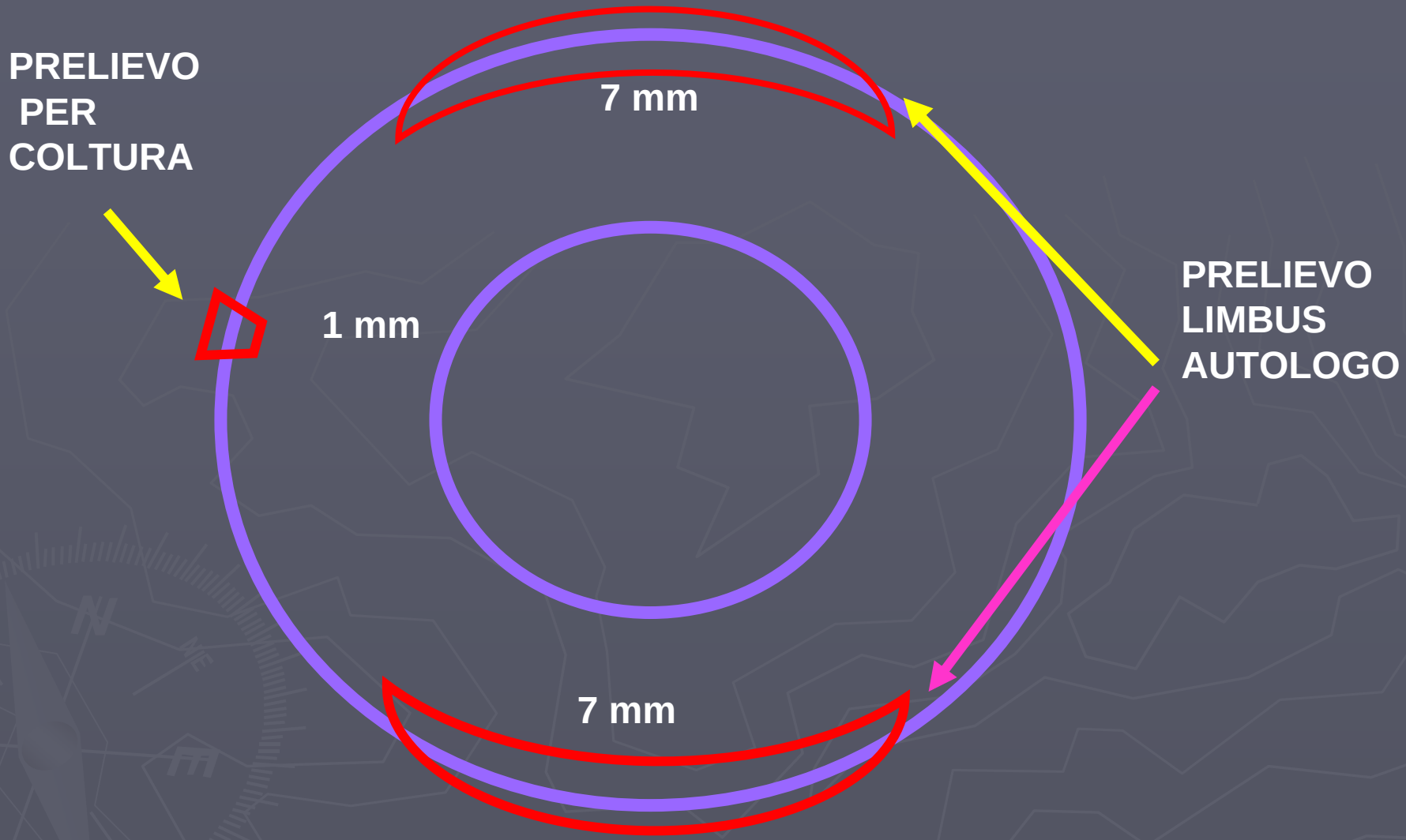
RICOSTRUZIONE DELLA SUPERFICIE OCULARE

TRAPIANTO DI CONGIUNTIVA

CHERATOEPITELIOPLASTICA

TRAPIANTO DI LIMBUS

INNESTO DI CELLULE COLTIVATE

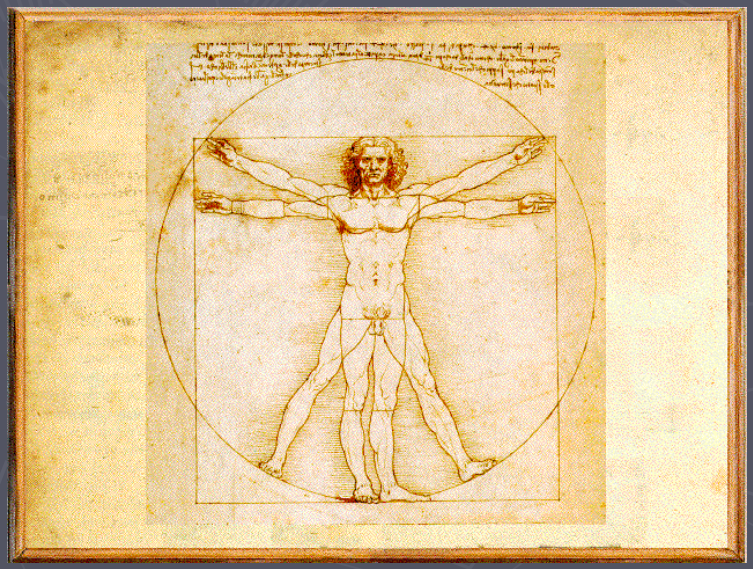

PRELIEVO
PER
COLTURA
7 mm
1 mm
PRELIEVO
LIMBUS
AUTOLOGO
7 mm

COLTURA DI CELLULE

-PRELIEVO PICCOLO

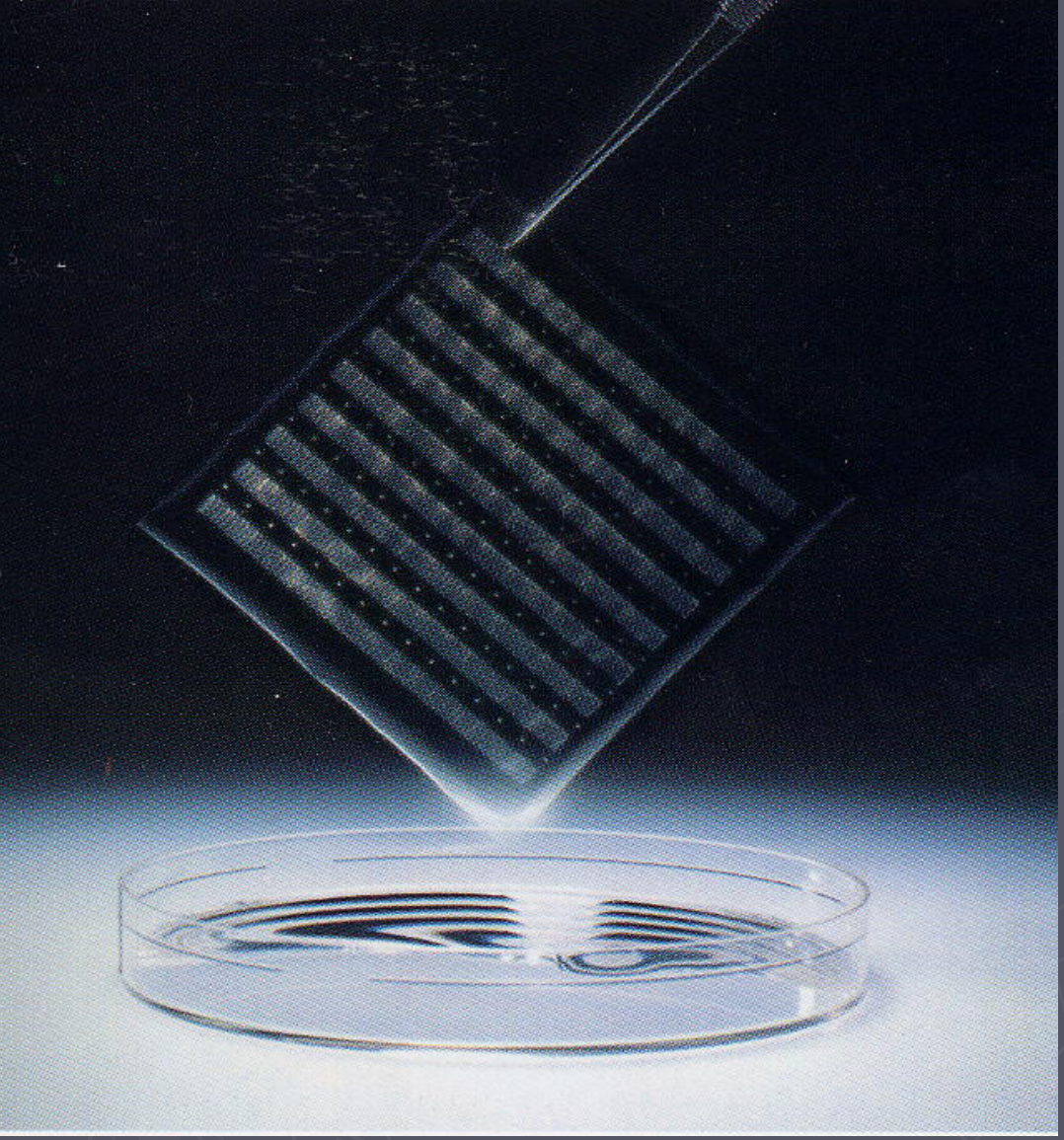
-PROCEDURA NON CONTESTUALE

-LABORATORIO PER LE COLTURE

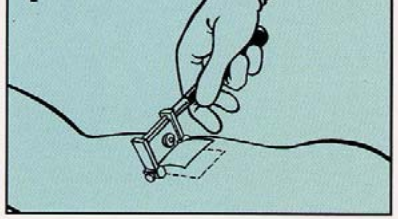


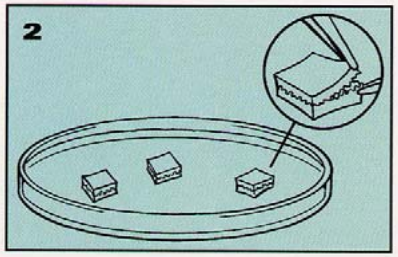
COLTURA DI CELLULE : CARRIER

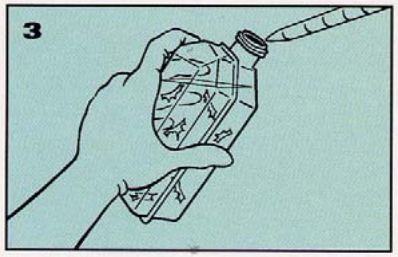
- LENTE A CONTATTO
- PETROLATUM GAUZE
- FIBRINA
- MEMBRANA AMNIOTICA
- SCHIELDS DI COLLAGENE
- ACIDO IALURONICO ESTERIFICATO (LASERSKIN)

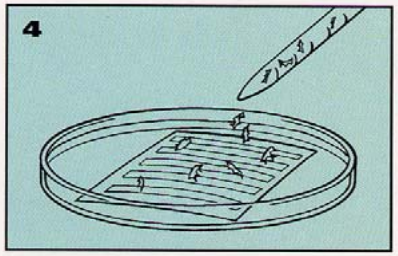


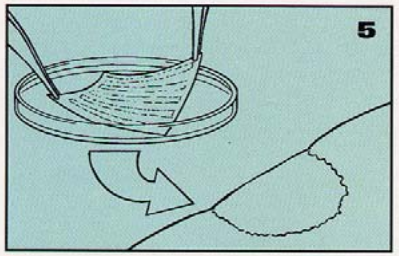
LASERSKIN
MEMBRANA DI ACIDO IALURONICO ESTERIFICATO CON FORI DI 40 MICRON

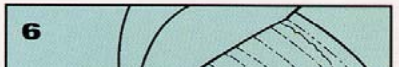












Shave and disinfect the skin surface prior to taking the biopsy. We recommend using a solution of povidone iodine or chlorhexidine in sterile water. Then, use a sterile gauze soaked in 70% isopropanol to leave the skin dry and ready for biopsy. 2-4 cm² biopsies are taken using a scalpel or dermatome. The latter technique is advisable since it provides skin specimens of a more uniform thickness. The skin biopsy should be sent immediately to the laboratory in antibiotic medium (95% DMEM, 5% FCS, 100 IU/ml penicillin, 100 IU/ml streptomycin). If the biopsy cannot be sent to the laboratory immediately, it should be kept at 4°C in transport medium until required. The keratinocyte cultures should be prepared within 24 hours of surgery, in order to avoid a significant decrease in cell viability.

2. Isolation of keratinocytes

Wash the skin biopsy with sterile PBS in a Petri dish. Spread the skin out in a Petri dish in the presence of few drops of PBS. Cut the skin biopsy into smaller fragments (about 0.5 cm²) and transfer them in a Petri dish containing 10 ml dispase solution (see Appendix 1 for preparation); incubate at 37°C and 5% CO_2 for [illegible] hours. Separate epidermis from dermis. Transfer the epidermal sheets into a tube containing [illegible] PBS, supplement with 0.05% trypsin/0.02% EDTA, stir gently at 37°C and 5% CO_2 for 15 minutes. To block the action of trypsin, add an equal volume of DMEM containing 20% fetal calf serum (FCS). Filter the digested epidermal sheets [illegible] cellular debris. Centrifuge the keratinocyte suspension (250 g) at 4°C for 10 minutes.

3. Primary culture of isolated keratinocytes (P0)

Resuspend the pellet in 5 or 10 ml serum-containing keratinocyte growth medium ("KM", see Table 1) *without EGF*. Cells are counted using trypan blue to identify dead cells and keratinocytes are seeded at a concentration of 20,000 viable keratinocytes/cm², in KM medium *without EGF*, in 75 cm² flasks in the presence of fresh irradiated 3T3 cells (20,000 cells/cm²). After 48-72 hours culture, substitute the medium with complete KM (*with EGF*). Grow the keratinocytes at 37°C in a humidified atmosphere at 5% CO_2, changing the medium every 2-3 days, until keratinocytes cover 75-80% of the flask bottom. This sub-confluence is normally reached in 7 days.

NOTE The 3T3 feeder-layer should be prepared 24 hours beforehand. (Appendix 2).

4. Secondary cultures of keratinocytes on Laserskin® (P1)

Laserskin® preparation

The day before splitting P0 keratinocytes, seed Laserskin® with irradiated fibroblasts (prepared according to Appendix 2) in the Petri dish supplied. Incubate at 37°C in a humidified atmosphere of 5% CO_2.

P0 keratinocytes trypsinization

Wash the flask with 5 ml PBS (CA^{++} and Mg^{++} free), aspirate and add 5 ml 0.1% glucose 0.02% EDTA, in order to obtain complete cell detachment (usually 5 minutes at 37°C with gentle shaking). Discard the supernatant and add 7 ml trypsin (0,05%) and EDTA (0,2%), incubate at 37°C in humidified atmosphere of 5% CO_2 for 10-15 minutes, in order to detach the keratinocytes from the plastic dish. Neutralize the trypsin solution with an equal volume of DMEM containing 20% FCS and centrifuge as above (250 g, 4°C, 10 minutes). Resuspend the cells in 10 ml complete KM medium, count the viable cells by trypan blue exclusion and seed the keratinocytes onto Laserskin® at a density of 20,000 cells/cm2. Culture medium should be renewed every 2-3 days until grafting.

Table 1: Composition of complete keratinocyte medium (KM) containing EGF

Basal medium	DMEM	75%	Cholera toxin	6 ng/ml
	Ham's F12	25%	Transferrin	5 µg/ml
Supplements	FCS	10%	3,3' triiodothyronine	1.3 ng/ml
	Adenine	24 µg/ml	Penicillin	100 IU/ml
	Hydrocortisone	0.5 µg/ml	Streptomicin	100 IU/ml
	Insulin	5 µg/ml	Epidermal Growth Factor	10 ng/ml

5. Grafting

Wound bed preparation

A chronic granulating bed may be "freshened", especially where it appears contaminated or contains significant devitalized tissue, by scraping or shaving necrotic material.

Laserskin® grafting

It is possible to graft keratinocytes grown on Laserskin® when they have reached semi-confluence (normally in 7 days). Clean the injured area and rinse the Laserskin® culture thoroughly with sterile saline. Lift the membrane with the aid of tweezers and place it directly onto the wound bed, making sure that the side exposed to the Petri dish is in contact with the wound bed.

Apply a non-adherent dressing and a support bandage, changing it at 5-7 days. The transparent nature of the Laserskin® membrane will allow inspection of the grafted bed as it heals.

Appendix 1

Dispase preparation

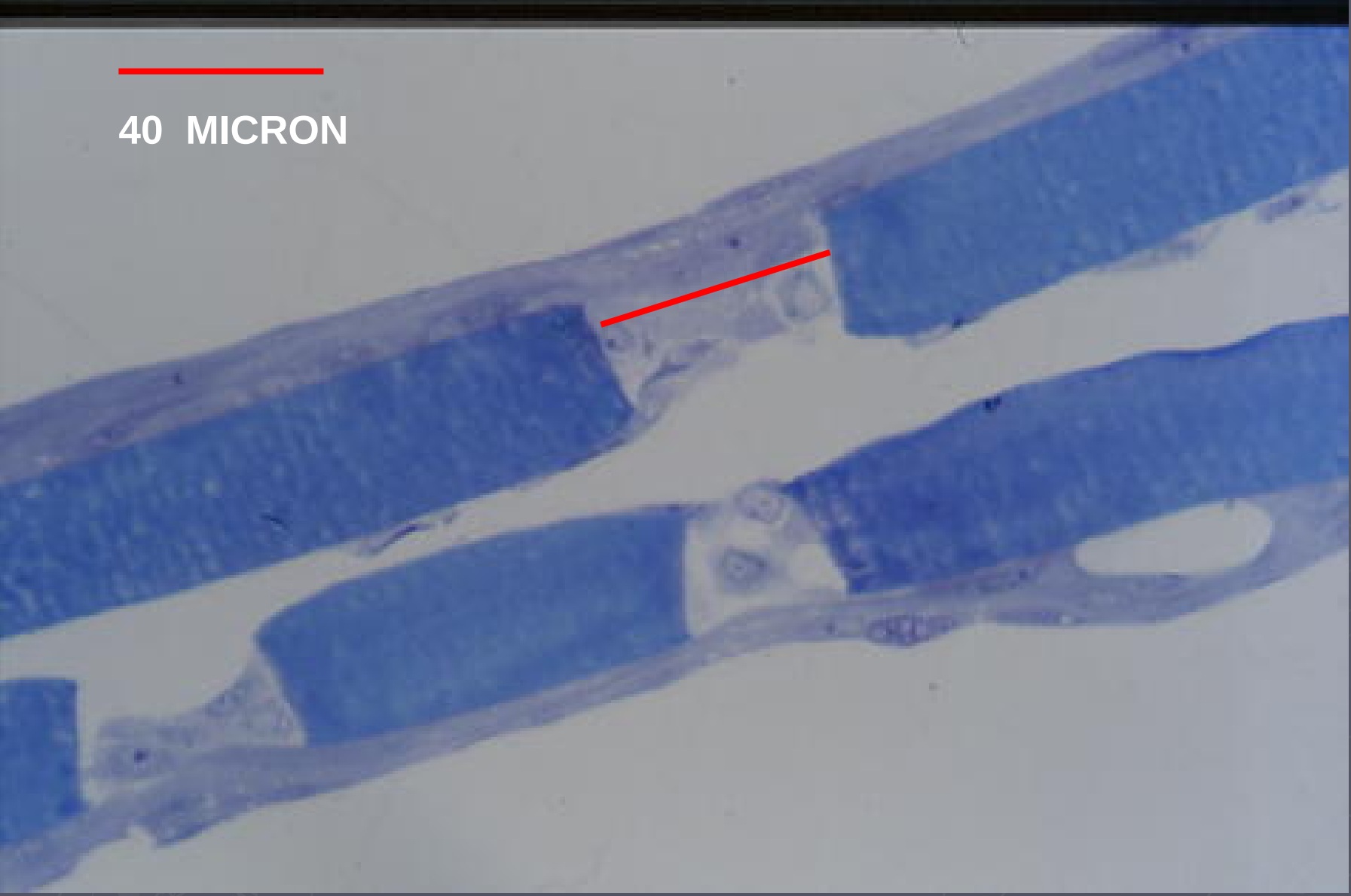
Dissolve 250 mg dispase in 50 ml Hank's Balanced Salt Solution, add 2 ml 1M HEPES. Sterlilize the solution by filtration. This solution can be stored in 10 ml aliquots at -20°C.

Appendix 2

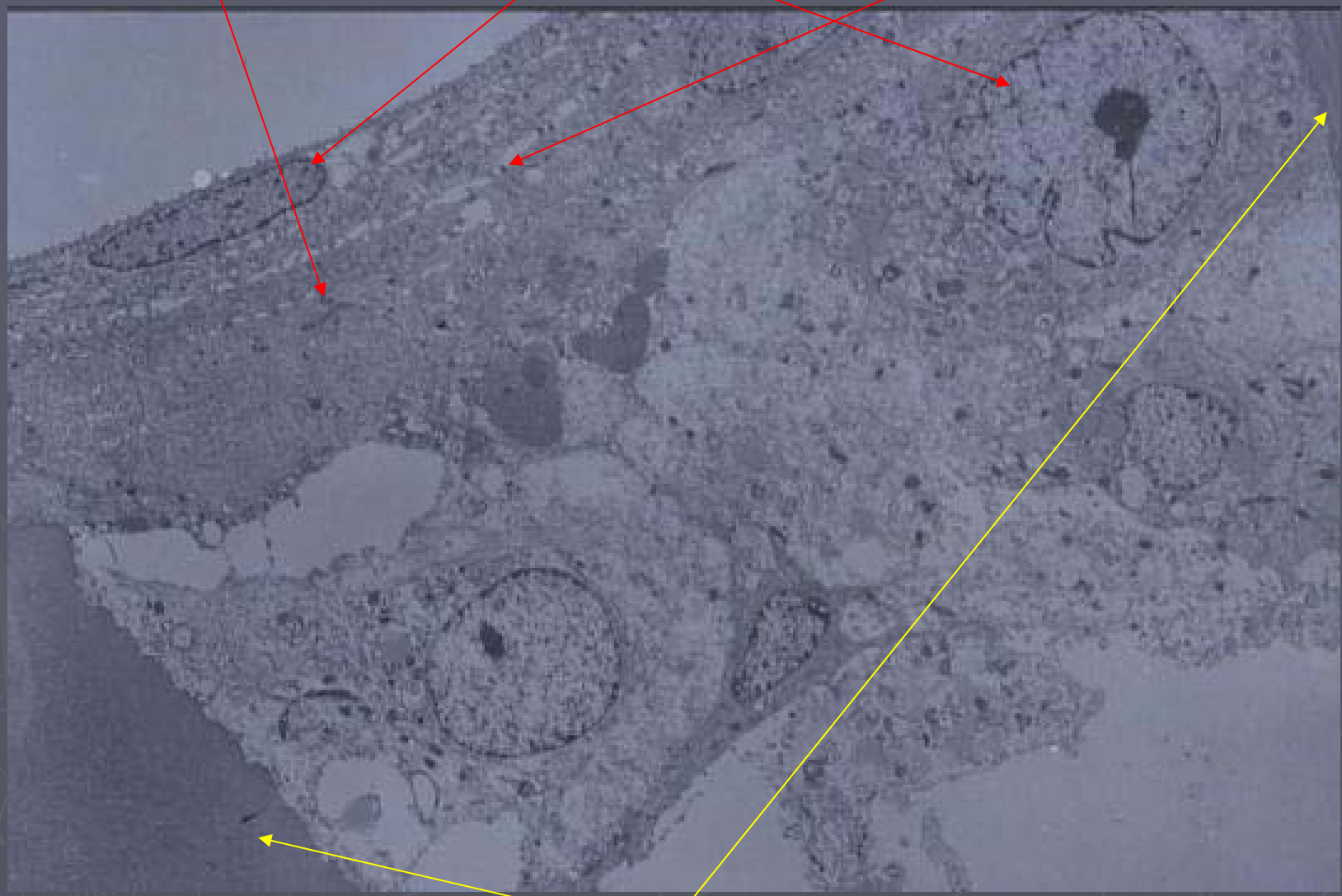
Preparation of 3T3 feeder-layer

1) Remove a 1 ml vial of 3T3 fibroblasts from liquid nitrogen and thaw rapidly.
2) Transfer the cells into a tube containing 9 ml complete medium (Table 2) and centrifuge at 250 g for 5 minutes.
3) Resuspend the cell pellet in 5 ml complete medium and count the cells by trypan blue exclusion staining.
4) Seed the cell suspension in 75 cm² tissue culture flasks, at a density of 20,000 viable cells/cm², in complete medium.
5) Incubate the cells at 37°C in 5% CO_2 for 48-72 hours to obtain a monolayer.
6) When confluent fibroblasts are trypsinized, centrifuged and trypan blue stained (as already described): they can be used for subsequent expansion or preparation of the feeder-layer. In the latter case irradiate the cell suspension with a γ

PREPARAZIONE DI COLTURA DI CELLULE EPITELIALI SU LASERSKIN



COLORAZIONE CON BLU DI TOLUIDINA DELLA MEMBRANA CON L'EPITELIO



MICROSCOPIA ELETTRONICA

LASERSKIN

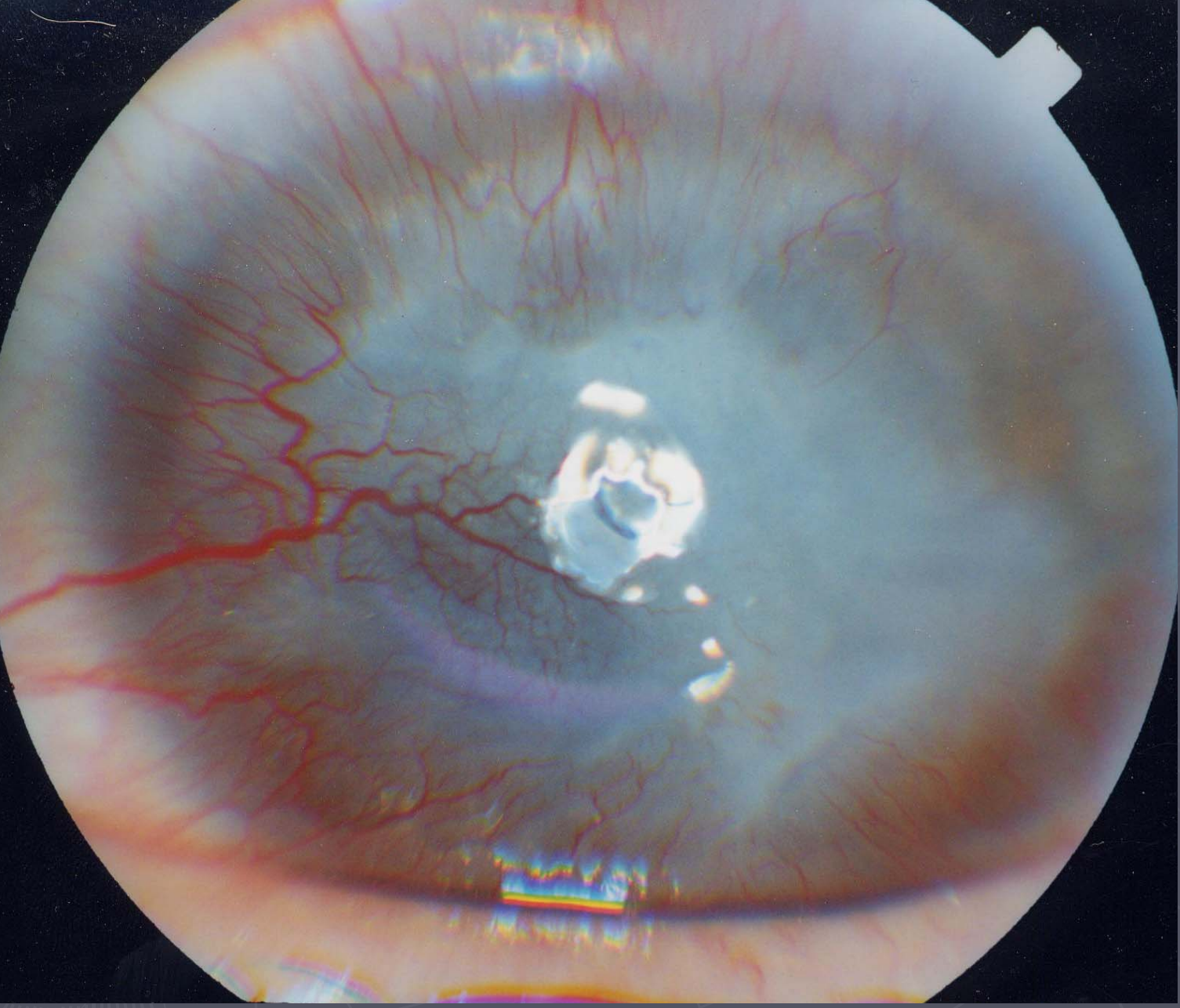
CASO CLINICO

F MAURO 25 ANNI

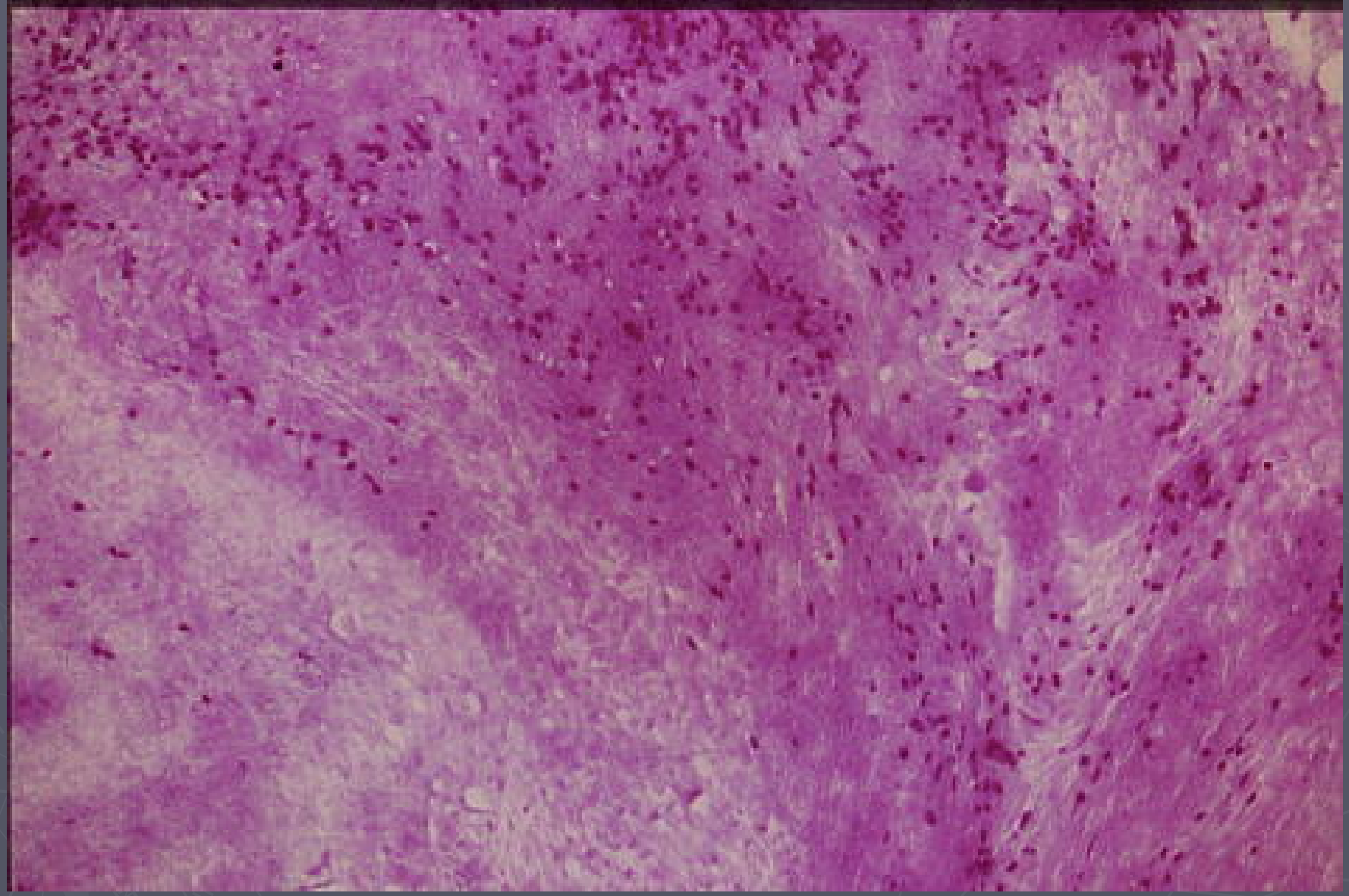
1997 OS CAUSTICAZIONE DA MISCELA DI ACIDI SUL LAVORO RIPARATA CON CONGIUNTIVALIZZAZIONE CORNEALE

2000 Osvisus motu manu

CITOLOGIA DA IMPRESSIONE PRESENZA DI CELLULE MUCIPARE



CASO CLINICO : F. M. 25 ANNI USTIONE DA MISCELA DI ACIDI
OSVisus motu manu



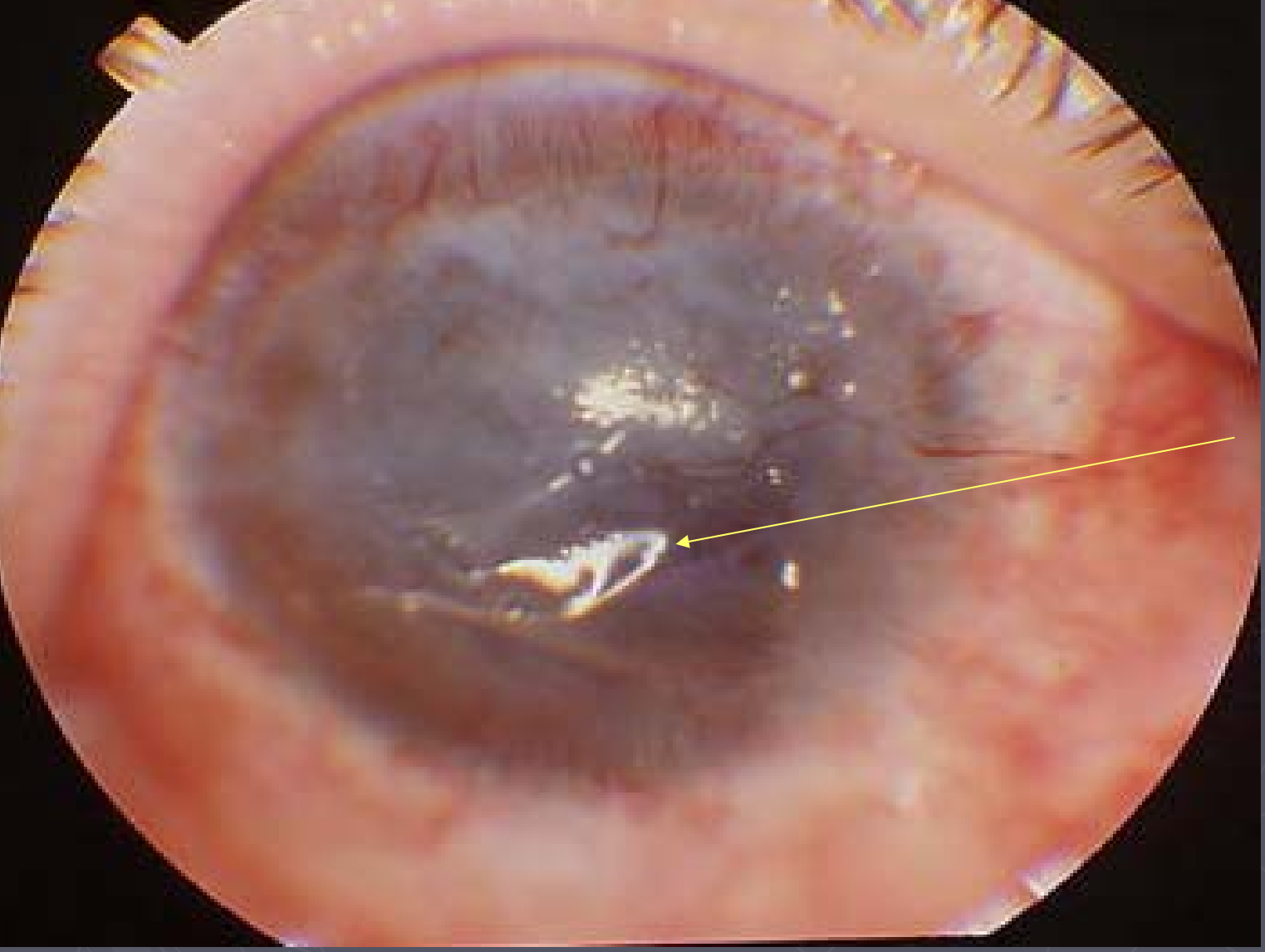
CITOLOGIA DA IMPRESSIONE
COLORAZIONE CON PAS : PRESENZA DI CELLULE MUCIPARE

CASO CLINICO

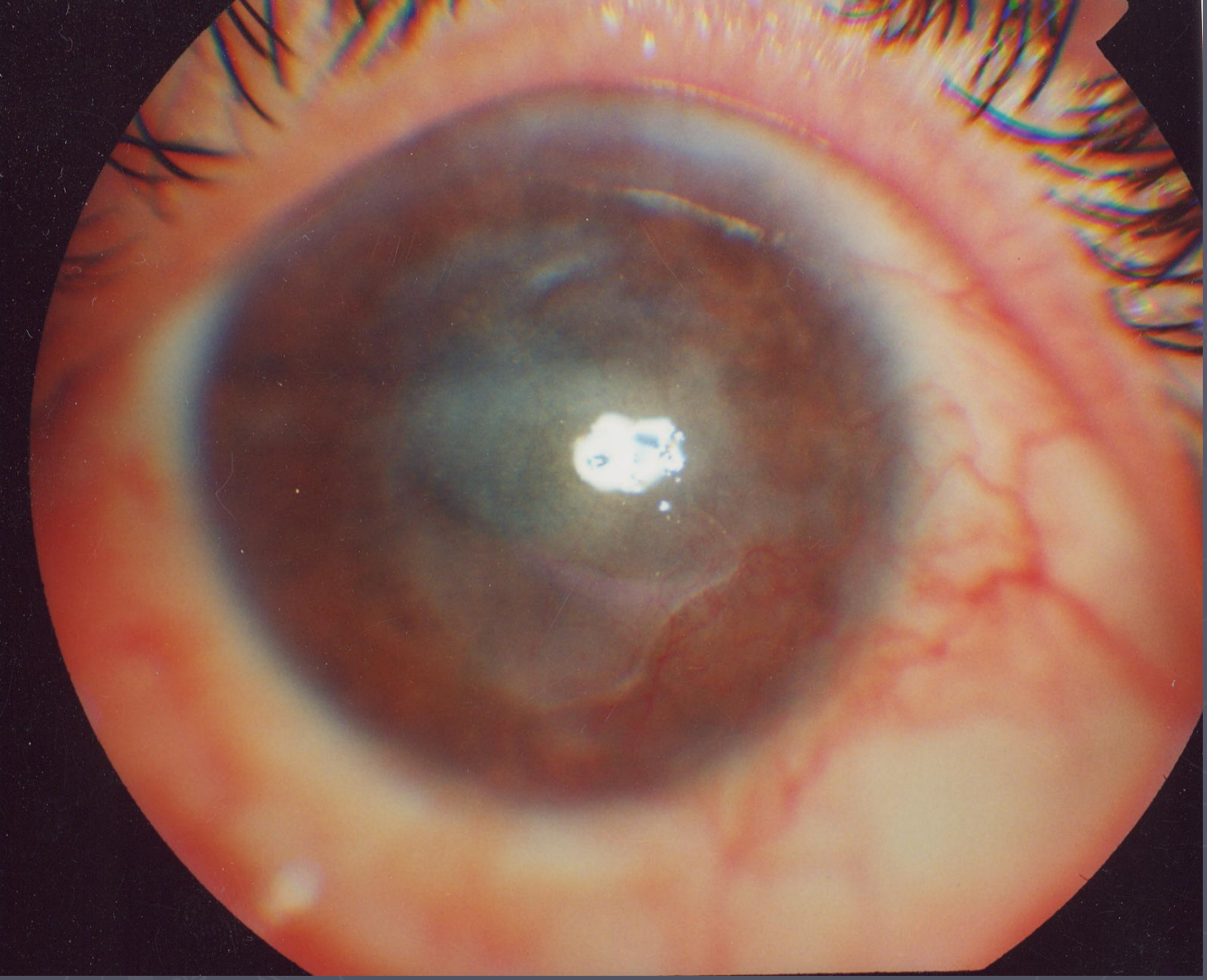
12 – 02 – 2000 PRELIEVO DI CIRCA 1 mm DI LIMBUS

01 – 03 – 2000 RECESSIONE DELLA CONGIUNTIVA
CHERATECTOMIA SUPERFICIALE
APPOSIZIONE DI LASERSKIN
BLEFARORAFFIA

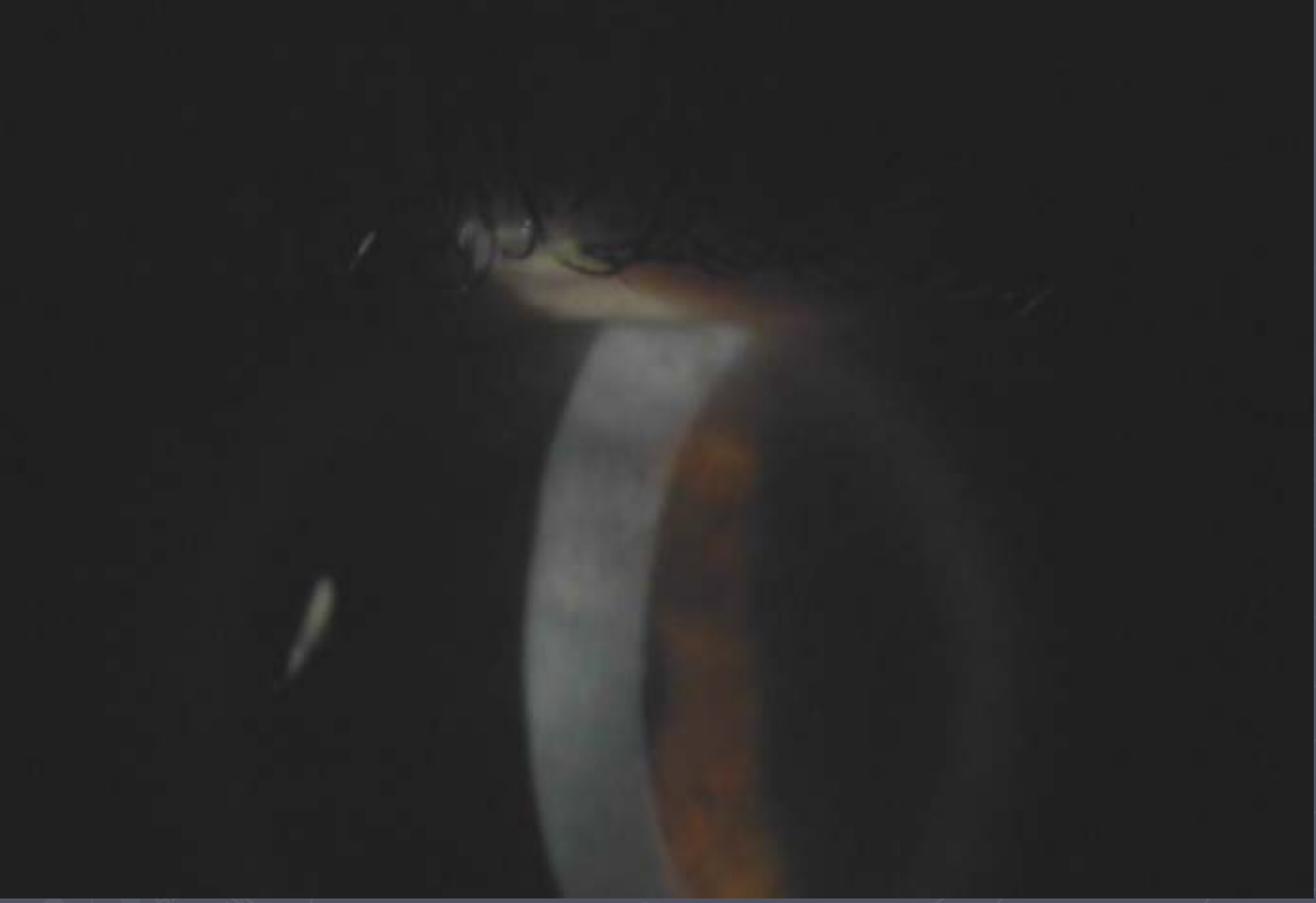
10 – 03 –2000 ASPORTAZIONE DELLA LASERSKIN



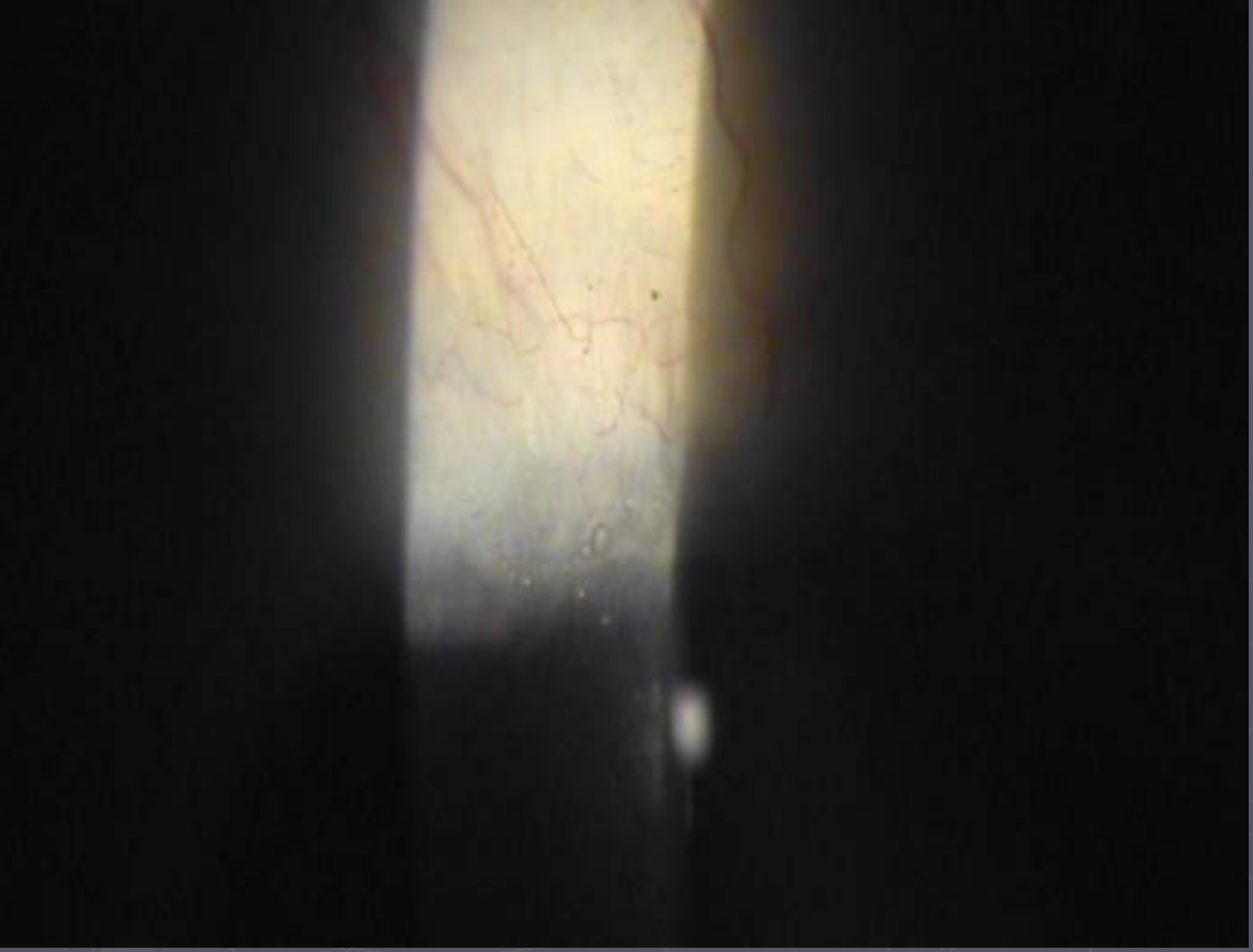
CASO CLINICO : 4 GIORNATA , PRESENZA DELLA MEMBRANA DI ACIDO IALURONICO



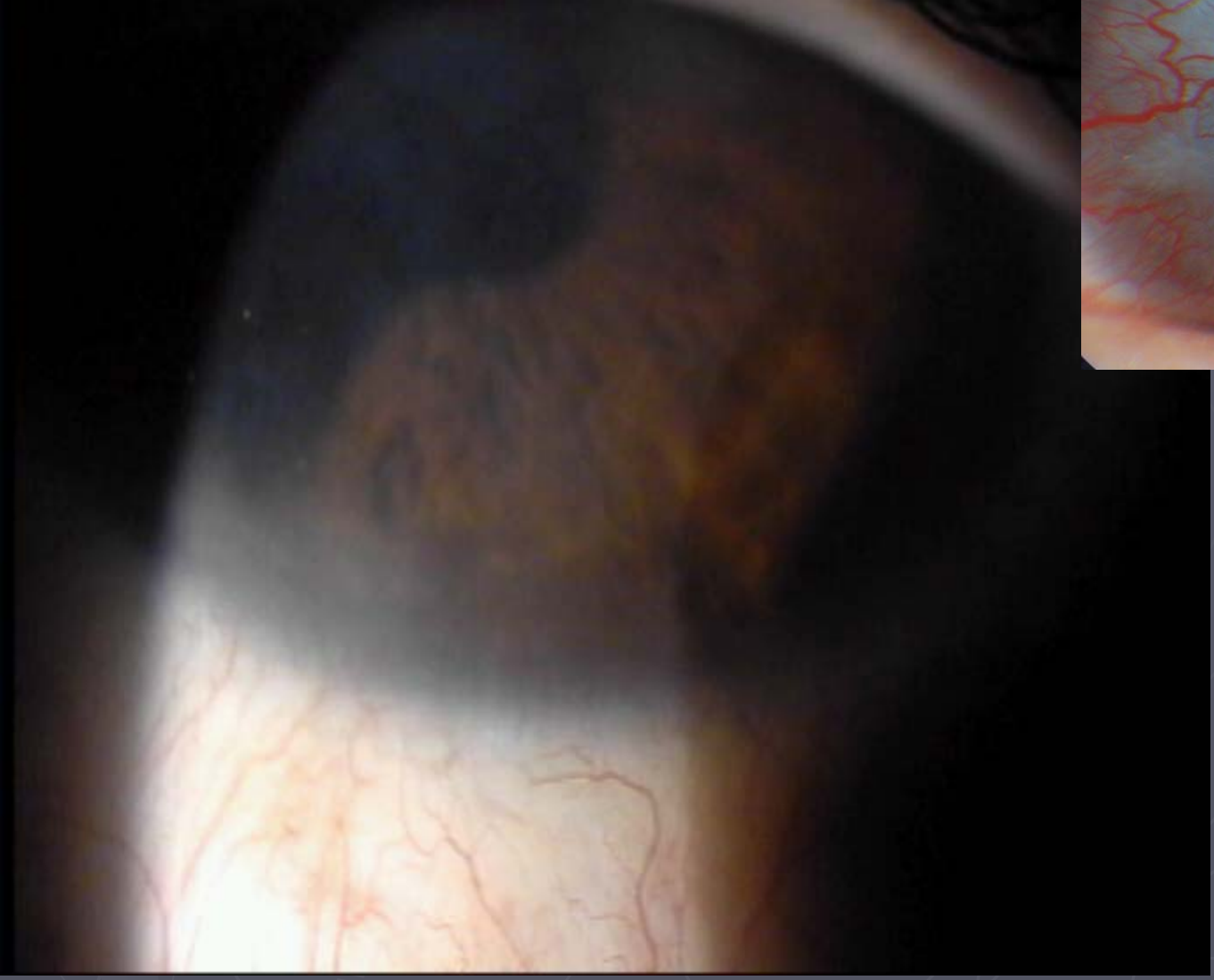
CASO CLINICO 20 GIORNATA DALL’INNESTO DELLE CELLULE COLTIVATE

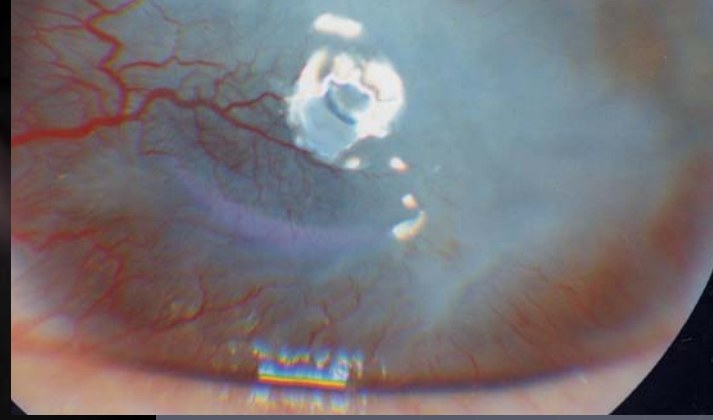


CASO CLINICO 3 MESI DALL'INTERVENTO
OS + 4 SF 2/10



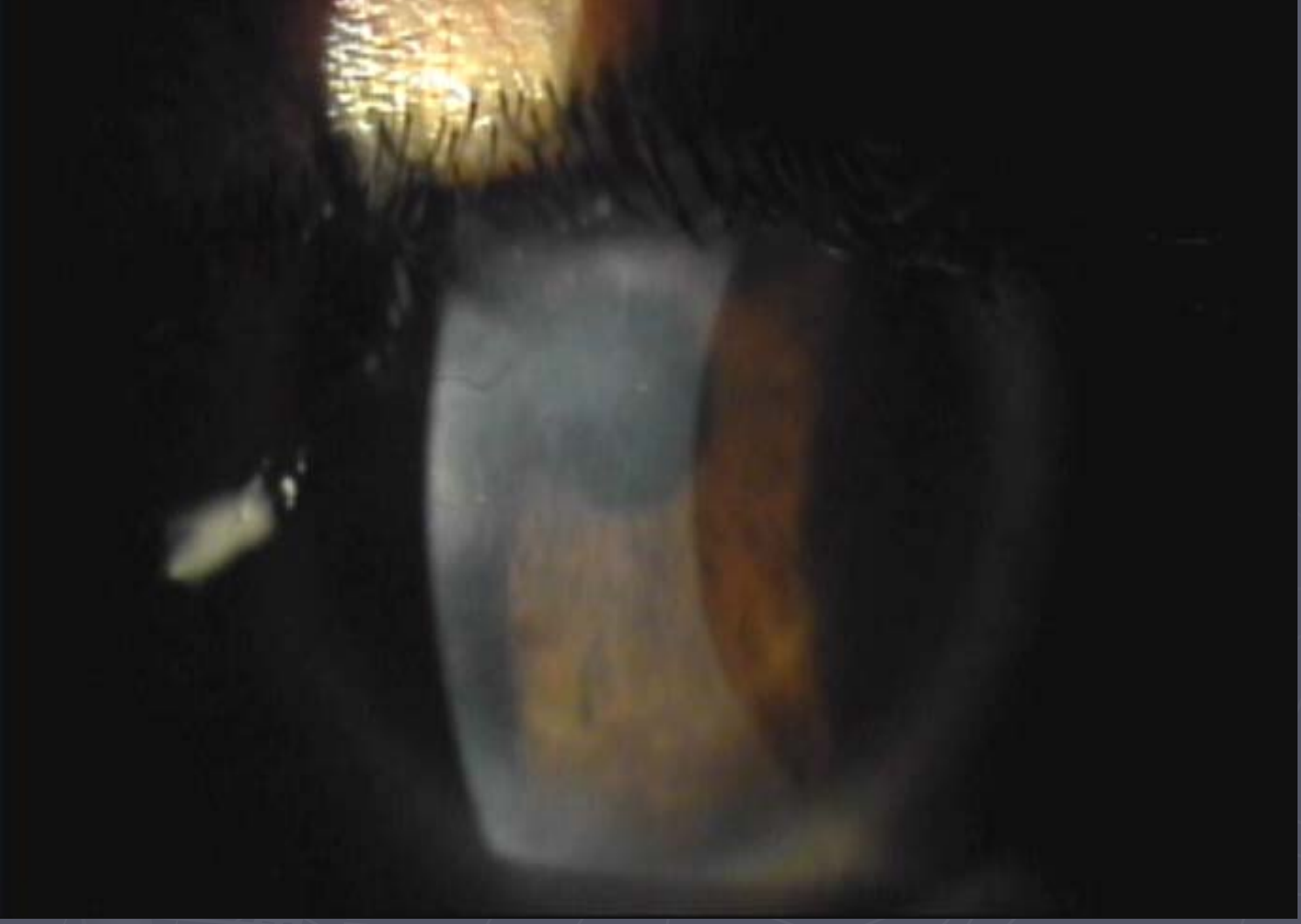
CASO CLINICO :OD SEDE DEL PRELIEVO DI 1MM A DISTANZA DI 6 MESI



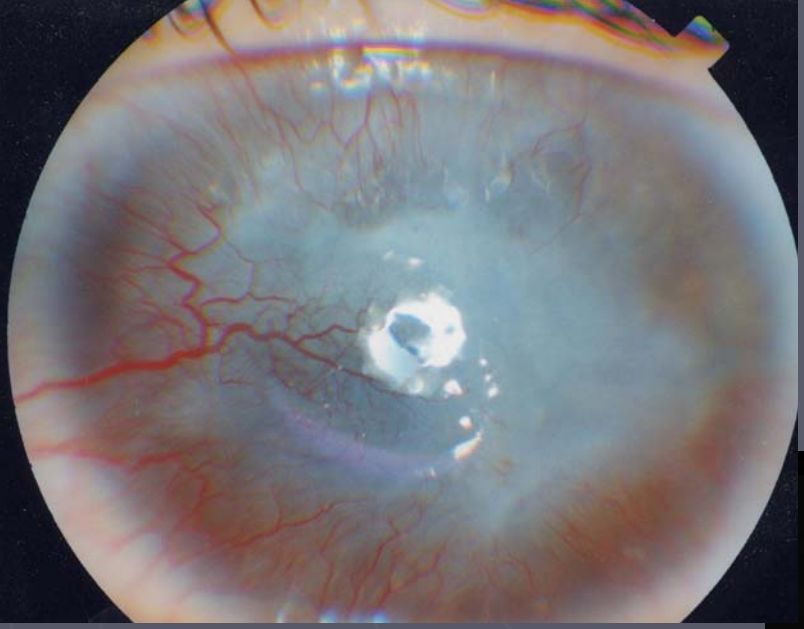


FEBBRAIO 2000

CASO CLINICO : OS LIMBUS INFERIORE A DISTANZA DI 6 MESI

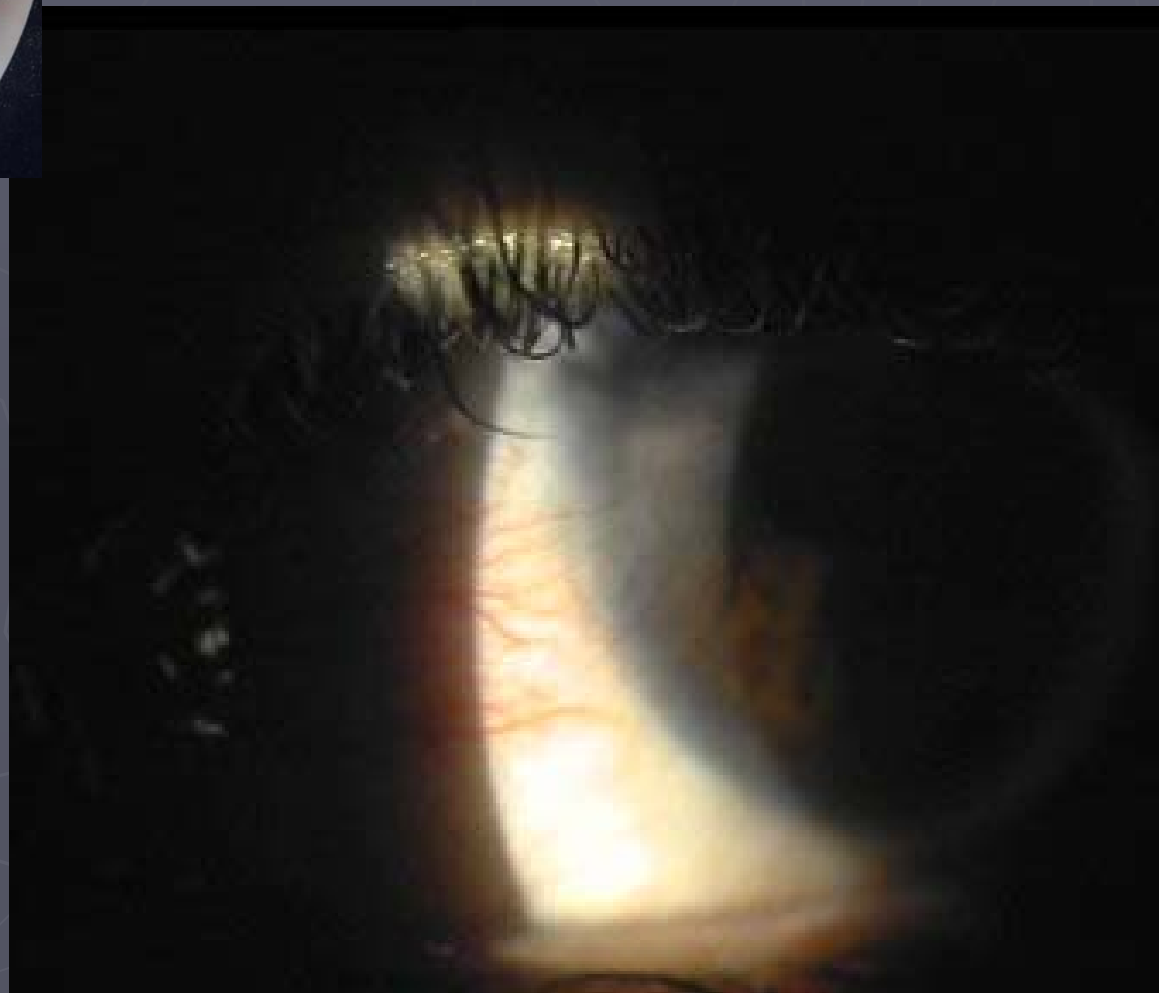


CASO CLINICO : OS A DISTANZA DI 6 MESI



Febbraio 2000 OS Visus motu manu

Settembre 2000 OSVisus +4 sf. 2/10



La “cell factory” oggi

- **Stesura del protocollo clinico** *(letteratura, studi sperimentali e preclinici)*
- ***Individuazione del Direttore Tecnico*** *(AIFA)*
- **Individuazione del sito di produzione** *(CellFactory autorizzata)* secondo Regolamento Europeo *(2007/1394 CE -in vigore da dic.2008)*
- Approvazione del **Comitato Etico** dei centri coinvolti Applicazione clinica
- **Convalida del processo e dei controlli di qualità** Approvazione: **ISS** *(fase I, first-in-man)* o **AIFA** *(fasi II, III, IV)*
- PRODUZIONE DI CELLULE PER TERAPIE SPERIMENTALI Report per la Banca dati ISS *(monitoraggio della terapia cellulare somatica-Decreto 2 marzo 2004)* Farmacovigilanza AIFA

DIRETTIVA 2009/120/CE DELLA COMMISSIONE del 14 settembre 2009 che modifica la direttiva 2001/83/CE del Parlamento europeo e del Consiglio recante un codice comunitario relativo ai medicinali per uso umano per quanto riguarda i medicinali per terapie avanzate