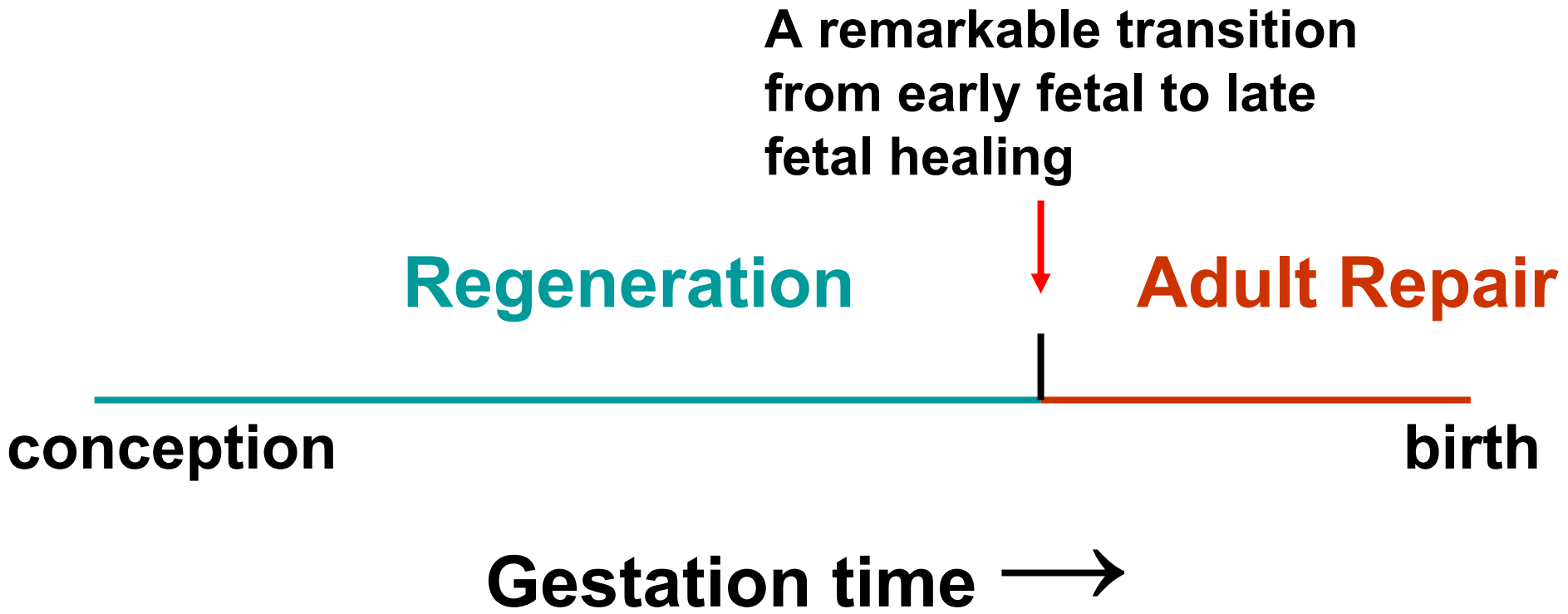
Cell-scaffold interactions during regeneration

1. Two questions.
2. Evidence supporting antagonistic relation between contraction and regeneration.
3. Similarity between early fetal regeneration and induced regeneration in adults.



Longaker et al.; Ferguson et al.

Mammals: Early fetal healing ⟶ Late fetal healing

Two major questions

1. What is the mechanism of induced organ regeneration in adults at the cell-biological and the molecular-biological levels?
2. Is the process of induced regeneration of organs in adults similar to spontaneous regeneration at the early fetal stage?

Three scales of understanding

- **Macroscopic---what we see with our eyes alone. Scale > 1 mm**
- **Cell biological----what microscopy shows. Scale > 1 μm**
- **Molecular biological---what biochemical assays or gene expression assays tell us is happening. Scale > 1 nm**

Spontaneous Regeneration in an Amphibian

Figure removed due to copyright restrictions.
See Figure 1.1 in [TORA].

[TORA] = Yannas, I. V. *Tissue and Organ Regeneration in Adults*. New York, NY: Springer-Verlag, 2001. ISBN: 9780387952147. [Preview in Google Books]

Unlike adult mammals, certain adult amphibians can regenerate arms and legs that have been amputated

Adult Repair of Injured Arm

Photo removed due to copyright restrictions.
See Figure 6 in Tomasek, J. J. et al. “Myofibroblasts and mechano-regulation of connective tissue remodelling.” *Nature Reviews Molecular Cell Biology* 3 (May 2002): 349-363.
http://dx.doi.org/10.1038/nrm809

Adult repair. Healing by contraction and scar formation in burn victim.

Tomasek et al., 2002

Adult Repair of Burned Skin

Photo removed due to copyright restrictions.

Spontaneous contraction and scar formation in burn victim.

Mass Gen Hospital, 1986

The tissue triad in skin and nerves

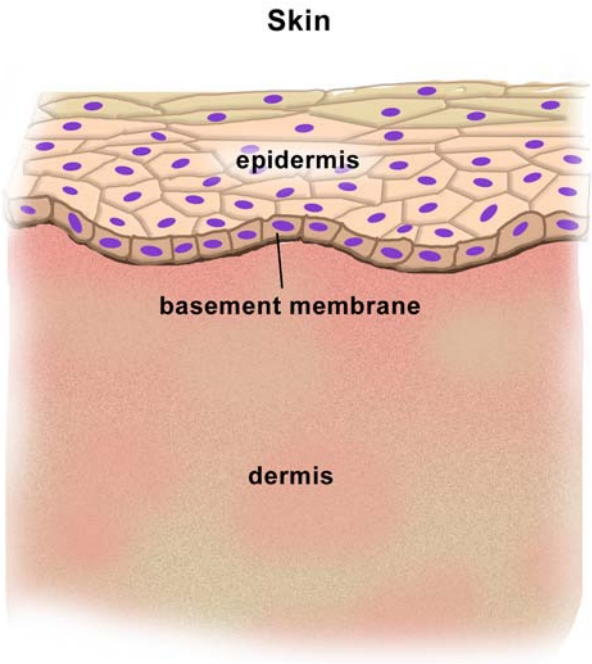


Figure by MIT OpenCourseWare.

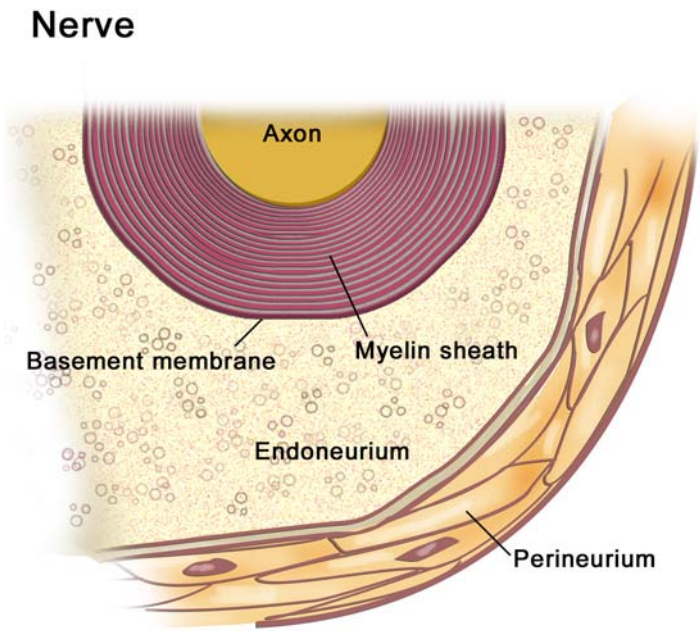
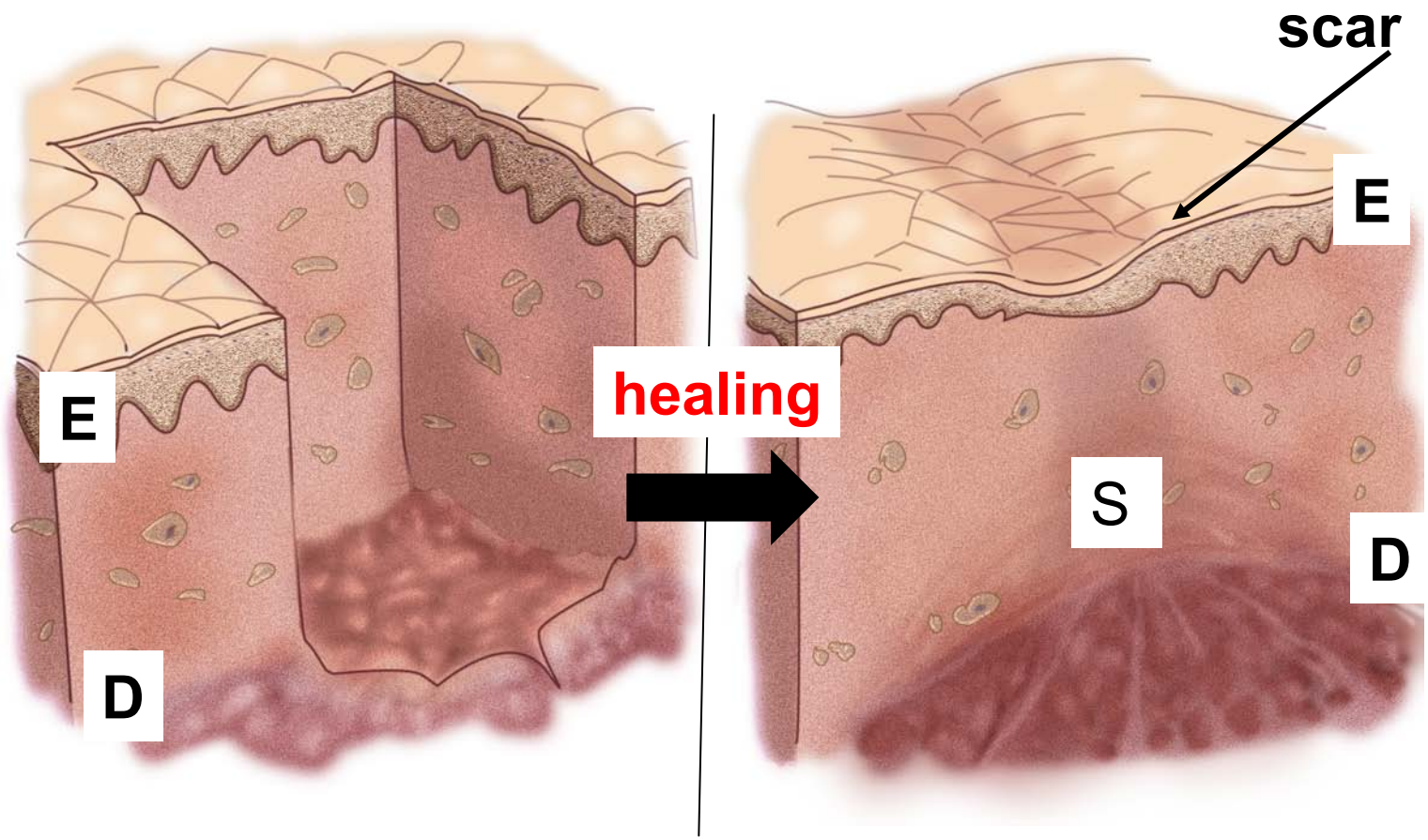


Figure by MIT OpenCourseWare.

Skin: Adult Repair



E: epidermis
D: dermis
S: scar

Figure by MIT OpenCourseWare.

The dermis does not regenerate spontaneously. Wound closes with contraction and scar formation.

Yannas, 2001

Peripheral nerve: Adult Repair of Injury

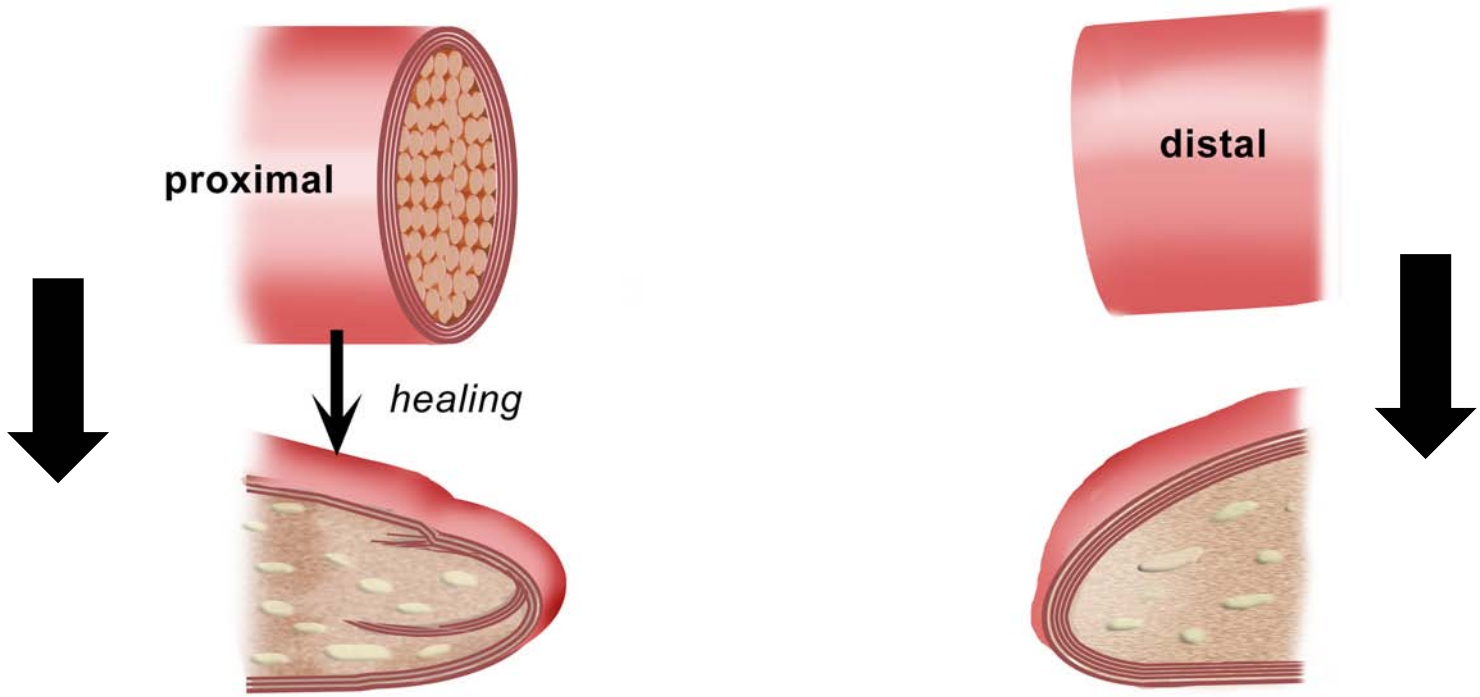


Figure by MIT OpenCourseWare.

Transected (cut) nerve heals by contraction and scar formation (paralysis)

Skin: Adult Repair of Injury (followup of original studies by Medawar and Billingham in early 1950s)

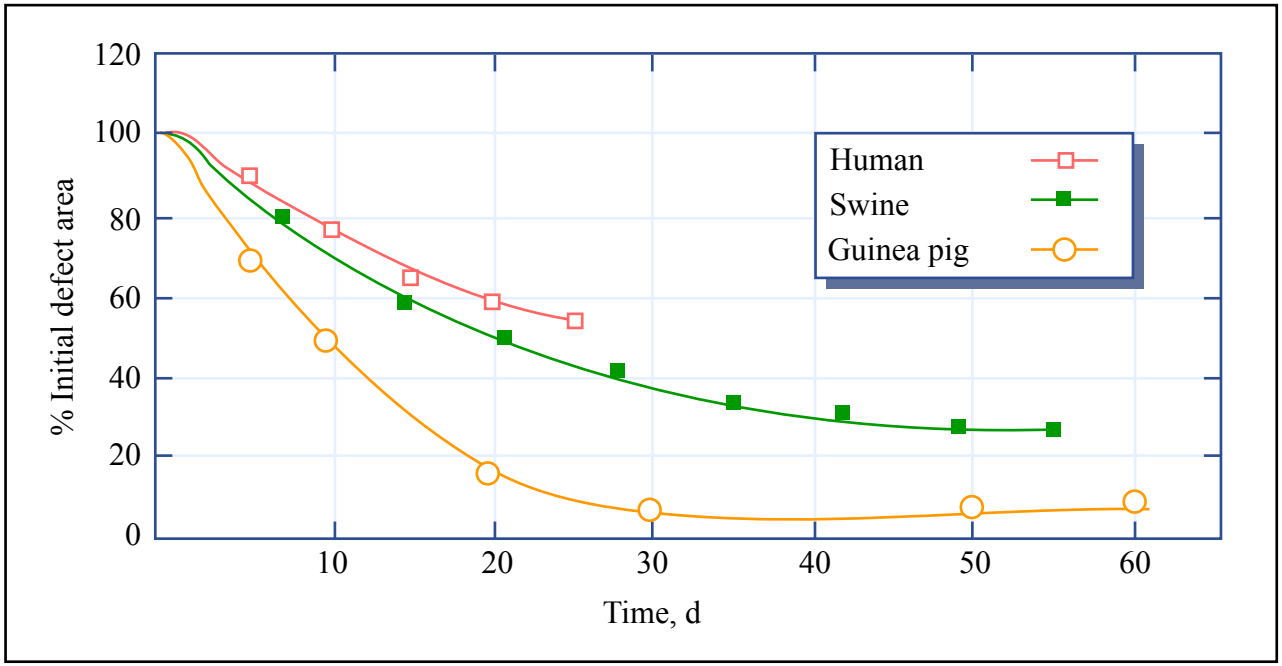
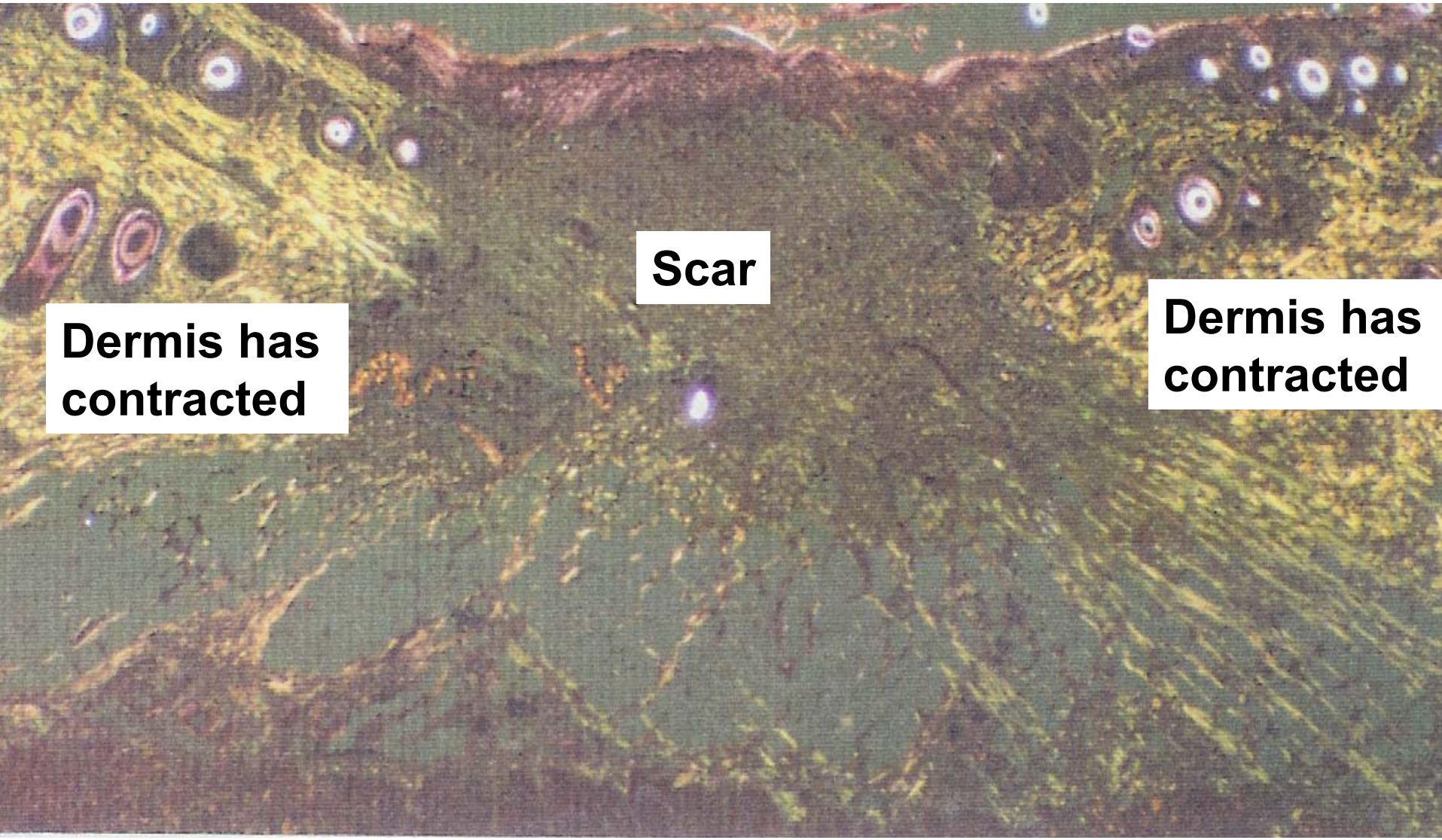


Figure by MIT OpenCourseWare.

Speed of contraction of skin wounds

Ramirez et al., 1969:
Rudolph, 1979;
Yannas, 1981

Skin: Adult Repair with Wound Contraction and Scar Formation (guinea pig)



Microscopy of a healed skin wound

Troxel, MIT Thesis, 1994

Normal skin wound healing

A short film by D. Tzeranis

Why does our adult body fail to regenerate when injured?

Prevalent explanation:

“Regeneration is blocked by scar formation”

An unexpected result in 1979 (“artificial skin”)

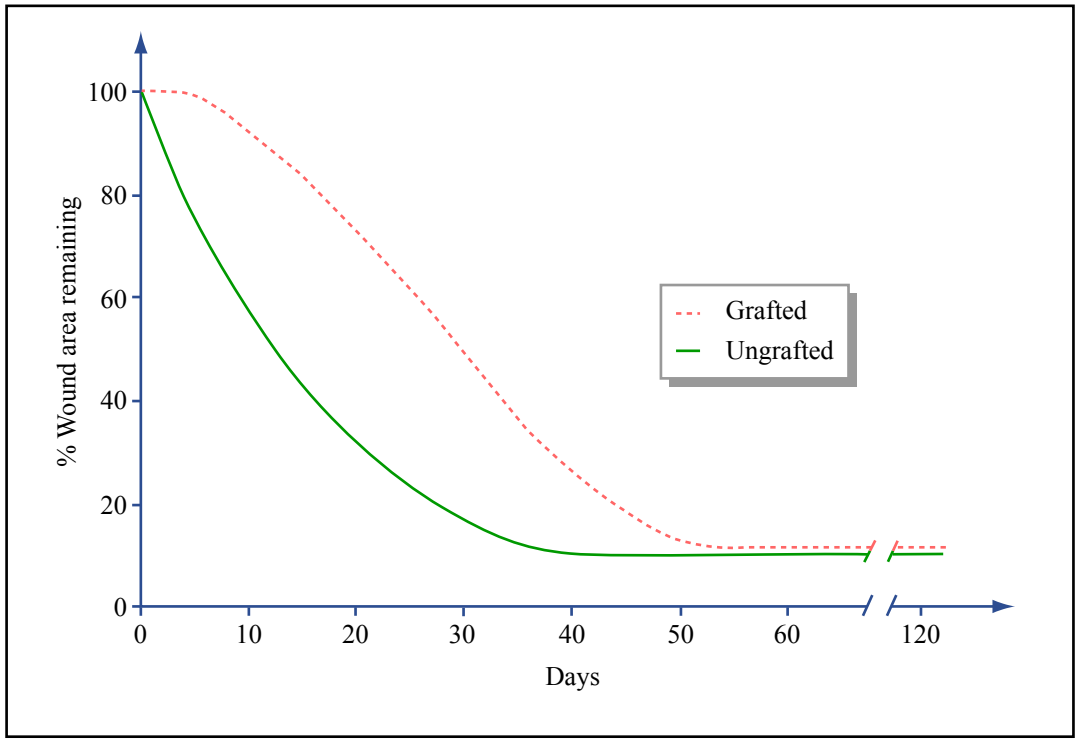


Figure by MIT OpenCourseWare. After Yannas, 1981.

Contraction dramatically delayed when full-thickness skin wounds grafted with a particular scaffold. Scar formation blocked. First observation of induced organ “regeneration” (partial) in adult mammal.

Yannas et al., 1981

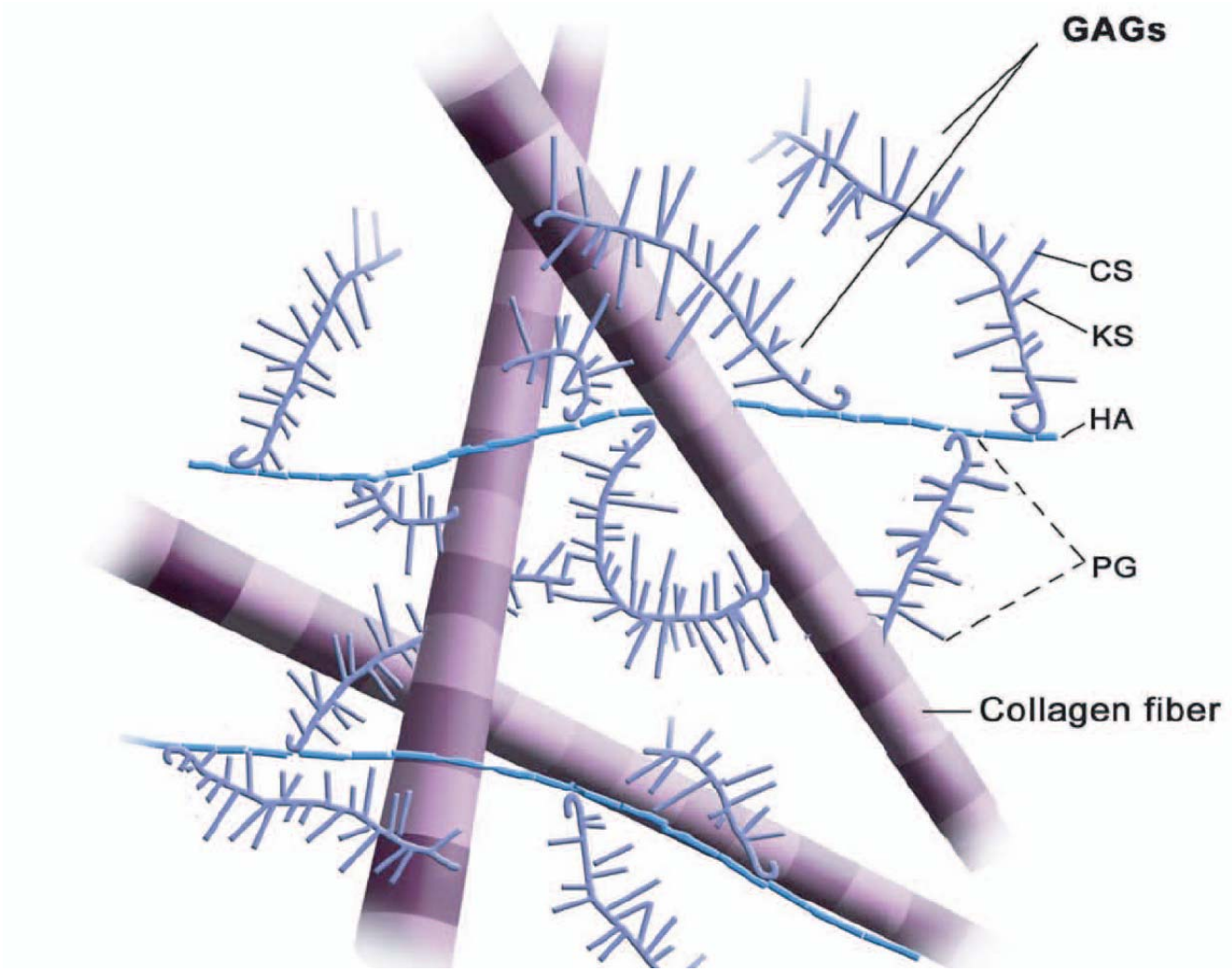
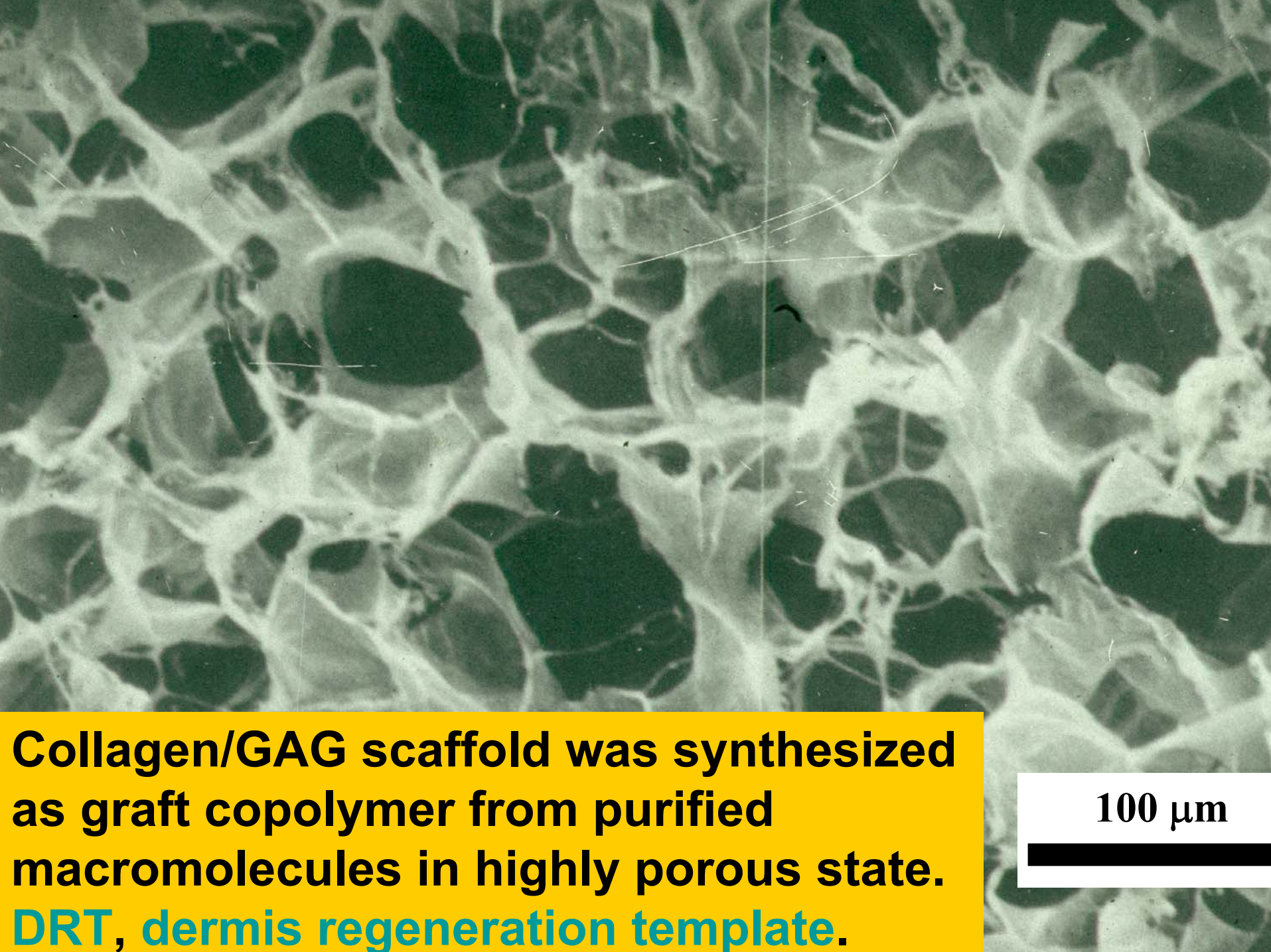


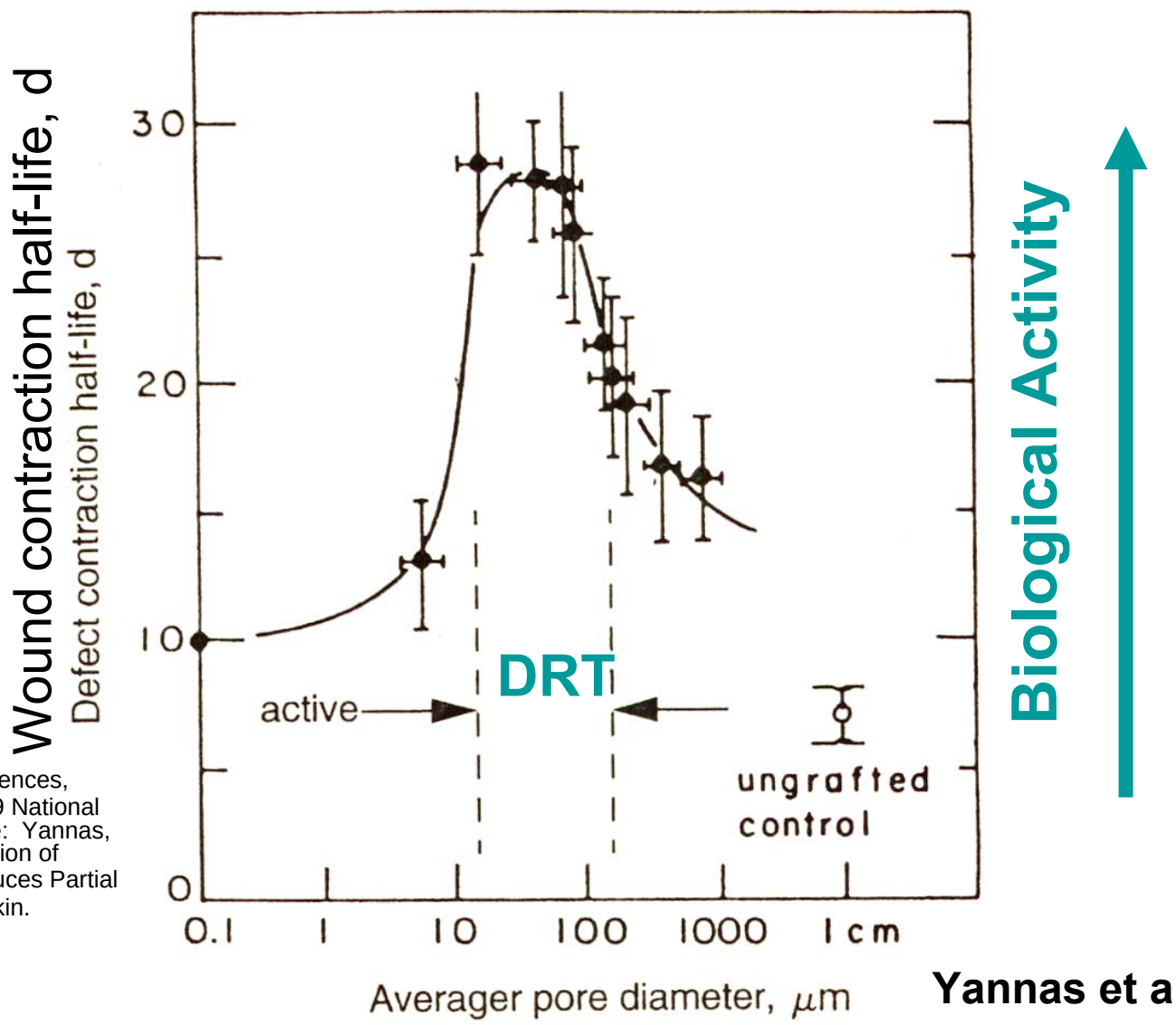
Image by MIT OpenCourseWare. After Ricci.

The scaffold was an analog of the Extracellular Matrix (ECM) based on collagen and a glycosaminoglycan (GAG).

Harley, 2006



Collagen/GAG scaffold was synthesized as graft copolymer from purified macromolecules in highly porous state. DRT, dermis regeneration template.



Yannas et al., 1989

Only scaffolds within narrow pore size range are active. DRT, dermis regeneration template, blocks contraction maximally.

What makes a scaffold active biologically ? Active scaffold reduces number of contractile cells in the wound---and binds these cells on “ligands” on the scaffold surface

1. chemical composition (ligand identity)
2. pore structure---pore volume fraction, pore size (ligand density)
3. orientation of pore channels (ligand spatial coordinates)

Diagram removed due to copyright restrictions.

4. macromolecular structure (duration of ligands on surface)

Yannas et al., 1989

Structural determinants of scaffold biological activity.

What does the scaffold do?

It blocks contraction and abolishes scar formation during wound healing. If used without cell seeding, it leads to regeneration of dermis.

Eventually, epidermal cells from wound edges regenerate over the new dermis, and synthesize basement menrane, to produce a new skin.

The scaffold DRT induces growth of a new dermis

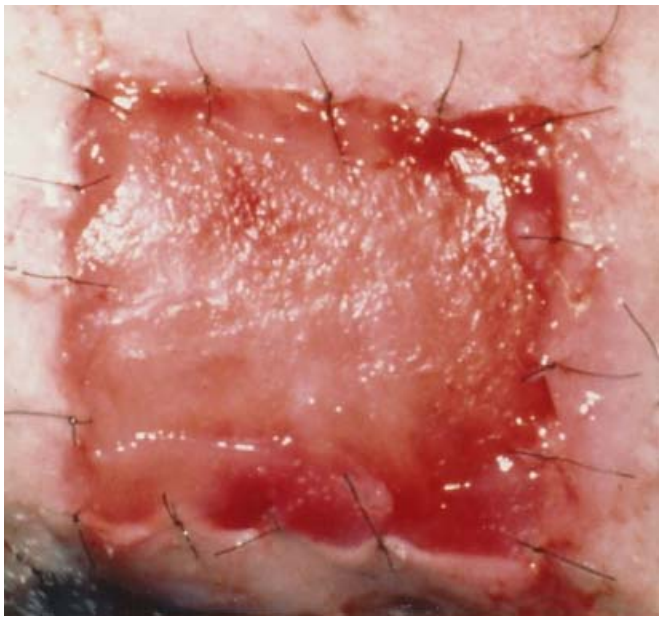
Examples from five organs. Blocking of adult healing response following grafting with active scaffold

1. Skin regeneration
2. Peripheral nerve regeneration
3. Conjunctiva (eye) regeneration
4. Scar inhibition in kidney
5. Blocking of wound contraction in liver

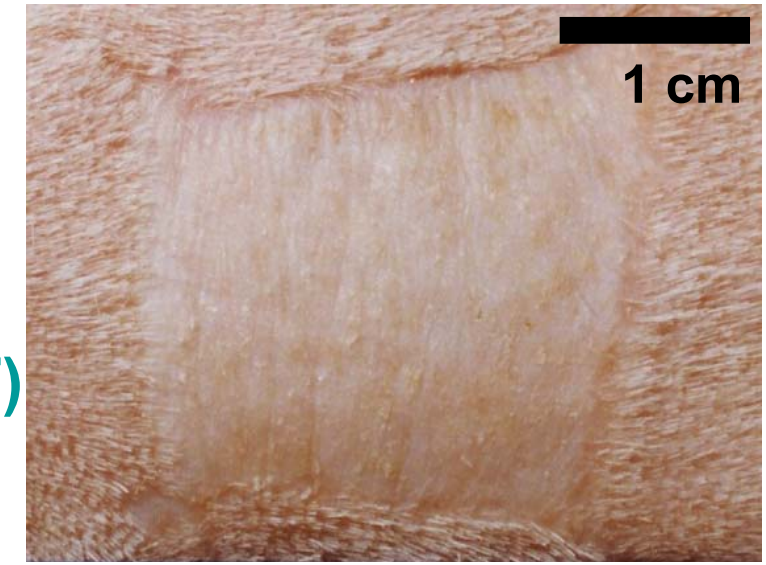
Organ #1. Skin Regeneration

Graft a skin wound with a scaffold that has been seeded with keratinocytes (KC), the cells of the epidermis. The scaffold induces dermis regeneration and eventually epidermis regeneration from wound edges. Seeding with KC speeds up formation of the epidermis.

Wound grafted with scaffold

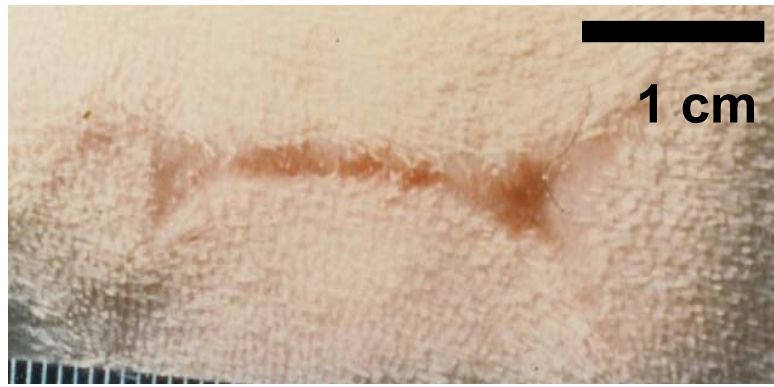


KC + active scaffold (DRT)



Blocked contraction. New tissue formed is skin, not scar.

KC + inactive scaffold



Contraction and scar

Simultaneous synthesis of epidermis and dermis using the cell-seeded scaffold.

Orgill, MIT Thesis, 1983

KINETICS OF SKIN SYNTHESIS II.

Three histology photos removed due to copyright restrictions.
See Butler, C. E. et al. “Effect of Keratinocyte Seeding of Collagen-Glycosaminoglycan Membranes on the Regeneration of Skin in a Porcine Model .” *Plast. Reconstr. Surg.* 101 (1998): 1572-1579.

Scaffold degraded; diffuses away

Butler et al., 1998

Partially regenerated skin is not scar.
Scar does not have capillary loops. Nor does scar have a wavelike border separating epidermis from dermis

Diagram removed due to copyright restrictions.

capillary loops

Normal skin has capillary loops and a wavelike border separating epidermis from dermis. Burkitt et al., 1992

Histology photo removed due to copyright restrictions.

rete ridges

capillary loops

75 μm

Partially regenerated skin in the swine. Compton et al., 1998

Partially regenerated skin is not scar.

Before the wound closes…in the absence of the scaffold

Myofibroblasts pull wound edges together and close wound

Contractile fibroblasts (myofibroblasts) in the skin wound stain brown red with antibody to α-actin.

K. S. Troxel,
MIT Thesis, 1994

Normal Dermis

Scar

Diagram removed due to copyright restrictions.
Schematic of laser beam passing through histologic slide.
See Fig. 4.7 in [TORA].

Images removed due to copyright restrictions.
Laser scattering patterns
See Fig. 4.7 in [TORA].

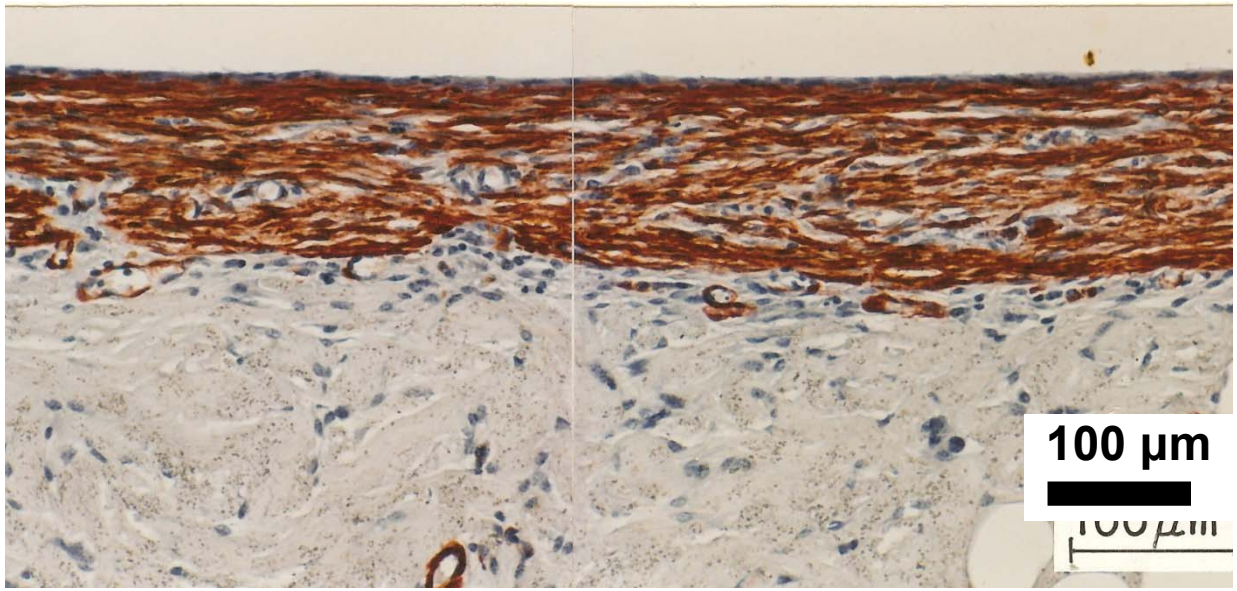
$$S = 2\left\langle \cos^2(a) \right\rangle - 1$$

	Dermis	Scar
$< \cos^2(a) >$	0.5	1
Orientation function, S	0	1

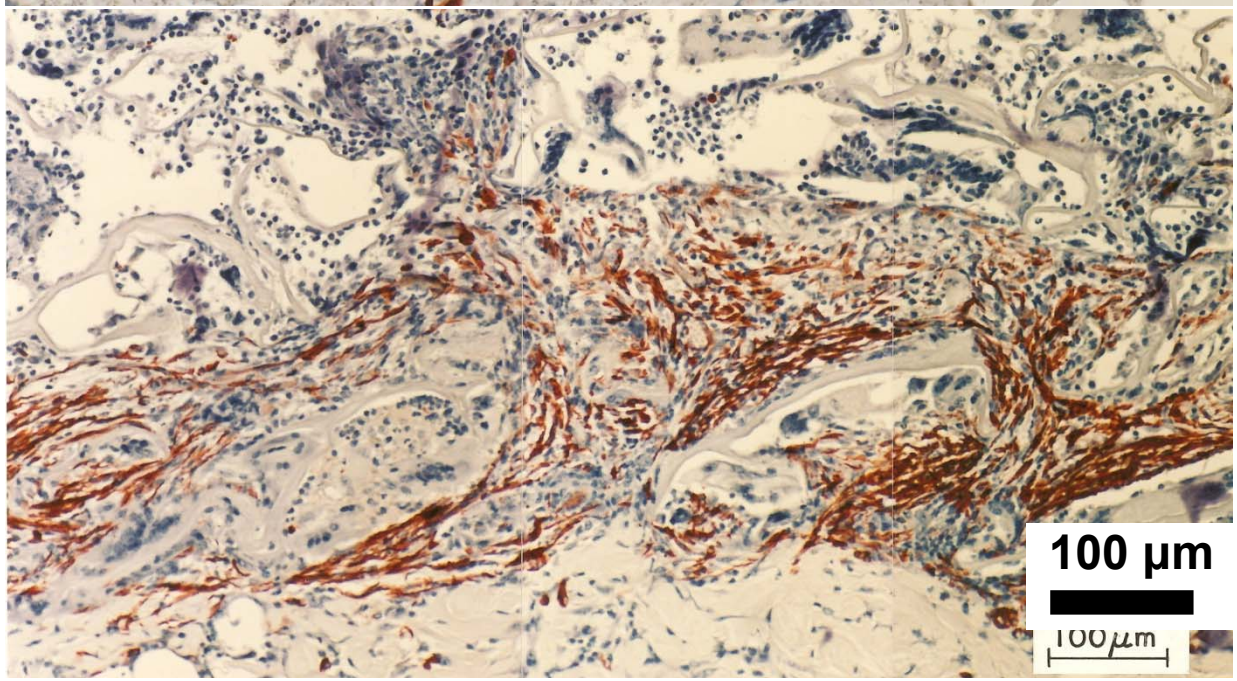
Identify scar using laser light scattering assay

Ferdman and Yannas, 1993

No scaffold
This wound is contracting vigorously

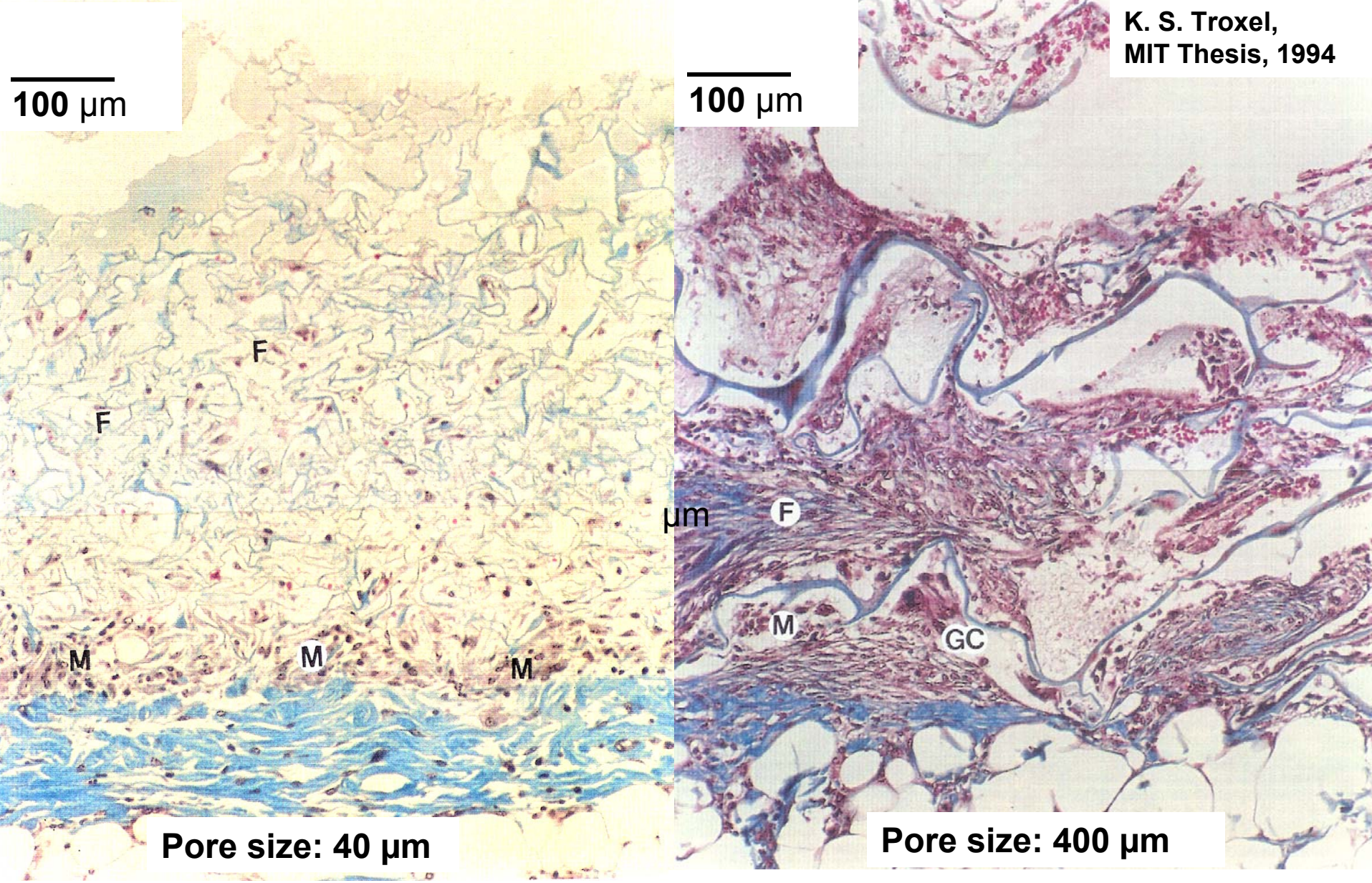


Scaffold
This wound is not contracting



Contraction blocked by active scaffold.

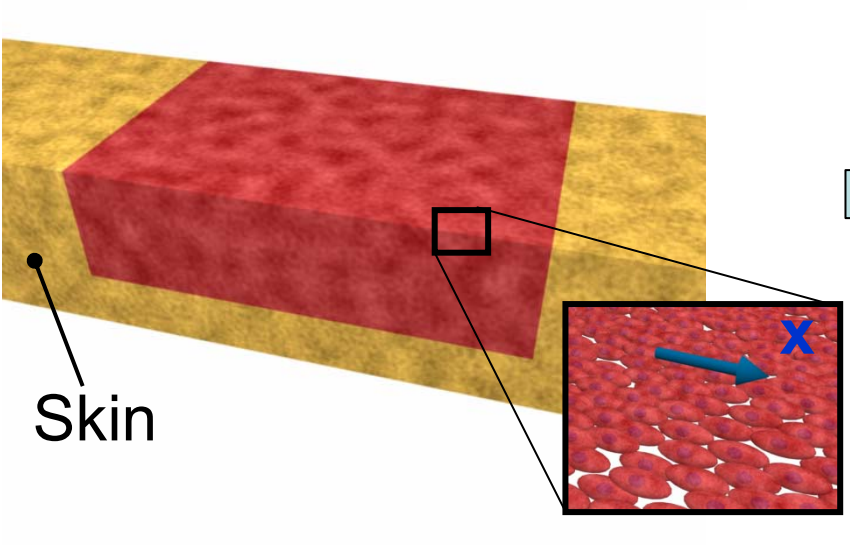
K. S. Troxel,
MIT Thesis, 1994

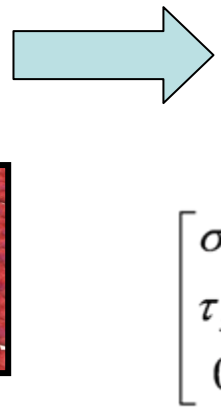


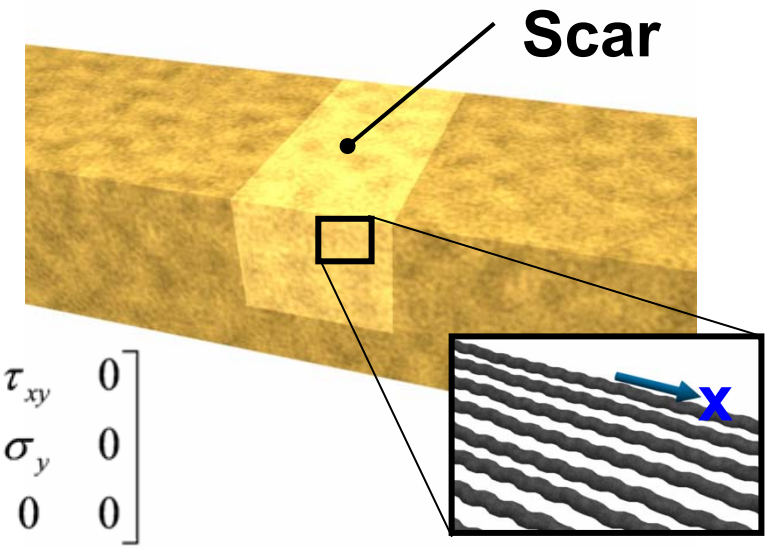
K. S. Troxel,
MIT Thesis, 1994

Cells inside an inactive collagen-GAG scaffold are densely clustered together inside the large pores, average size 400 µm, while being isolated inside the active scaffold with average pore size 40 µm. M, macrophages. F, fibroblasts.

Skin wound - No scaffold

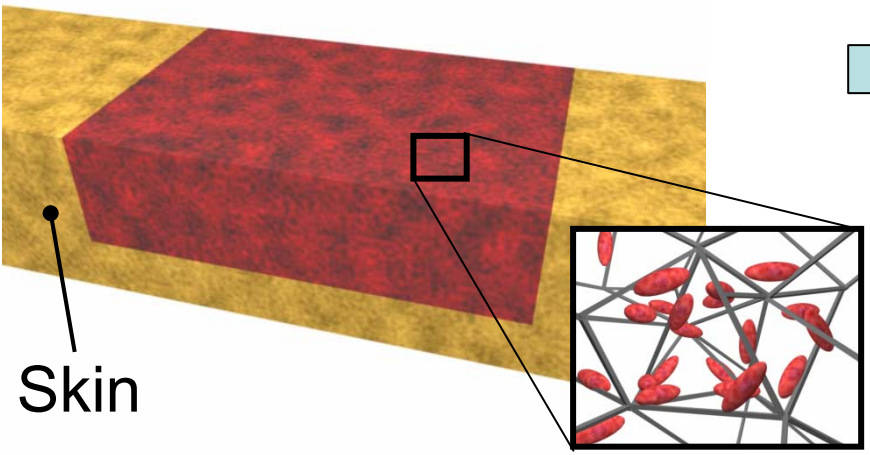


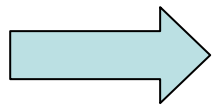


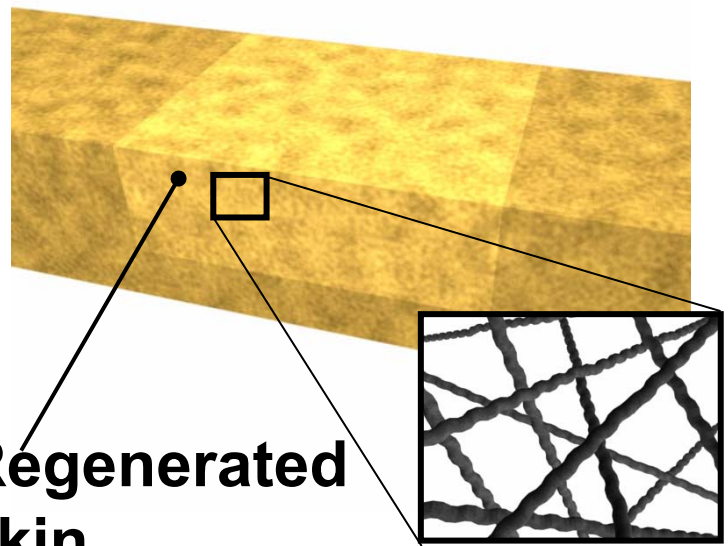


$$\begin{bmatrix} \sigma_x & \tau_{xy} & 0 \\ \tau_{xy} & \sigma_y & 0 \\ 0 & 0 & 0 \end{bmatrix}$$

Skin wound with scaffold







Hypothetical Stress Fields in Skin Wound

Graphic by Tzeranis

Organ #2. Peripheral Nerve Regeneration

If a scaffold can induce regeneration of the dermis can another scaffold do the same thing for a peripheral nerve?

Nervous system = central nervous system (CNS) + peripheral nervous system (PNS)

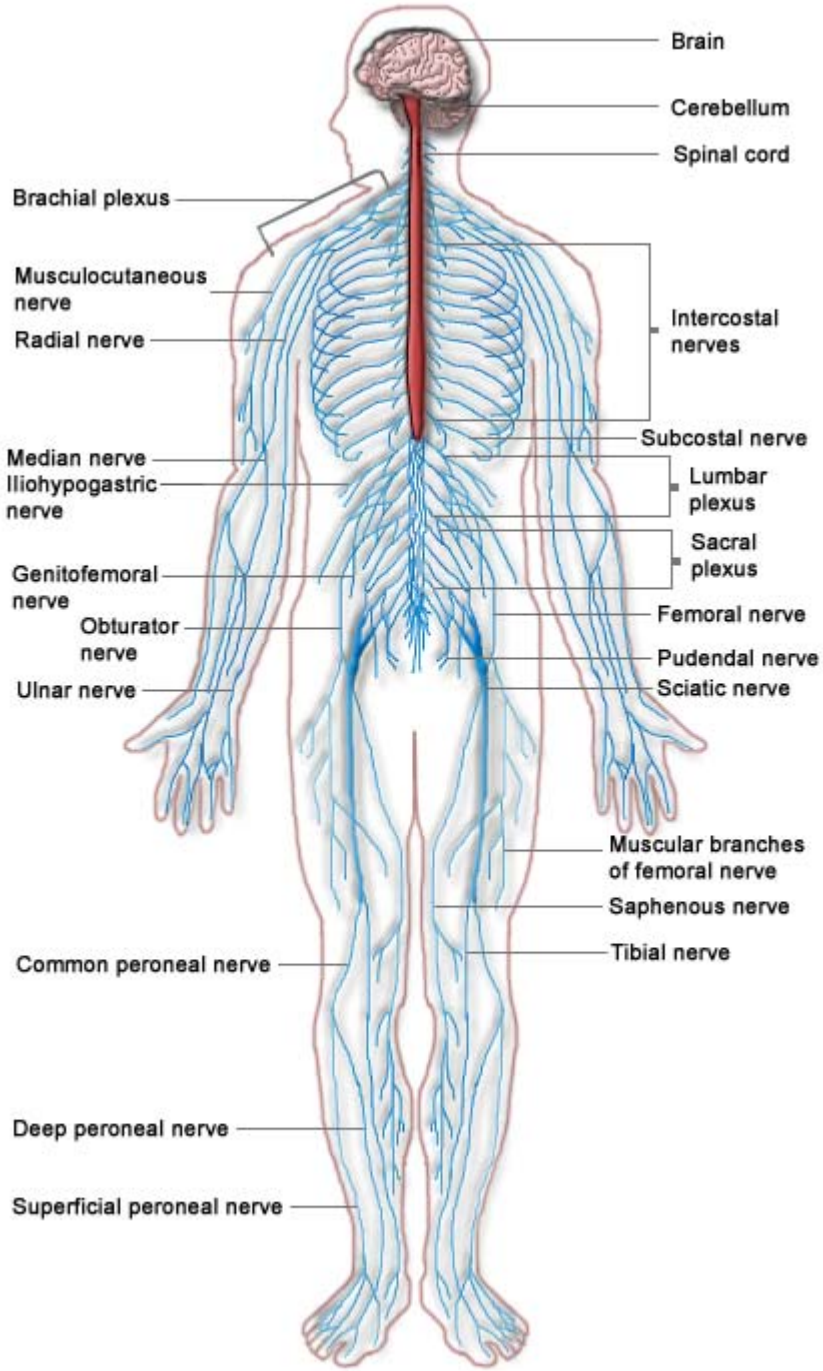
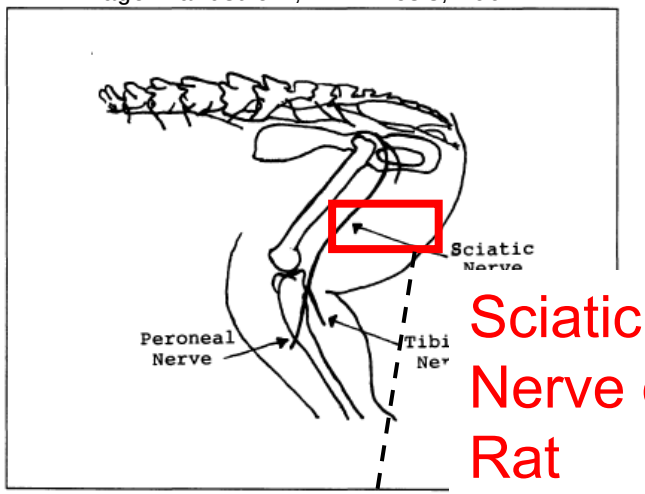
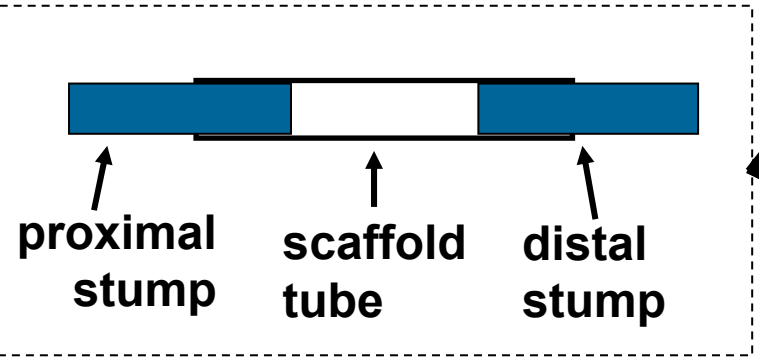


Image: public domain (by Wikipedia User: Persian Poet Gal)

Image: Landstrom, MIT Thesis, 1994



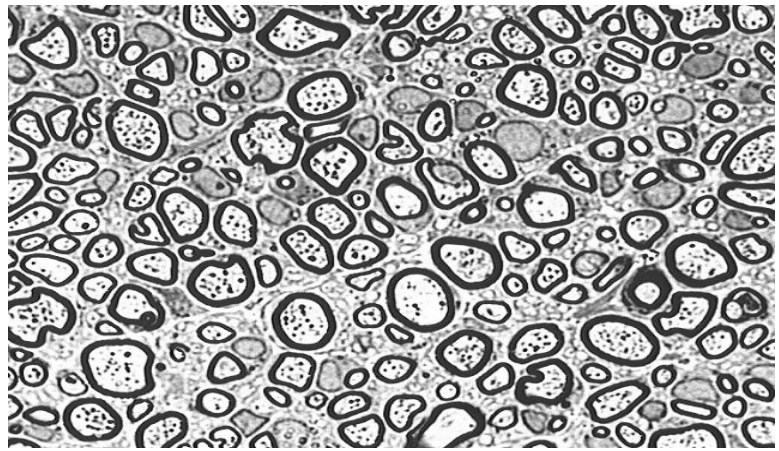
Sciatic Nerve of Rat



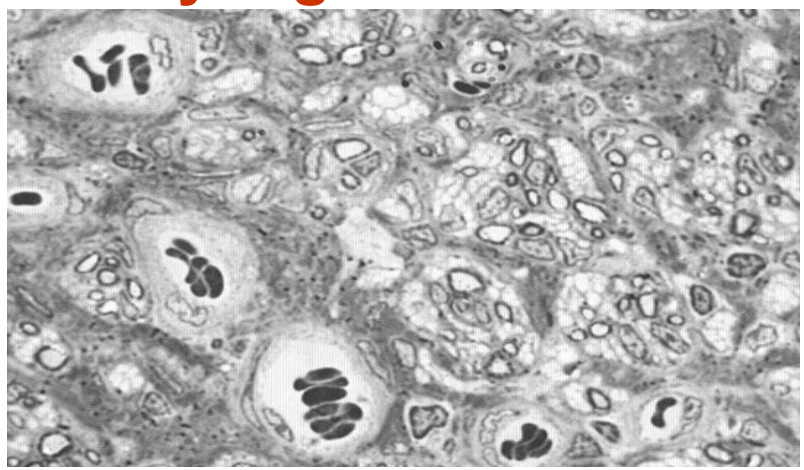
Active scaffold tube

Inactive scaffold tube

Well-regenerated nerve



Poorly regenerated nerve

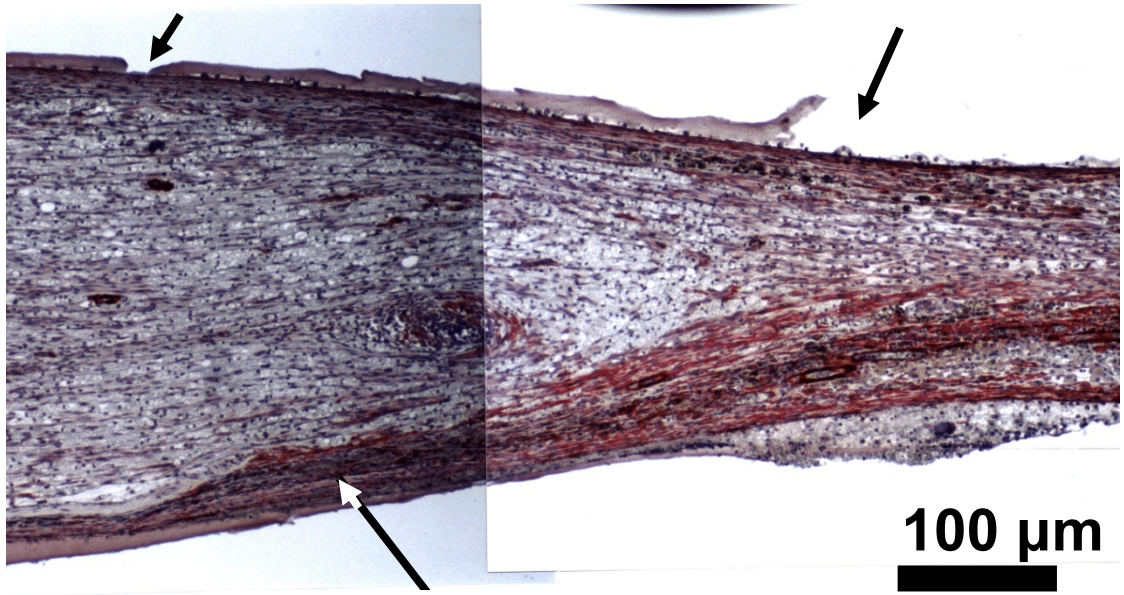


Courtesy of Brendan Harley. Used with permission.

Stumps inserted in active or inactive scaffold tube.

Harley et al., 2003

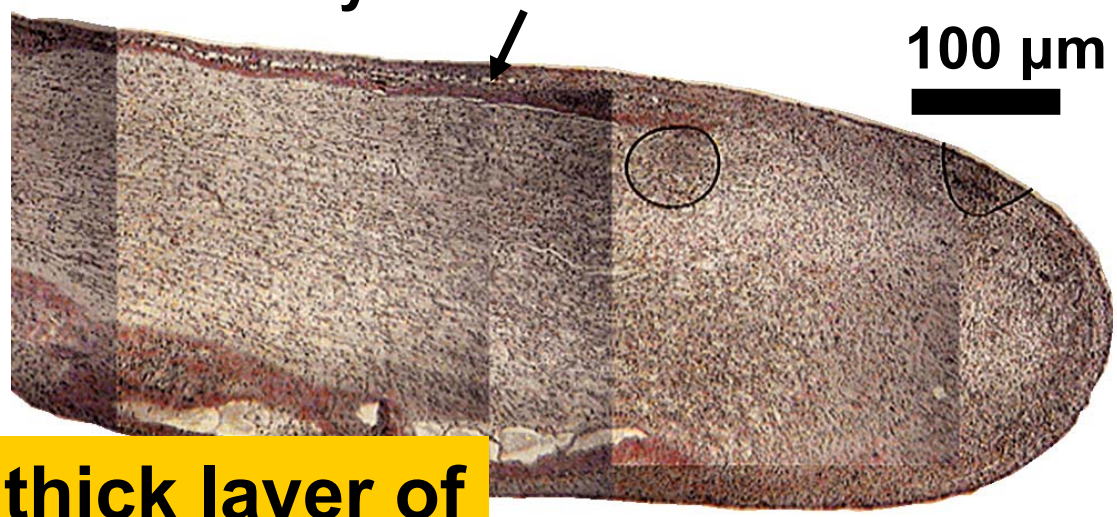
Very poorly regenerated nerve



Red-brown: stained with antibody to α-SM actin.

myofibroblasts

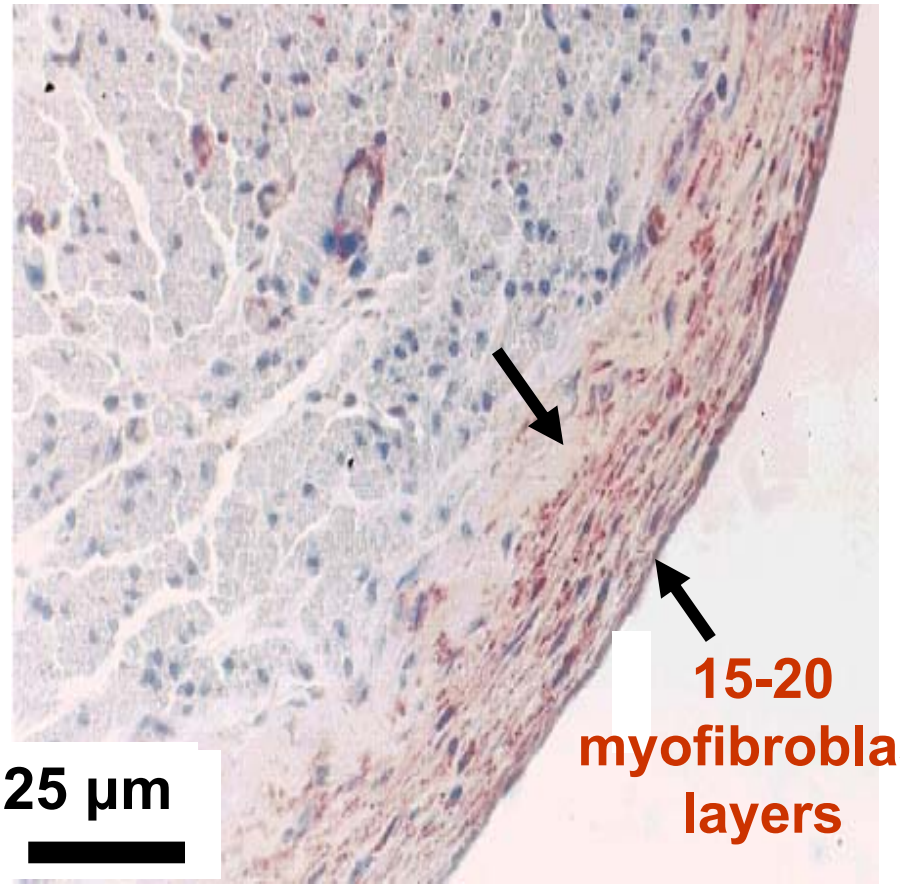
No regeneration (neuroma)



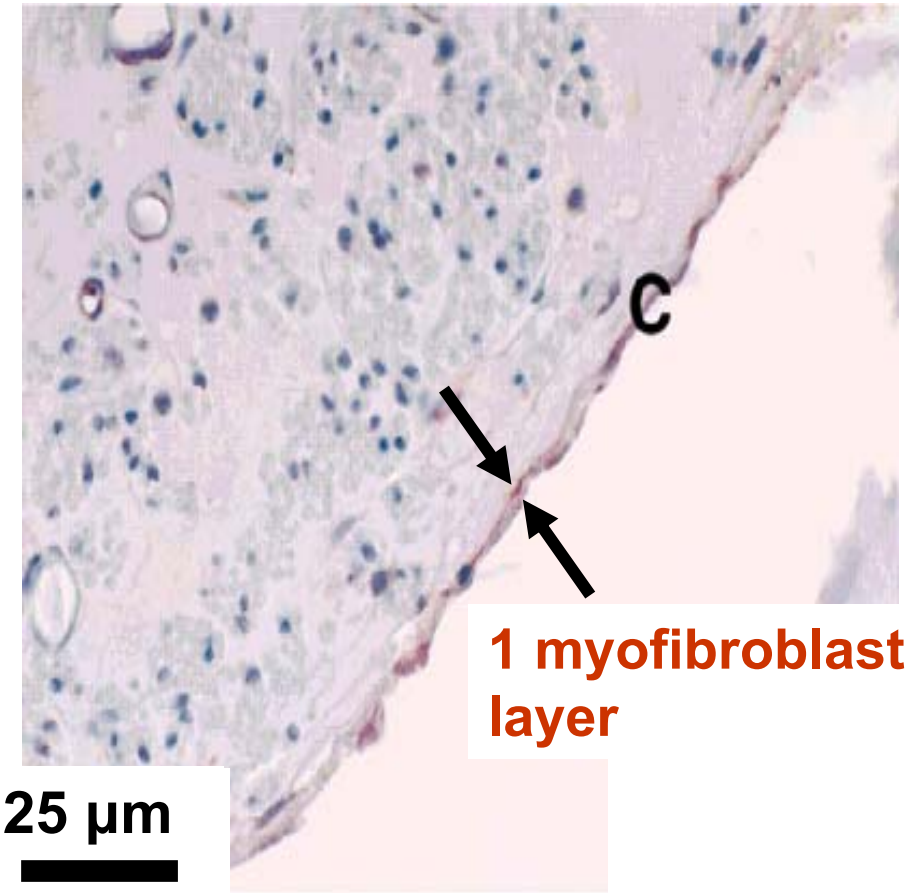
Poor regeneration. A thick layer of myofibroblasts (contractile cells) surrounds the cut nerve.

Spilker and Chamberlain, 2000

Poorly regenerated nerve

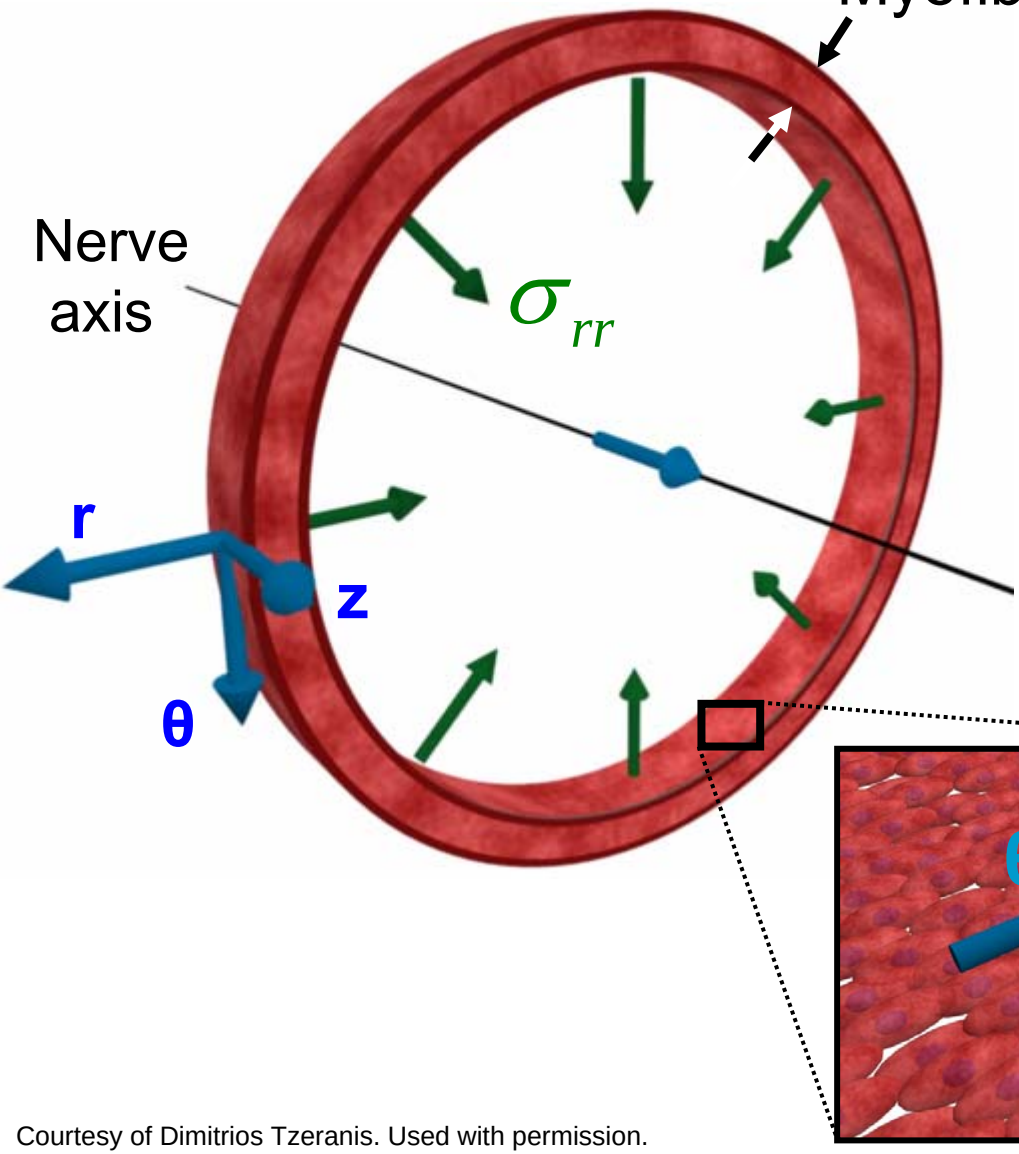


Well-regenerated nerve



A poorly regenerated nerve is surrounded by a thick layer of contractile cells.

Chamberlain et al., 2000



$$\varepsilon_{rr} = \frac{1}{E}(1-\nu)\sigma_{rr}$$

Myofibroblasts align with θ axis

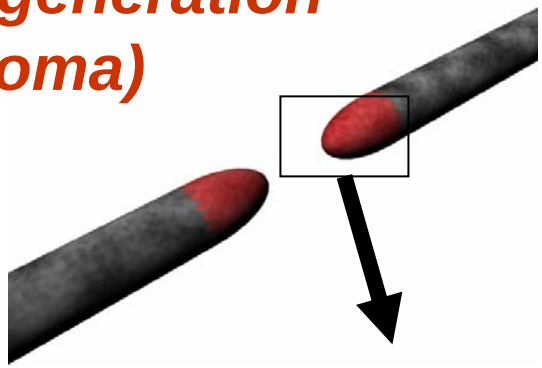
Courtesy of Dimitrios Tzeranis. Used with permission.

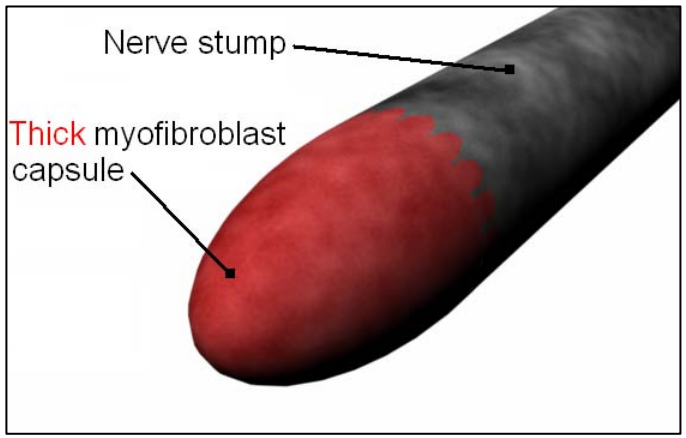
Stress Fields – Peripheral Nerves

Zhang and Yannas, 2003

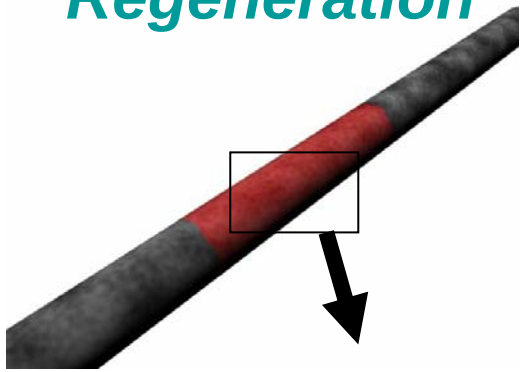
Graphic by Tzeranis, 2006

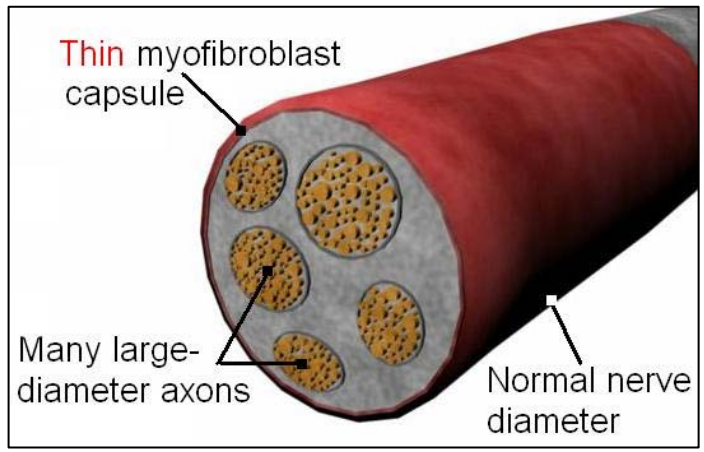
No regeneration (neuroma)





Regeneration





Courtesy of Dimitrios Tzeranis. Used with permission.

- **Thickness of contractile cell layer controls mechanical stress acting around the regenerating nerve**
- **Thick layer generates large mechanical stress and blocks synthesis of new tissue → no reconnection of nerve stumps**

The pressure cuff theory

Graphic by Tzeranis, 2006

Nerve Regeneration

A short movie:

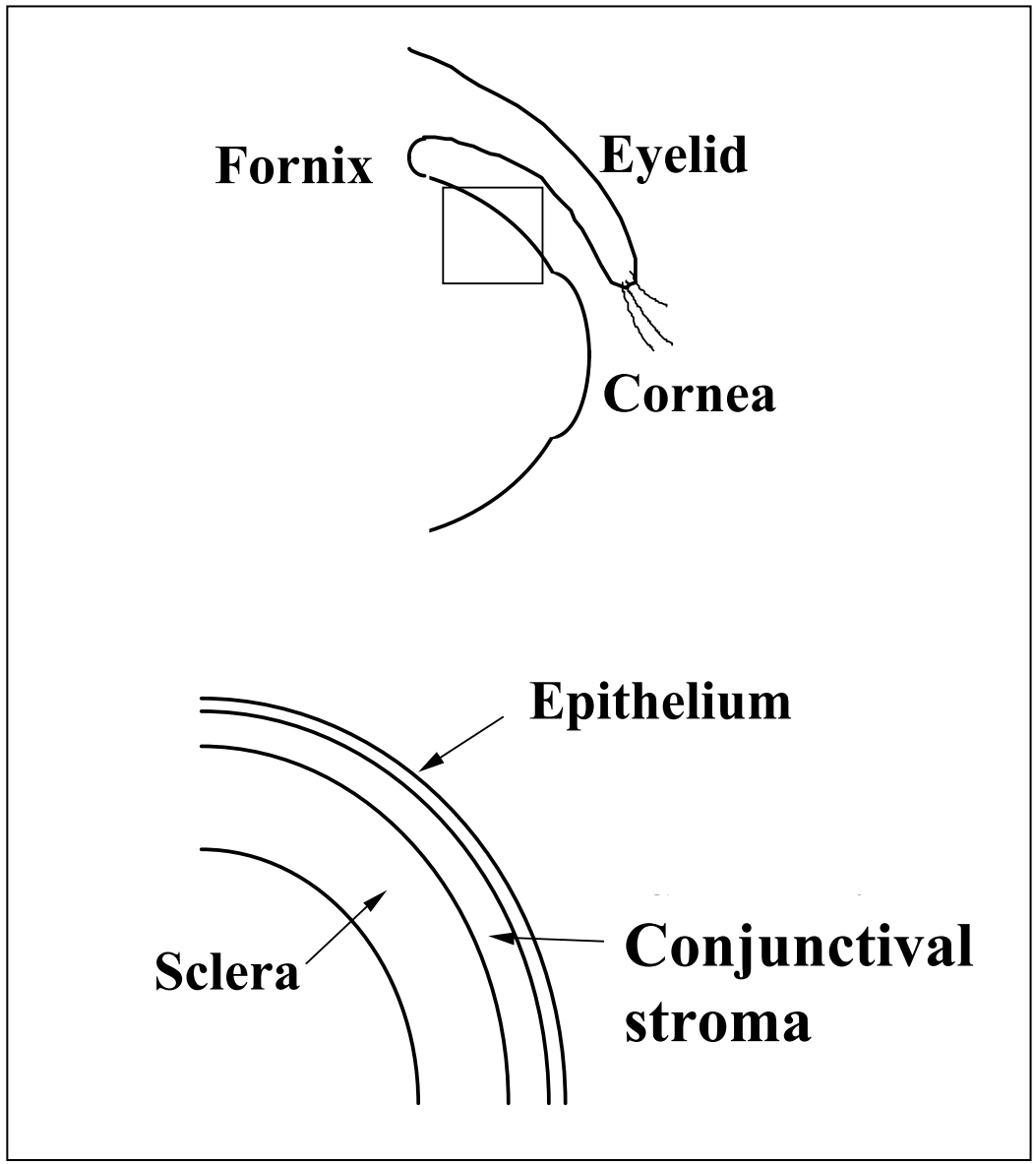
Peripheral Nerve Regeneration

Animation by Dimitrios Tzeranis, Yannas Lab, 2007

Organ #3. Conjunctiva Regeneration

Induce regeneration of conjunctiva using DRT

Anatomy of the conjunctiva



Effect of DRT on contraction kinetics of conjunctival defect. It is experimentally convenient to study contraction of the fornix, a tissue attached to the conjunctiva.

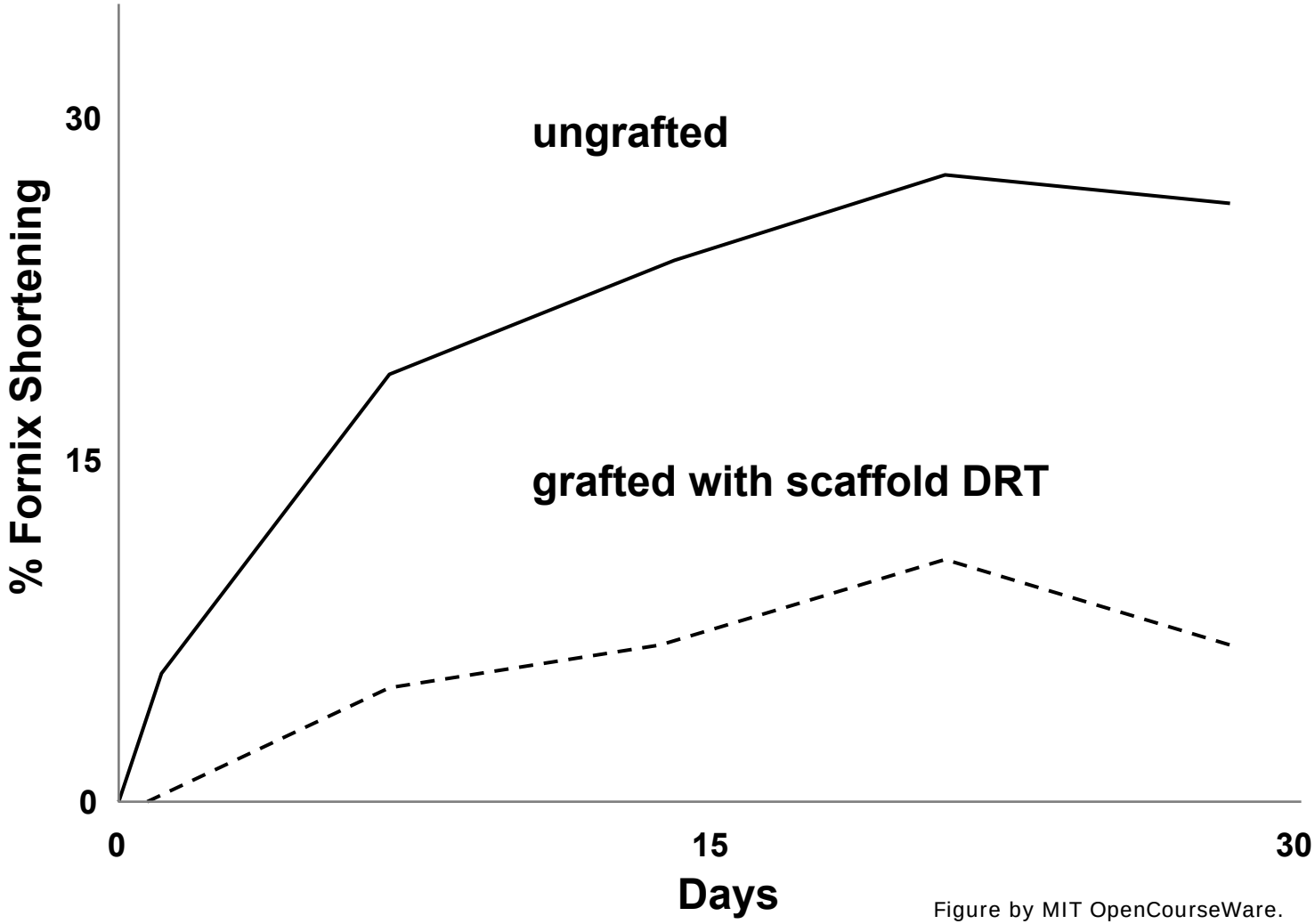


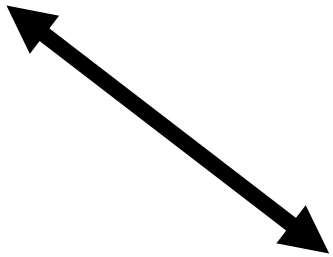
Figure by MIT OpenCourseWare.

Hsu et al, 2000

Conjunctival scar

Normal conjunctiva

Three images removed due to copyright restrictions.
See Figure 7 in Hsu, W.-C., M. H. Spilker, I. V. Yannas, and P. A. D. Rubin (2000). “Inhibition of conjunctival scarring and contraction by a porous collagen-GAG implant.” *Invest. Ophthalmol. Vis. Sci.* 41:2404-2411.



Regenerated conjunctiva (rabbit)

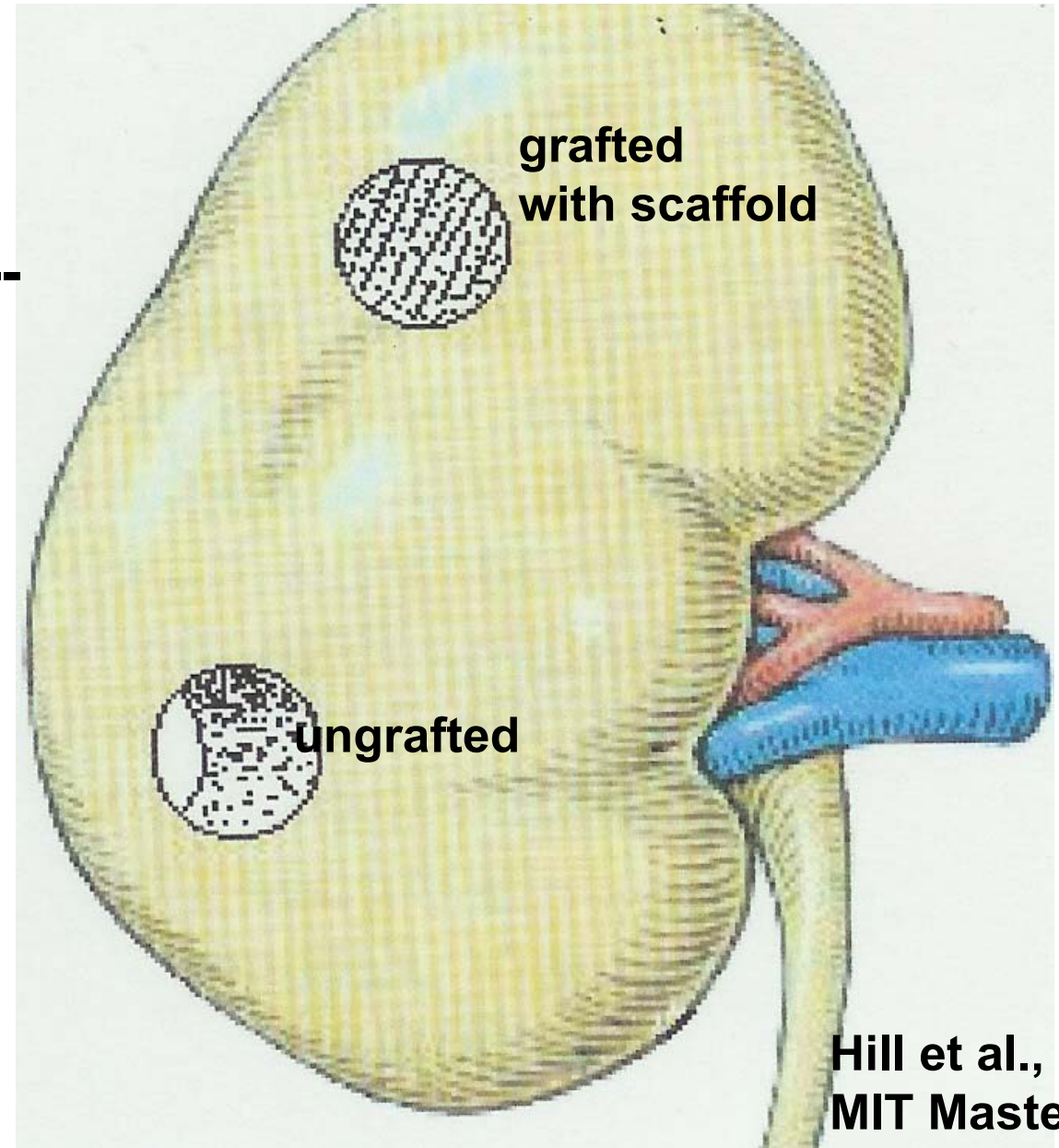
Orientation of collagen fibers in conjunctival stroma using polarization microscopy.

Hsu et al., 2000

Organ #4. Blocking of wound contraction and scar inhibition in adult rat kidney

EXPERIMENTAL ARRANGEMENT FOR STUDY OF SCAR INHIBITION IN KIDNEY

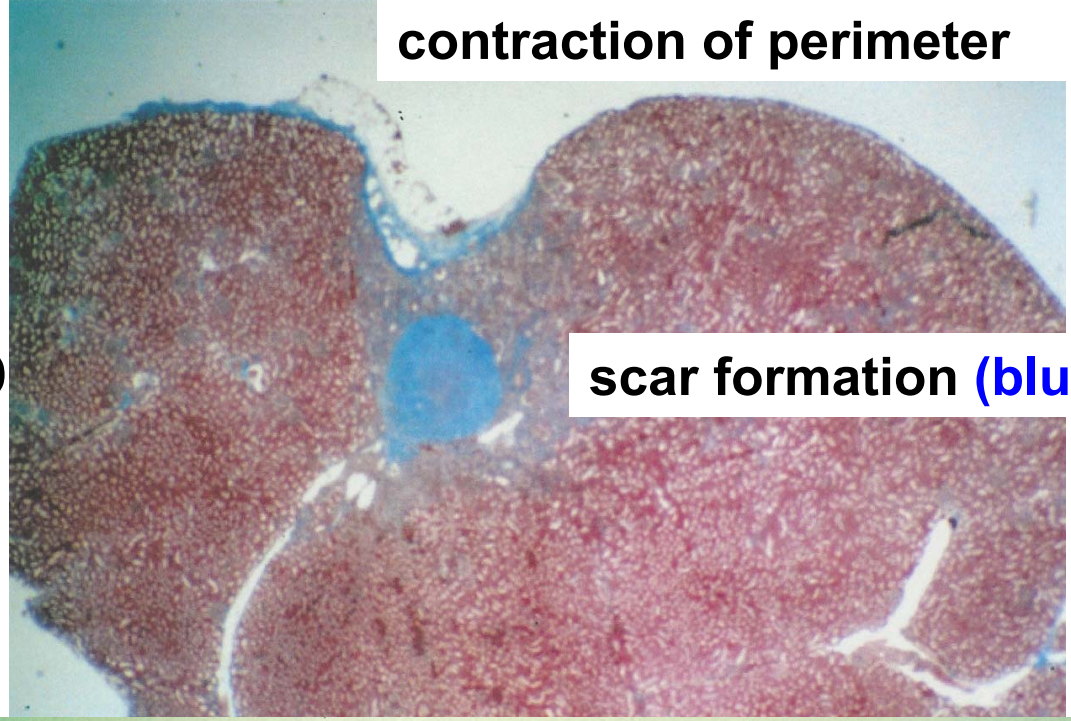
rat kidney wound model--- 3-mm diam. perforations



Hill et al., 2003
MIT Masters Thesis

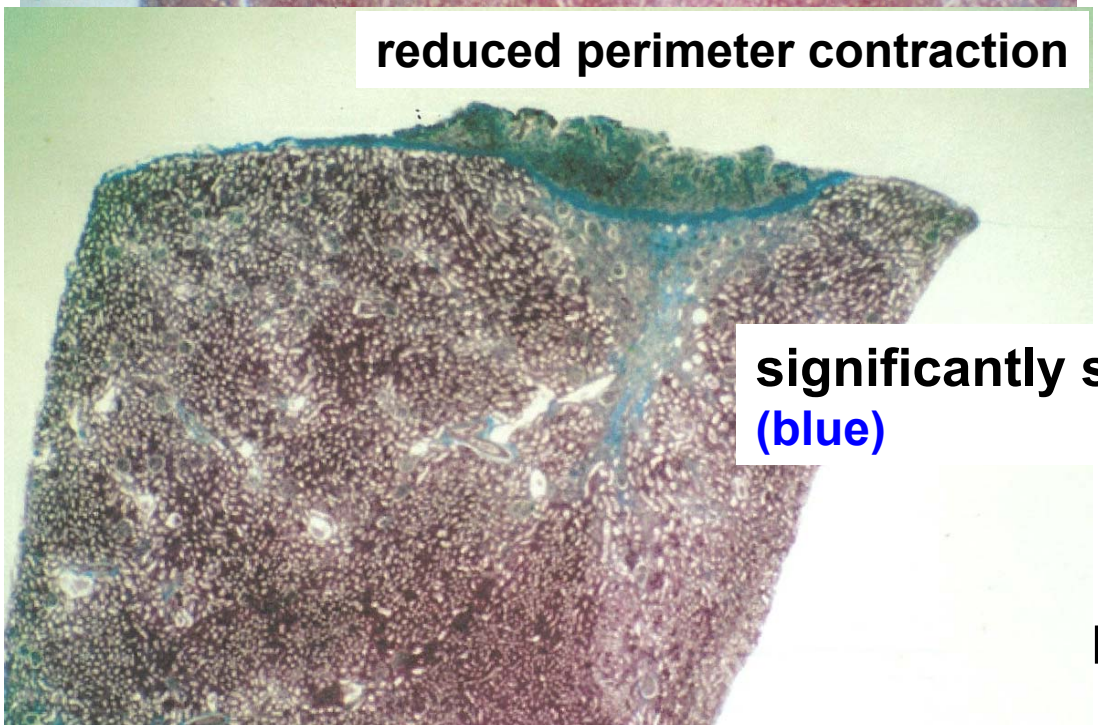
Rat kidney

UNTREATED



fibrotic tissue stains blue

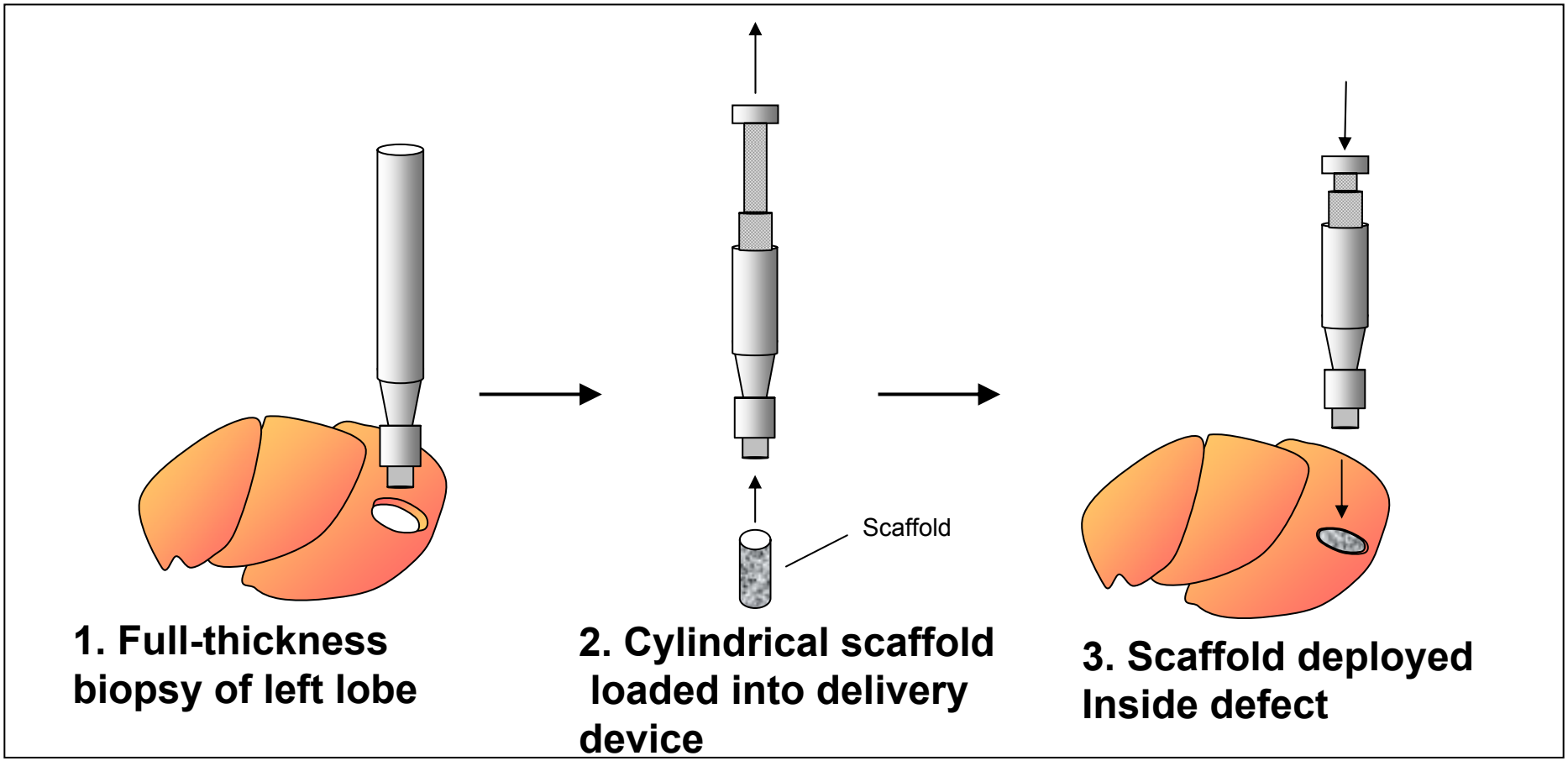
TREATED WITH SCAFFOLD



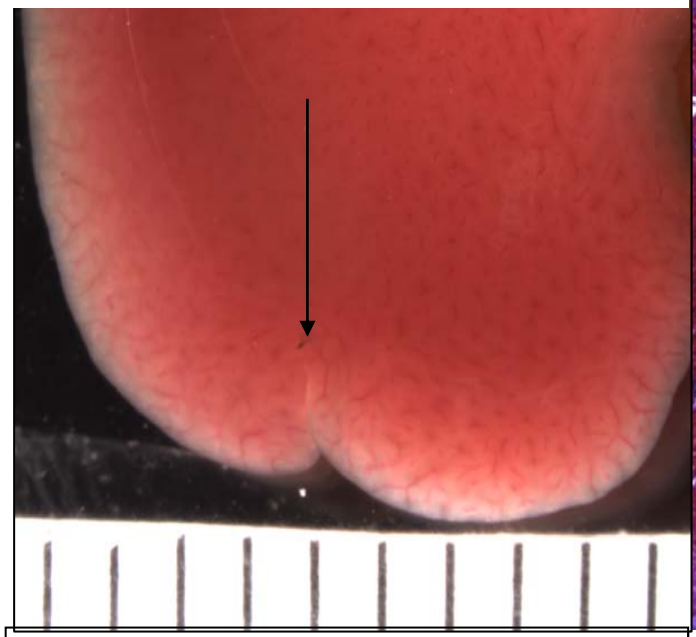
Hill et al., 2003

Organ #5 Blocking of wound contraction in adult mouse liver

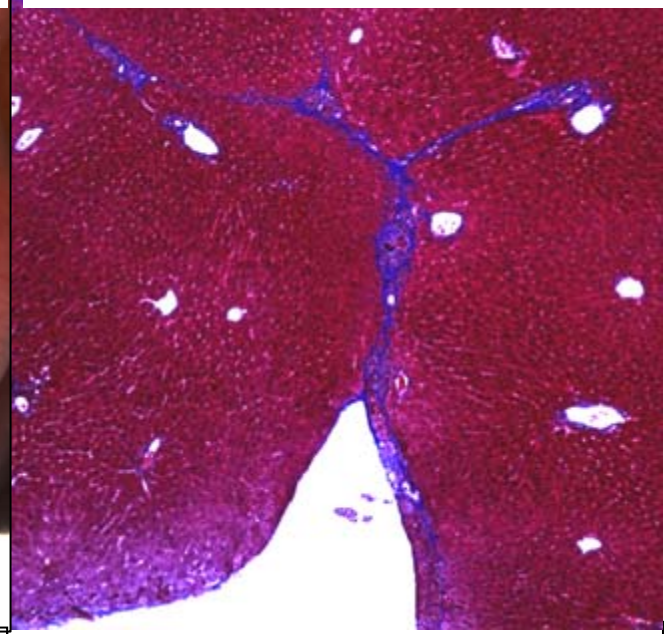
Schematic of Wound Model in Adult Mouse Liver

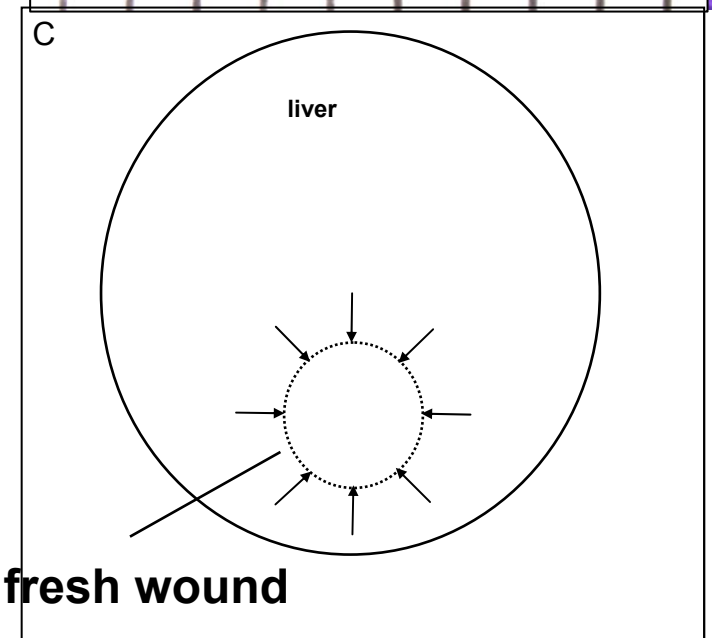


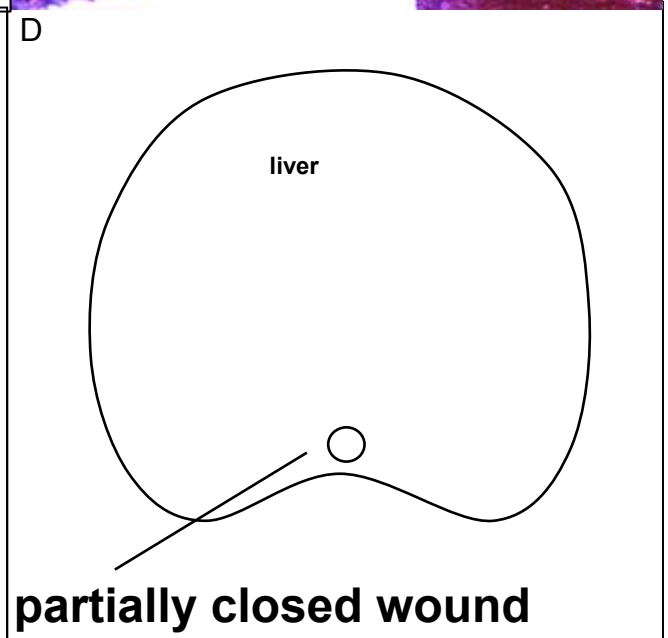
Spontaneously healed mouse liver 4 weeks following dissection of lobe. Gross view.



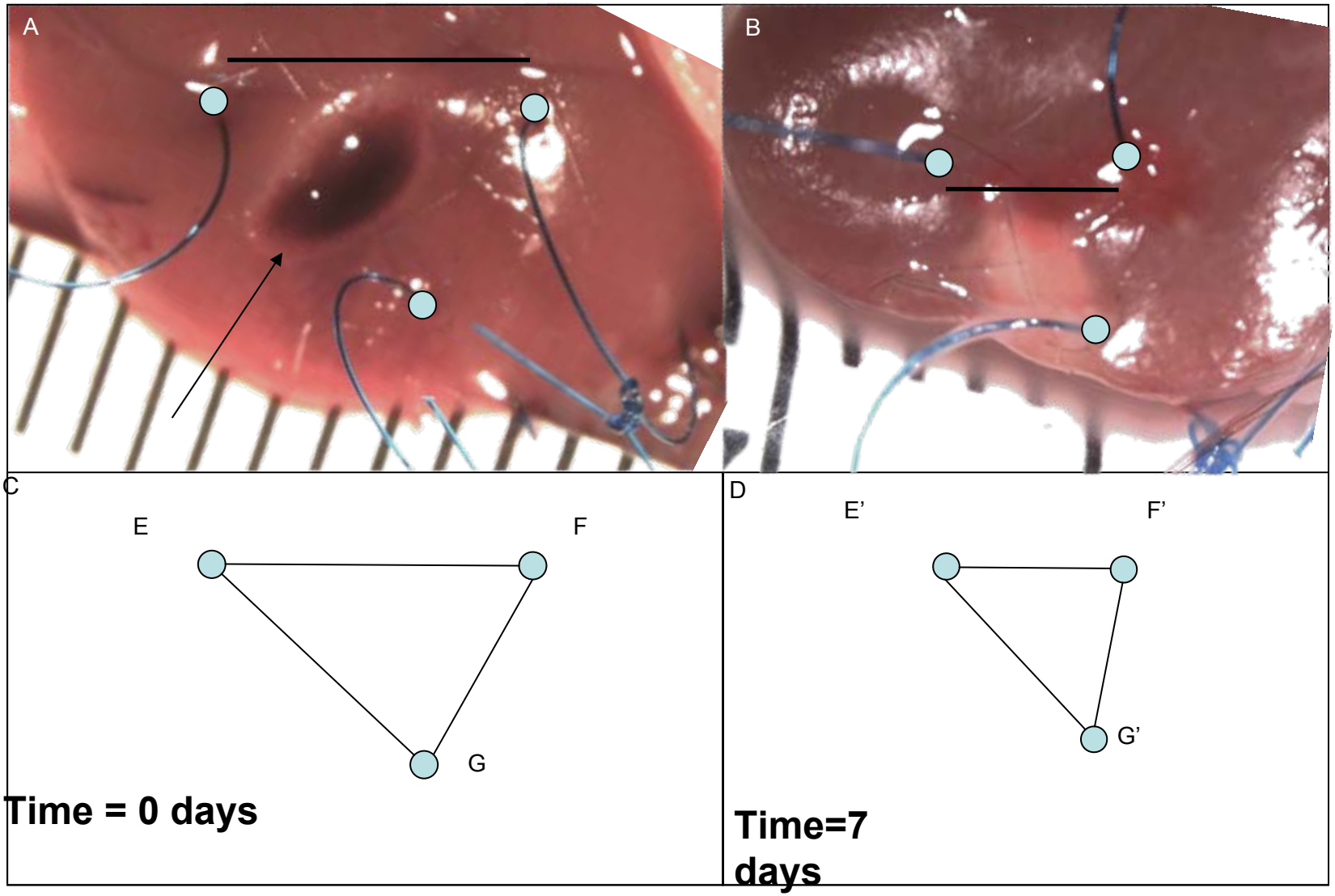
Histology (trichrome stain) shows fibrotic tissue (blue) lining edges of closed wound.



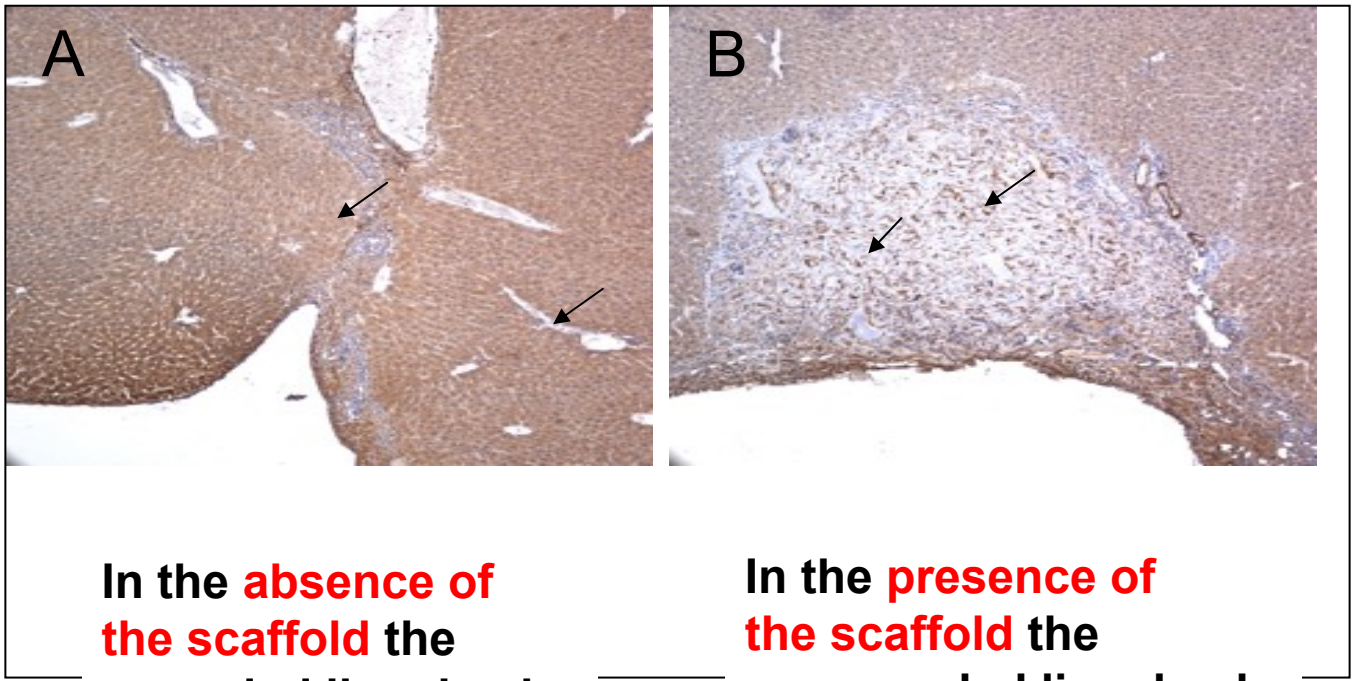




Sutures are used to monitor wound contraction in adult mouse liver



Contraction of wounded adult mouse liver is blocked following grafting of scaffold (4 weeks' data). Scaffold is extremely compliant; does not act as a mechanical splint.



In the absence of the scaffold the wounded liver heals by contraction and scar formation (blue)

In the presence of the scaffold the wounded liver heals with little contraction and no scar (blue absent)

Summarize evidence from healing in five adult organs
Back to the old question: Does scar block regeneration?

- **No. Wounds in most organs close spontaneously primarily by contraction.**
- **Scar is the by-product of contraction. It is highly oriented stroma that is synthesized in the presence of the mechanical field that dominates wound contraction.**
- **Regeneration requires blocking of contraction. When contraction is blocked by a scaffold, even partly, scar formation is cancelled.**

What is the mechanism of contraction-blocking by an active scaffold at the

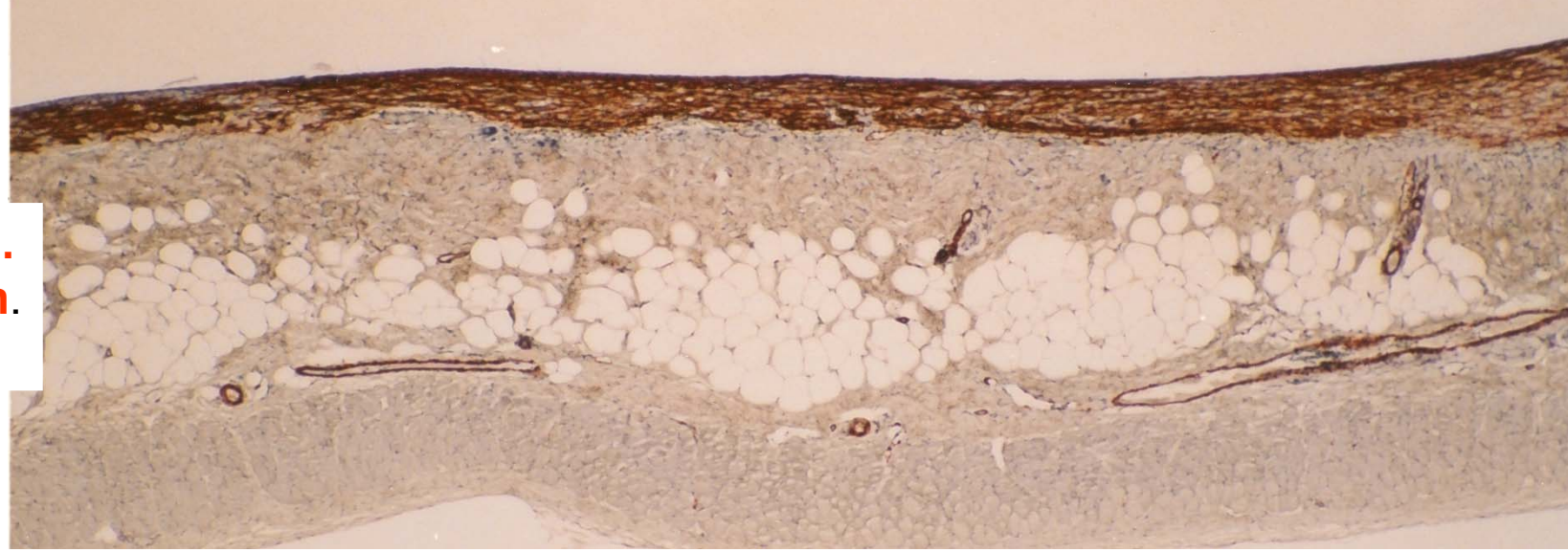
- **Scale of the cell (1 μm)?**
- **Scale of the molecule (1 nm)?**

Diagram removed due to copyright restrictions.
Figure 2 in Tomasek, J.J., et al. (2002). "Myofibroblasts and mechano-regulation of connective tissue remodeling. " *Nature Reviews Molecular Cell Biology* 3: 349-363.

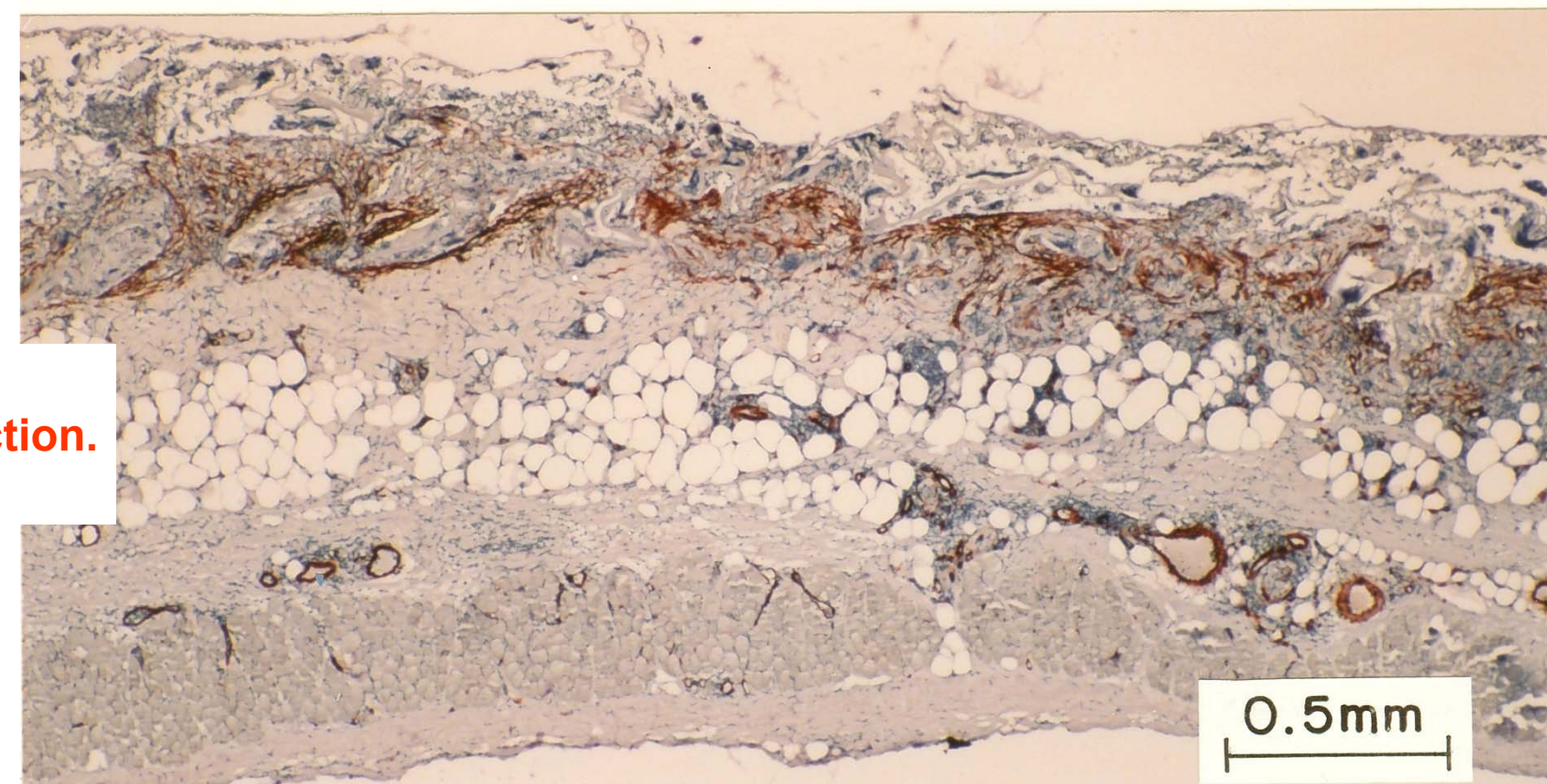
The contractile fibroblast (myofibroblast. MFB) is expressed during wound healing and childbirth. Differentiation of fibroblast to MFB requires the presence of TGFβ1, a fibronectin fragment and mechanical force

Tomasek et al., 2002

No scaffold.
Contraction.
Day 10.



Scaffold.
No contraction.
Day 10.



Does the scaffold act as a mechanical splint?

- Assume a contractile force F_c = 0.1 N acts on a scaffold that has grafted a wound measuring 10mm X 10mm in surface area with 1 mm depth. Acting on an area A = 10 mm X 1 mm = 10 mm^2 = 10^{-5} m^2, the force applies a compressive stress

  $\sigma_c = F_c/A = 0.1N/ 10^{-5} m^2 = 10^4 N/m^2 = 10^4$ Pa.
- At equilibrium the compressive stress applied by the cells hypothetically equals the splinting stress, σ_s , with which the scaffold resists wound contraction.
- Hypothesize that the scaffold is a relatively stiff splint that has been deformed compressively by no more than 10%, or $\varepsilon = 0.1$. It must possess a Young's modulus that is not less than $E = \sigma_s/\varepsilon = 10^4$ Pa/0.1 = 10^5 Pa. However, measured values of E for scaffolds with pore volume fraction of 0.99 are only 10^2Pa. Such scaffolds are 1000X less stiff than required for mechanical splinting. The hypothesis that the scaffold works as a splint is rejected.

Model of wound contraction by myofibroblasts (MFB)

$$F_c = N f_i \varphi$$

F_c = Total macroscopic force exerted by MFB, which suffices to close wound by contraction (approx. 0.1 N)

N = Total number of MFB.

f_i = Contractile force exerted per cell.

φ = Fraction of MFB which are oriented with their contractile axes in the plane of the wound.

$$F_c = N \quad f_i \quad \varphi$$

Macroscopic Contraction force

No. cells

Force per cell

Orientation factor

- **If myofibroblasts act independently and are oriented in the plane, the macroscopic force is:**

$$F_c = 3 \times 10^5 \text{ cells} \times 10 \text{ nN/cell} \times 1 = 3 \times 10^{-3} \text{ N}$$

$$0.1 \text{ N} >> 3 \times 10^{-3} \text{ N}$$

- **Measured macroscopic force is about 33 times higher!!**
- **Do cells generate larger force by acting cooperatively?**

In the absence of the scaffold, the macroscopic force that contracts wound cannot be easily explained by hypothesizing independent (noncooperative) cell contractile activity.

Mechanism of scaffold activity # 1. Scaffold reduces the number of contractile cells

- **Fact: In absence of scaffold, 50 % of fibroblasts in wound are MFB. In presence of scaffold only 10% are MFB.**
- **The structure of collagen fibers in active scaffolds has been modified by acid treatment to reduce significantly the % banded collagen fibers.**
- **Loss of banding blocks thrombogenicity (platelet aggregation) of collagen fibers which in turn leads to TGFb1 depletion in wound.**
- **TGFb1 depletion prevents MFB differentiation.**
- **$F_c = N\varphi f_i$. According to this mechanism, the number of MFB, N, goes down in the presence of the scaffold. The macroscopic contractile force, F_c, is correspondingly reduced.**

Mechanism of scaffold activity # 2. Scaffold disorients MFB thereby reducing sum of forces generated by MFB

- **Fact: MFB bind avidly on scaffold surface with their long (contractile) axes quasi randomly oriented in space. Only a small fraction are oriented in the plane.**
- **Fact: Bound MFB lose many connections with neighboring cells.**
- **Lacking orientation in the plane of the wound, MFB lose most of their ability to pull wound edges in the plane and close the wound by contraction.**
- **$F_c = N\varphi f_i$. According to this mechanism, the fraction of cells, φ, bound to the matrix and capable of applying traction, is reduced following grafting with an active scaffold. The macroscopic contractile force, F_c, is correspondingly reduced.**

Hypothesis of loss of cell-cell cooperativity

- In the absence of an active scaffold, MFB are connected and oriented. They function cooperatively during wound contraction. The sum of forces exerted is higher than the sum of forces exerted by individual cells acting independently (noncooperatively).
- An active scaffold separates and disorients the MFB. It causes loss of cell-cell cooperativity.

Two examples of clinical application of the dermis regeneration template, DRT

Bilayer medical device approved by FDA

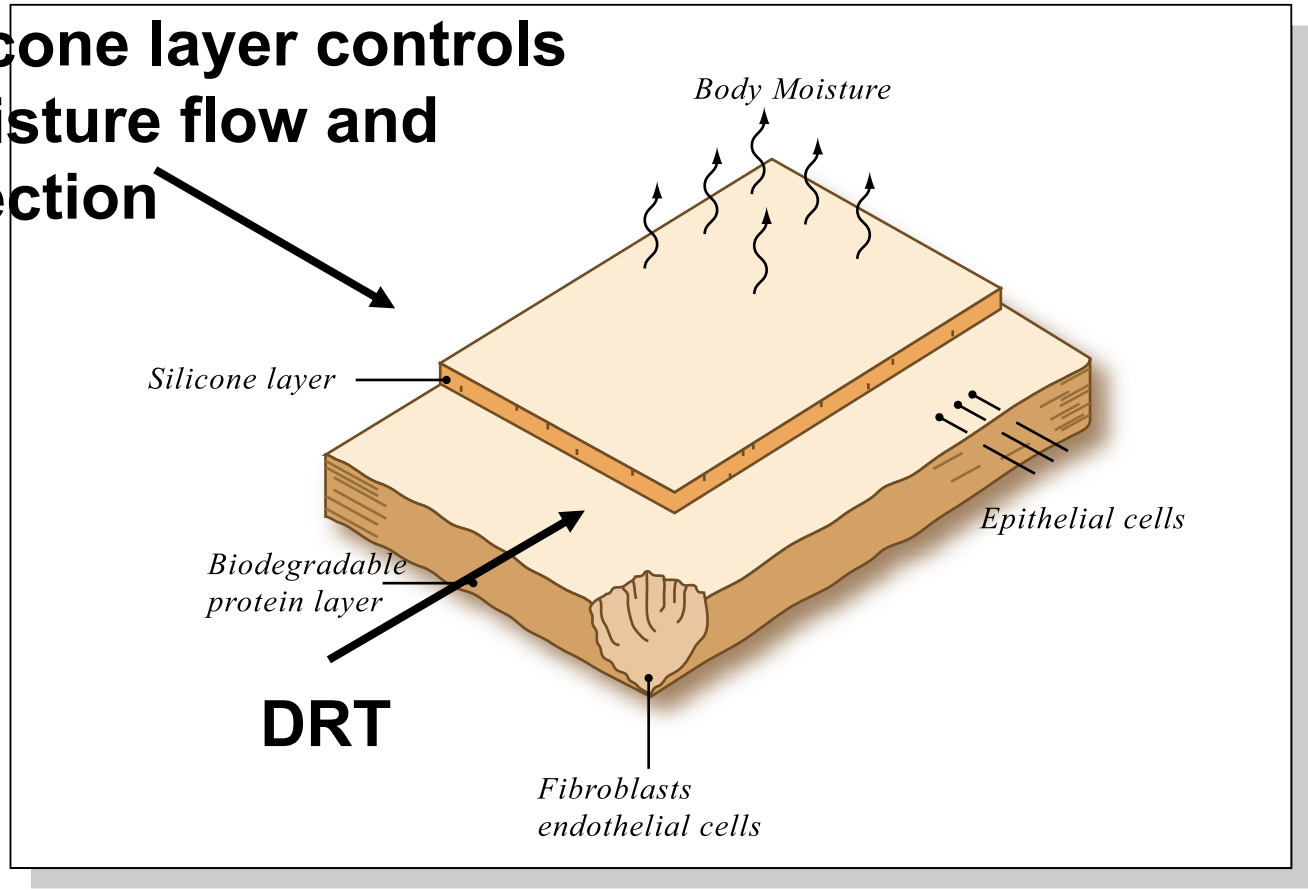


Figure by MIT OpenCourseWare.

Yannas et al., 1982

In clinical applications where skin is severely injured the active scaffold is used as a bilayer. Top layer is a thin silicone film. Bottom layer is the DRT scaffold.

CASE 1. Patient: Female teenager with large scars on skin.

Treatment:

Step 0: Evaluate scars

Step 1: Excision of burn scar

Step 2: Grafting of a biologically active scaffold (template)

Step 3: Partial regeneration of skin in place of burn scar

Photos by Dr. Andrew Byrd, M.D., Bristol, UK

scar

Photos removed due to copyright restrictions.

Step 0: Female teenager with scars from burns.

Left breast failed to develop due to mechanical stresses of scar

Photo removed due to copyright restrictions.

Step 1: Excision of scar → generation of a new wound.

Surgeon has excised the entire scar around breast generating a deep skin wound

Photo removed due to copyright restrictions.

Step 2: Graft open wound with DRT device.

Wounds have been grafted with the bilayer device (silicone layer outside; scaffold inside). Side view shows that left breast has now erupted.

Photo removed due to copyright restrictions.

Step 2: Graft open wound with DRT bilayer.

Top view emphasizes the shiny silicone layer outside

Photo removed due to copyright restrictions.

New skin with blood vessels and nerve endings has grown three weeks after grafting of scaffold. “Alligator” pattern disappears later.

Step 3: New skin has been partly regenerated in 21 days.

CASE 2. Care of Chronic Skin Wounds

- A 79-year-old woman presented with a chronic skin ulcer in the foot.
- The surgeon excised the ulcerous wound bed and generated a fresh wound.
- A scaffold based on the dermis regeneration template was then applied to the fresh wound.

- Data from 111-patient clinical trial in Phoenix, AZ by M.E. Gottlieb, and J. Furman. J Burns & Surg Wound Care 2004;3(1):4.

Example of induced regeneration in a chronic skin wound. "A 79 year old woman presented with an ankle ulcer of several years duration. …It was excised, including bone fragments and the arthrosis, and (a biologically active scaffold) was used to close the wound and the structures underneath. Seen here 11 days later, periwound inflammation is gone. The wound healed and has remained so, seen here a year later."

Photos removed due to copyright restrictions.

Chronic wound **Post-excision** **11 days after grafting**

Gottlieb and Furman, 2004

What is the mechanism of scaffold activity at the molecular scale?

Explore phenotype changes following specific binding of MFB to ligands on scaffold surface.

Changes in gene expression? Protein translation?

Does the difference between very good and very poor regeneration appear in <u>protein transcription or protein translation?</u>

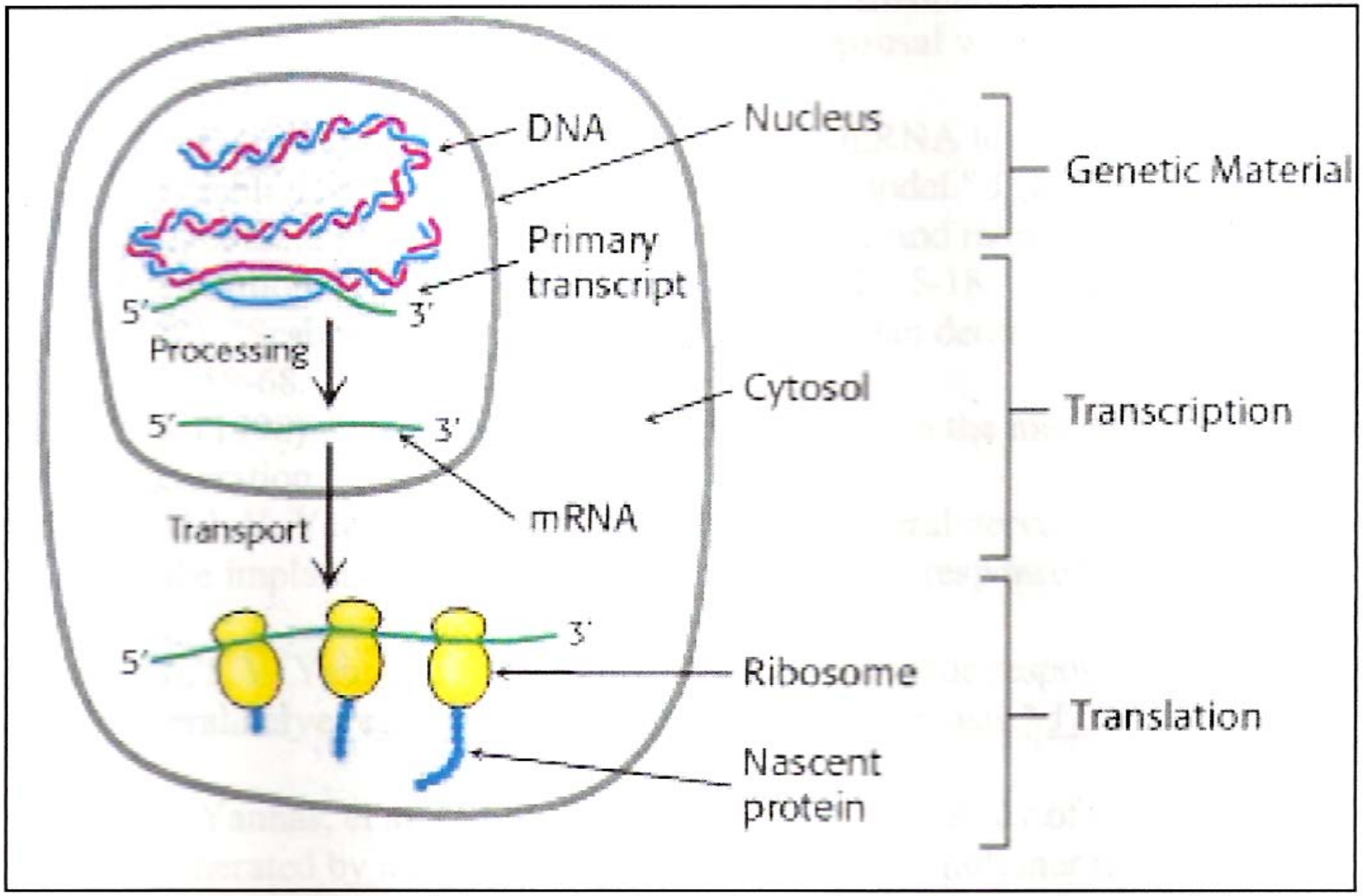
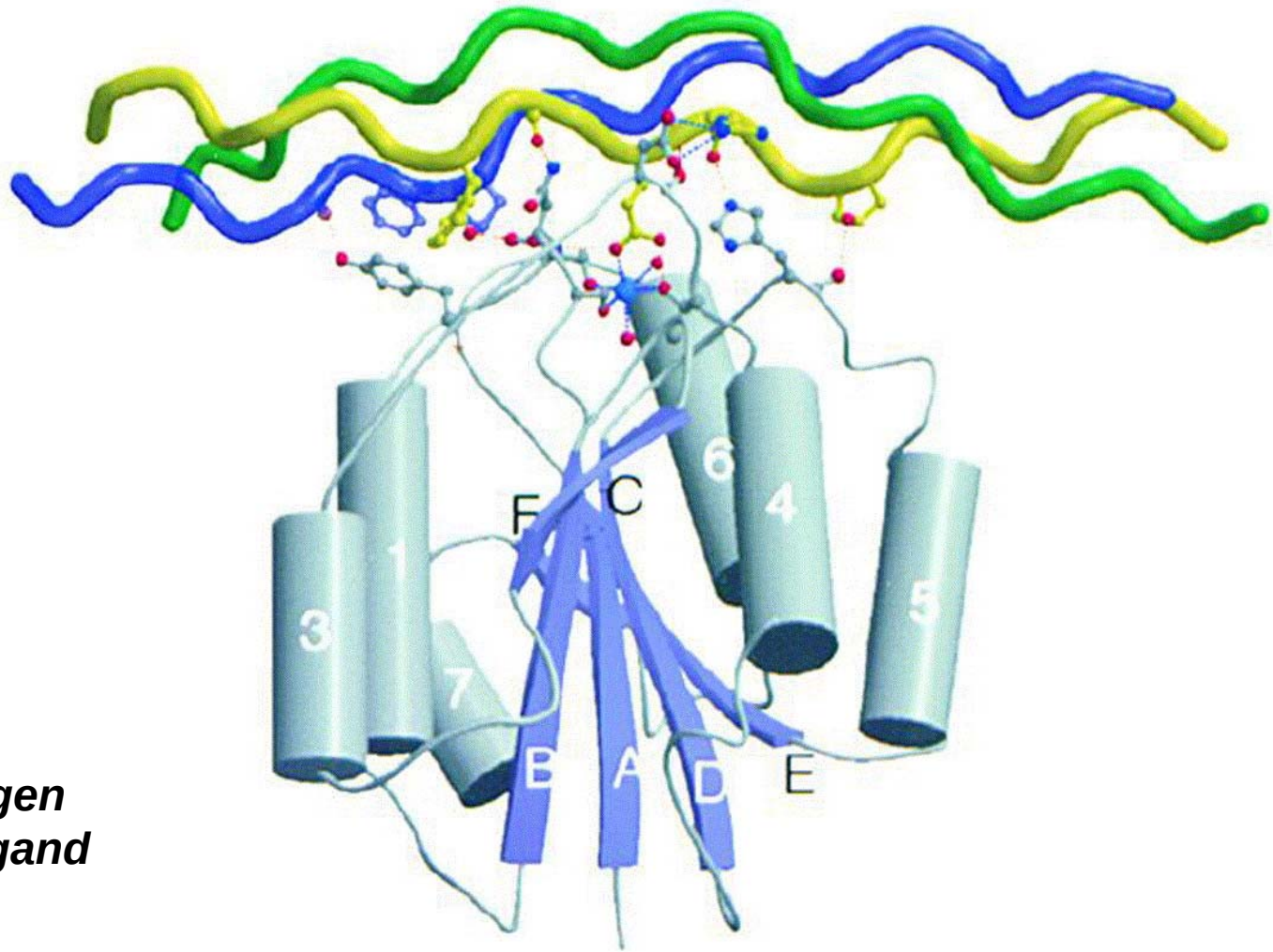


Figure 5.1 A graphic summary of the production of protein from genetic material through the processes of transcription and translation.

Wong, M. Q. MIT Masters Thesis, 2007

Phenotype change: fibroblast α2β1 integrin binds to collagen ligand GFOGER (hexapeptide)



Integrin (blue) adhesion to collagen at the GFOGER ligand

Emsley et al., 2000
Knight et al., 2000

Compare cytokine expression during healing of peripheral nerves inside a poorly regenerating wound (silicone tube) and a successfully regenerating tube (collagen tube)

mRNA expression and protein expression measured (at 14 days of healing) in two established models of regenerative activity: poor/successful			
Protein	**Measurement method**	**mRNA expression**	**Protein concentration**
TGFβ-1	PCR and ELISA	2.95	4.42
TGFβ-1	immunoblot	-----	3.96
TGFβ-2	immunoblot	2.86	3.33
TGFβ-3	immunoblot	1.00	0.91
α-SMA	immunoblot	3.71	13.6

Conclusions from transcriptional analysis and proteomics analysis in poorly vs successfully regenerating models

- TGFb1 and TGFb2, but not TGFb3, are expressed at significantly higher levels in the model of poor regeneration (relative to model of successful regeneration). Same for the mRNA of these proteins.
- α-smooth muscle actin is expressed at much higher levels during poor regeneration (relative to successful regeneration). Same for the mRNA of this protein.
- These results support the theory that contraction antagonizes regeneration.

How does wound healing induced in the early fetal stage (regeneration) differ from wound healing in late fetal stage (no regeneration)?

In early fetal healing (relative to late fetal healing):

1. **Very little, if any, wound contraction. No scar. Regeneration.**
2. **Very low TGFβ-1 and TGFβ-2 levels. High TGFβ-3 levels.**
3. **(a-SMA not measured by investigators in fetal models)**

Photos removed due to copyright restrictions.
See Soo, C., et al. "Ontogenetic Transition in Fetal Wound Transforming Growth Factor-Beta Regulation Correlates with Collagen Organization." *Am J Pathol* 163, no. 6 (2003): 2459-76.
http://www.ncbi.nlm.nih.gov/pmc/articles/PMC1892380/

EARLY fetal healing. No wound contraction. LOW TGFβ1 concentration.

[Fig. 2D]

Healing with skin regeneration including hair follicles (open arrows) (rat)

LATE fetal healing. Wound contraction occurs. During healing HIGH TGFβ1 Concentration.

[Fig. 3D]

Healing with no hair follicles (left). [Uninjured skin (right) has hair follicles]

Soo et al., 2001, 2003

regenerated skin
but no beard

Photo removed due to copyright restrictions.

Patient of Dr. John Burke
MGH, Boston

Regeneration induced in adult is partial, not perfect, as in early fetal healing. Severely burned patient was treated with DRT. Skin was regenerated on right side of face. However, the beard was not regenerated.

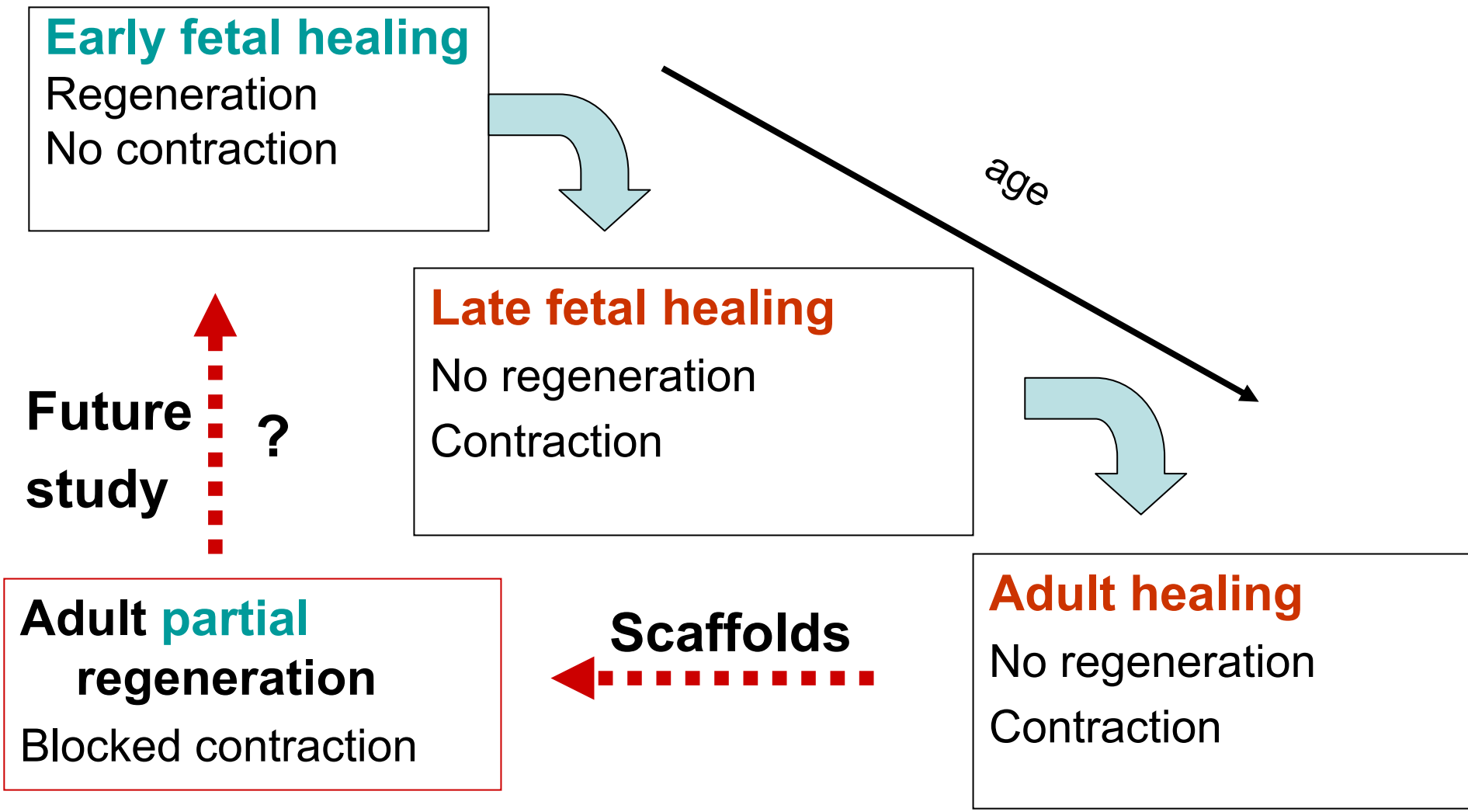
Question 1. What is the best current mechanism of induced organ regeneration in adults using active scaffolds at the cell-biological and the molecular-biological levels?

1. **Strong blocking of wound contraction, which leads to cancellation of scar formation.**
2. **Contraction blocking follows reduction of density of contractile cells and disorientation of their contractile axes.**
3. **Phenotype changes hypothetically due to regulation following binding of contractile cells on specific ligands of insoluble surface of scaffold.**

Question 2: Is the process of induced regeneration of organ in adults similar to spontaneous regeneration at the early fetal stage?

Partly, yes. Common features include: Downregulation of: wound contraction, density and function of contractile cells, cytokine (TGFβ-1) credited with induction of phenotype of contractile cells. However, myofibroblasts present in adult but not in early fetal wounds. Role of TGFβ-2 unclear. Role of TGFβ-3 is under study.

A hypothesis



The early fetal healing response is dormant in adults

Credit to early references in the literature…Prometheus discovers liver regeneration. Reported by Aeschylus, in *Prometheus Bound*, ca. 480 B.C.

20.441J / 2.79J / 3.96J / HST.522J Biomaterials-Tissue Interactions

Fall 2009