B. Conjunctiva synthesis in vivo (regeneration)

1. Structure and function of conjunctiva.
2. Clinical effects of conjunctival scarring.
3. Anatomically well-defined defect.
4. Synthesis of conjunctival stroma, followed by re-epithelialization.

article handed out

Inhibition of Conjunctival Scarring and Contraction by a Porous Collagen-Glycosaminoglycan Implant

Hsu et al., in Investigative Ophthalmology and Visual Science 41:2402-2411

1. Structure and function of the conjunctiva

The conjunctiva covers the exposed part of the sclera (opaque part of eye) and the inner surface of the eyelids.

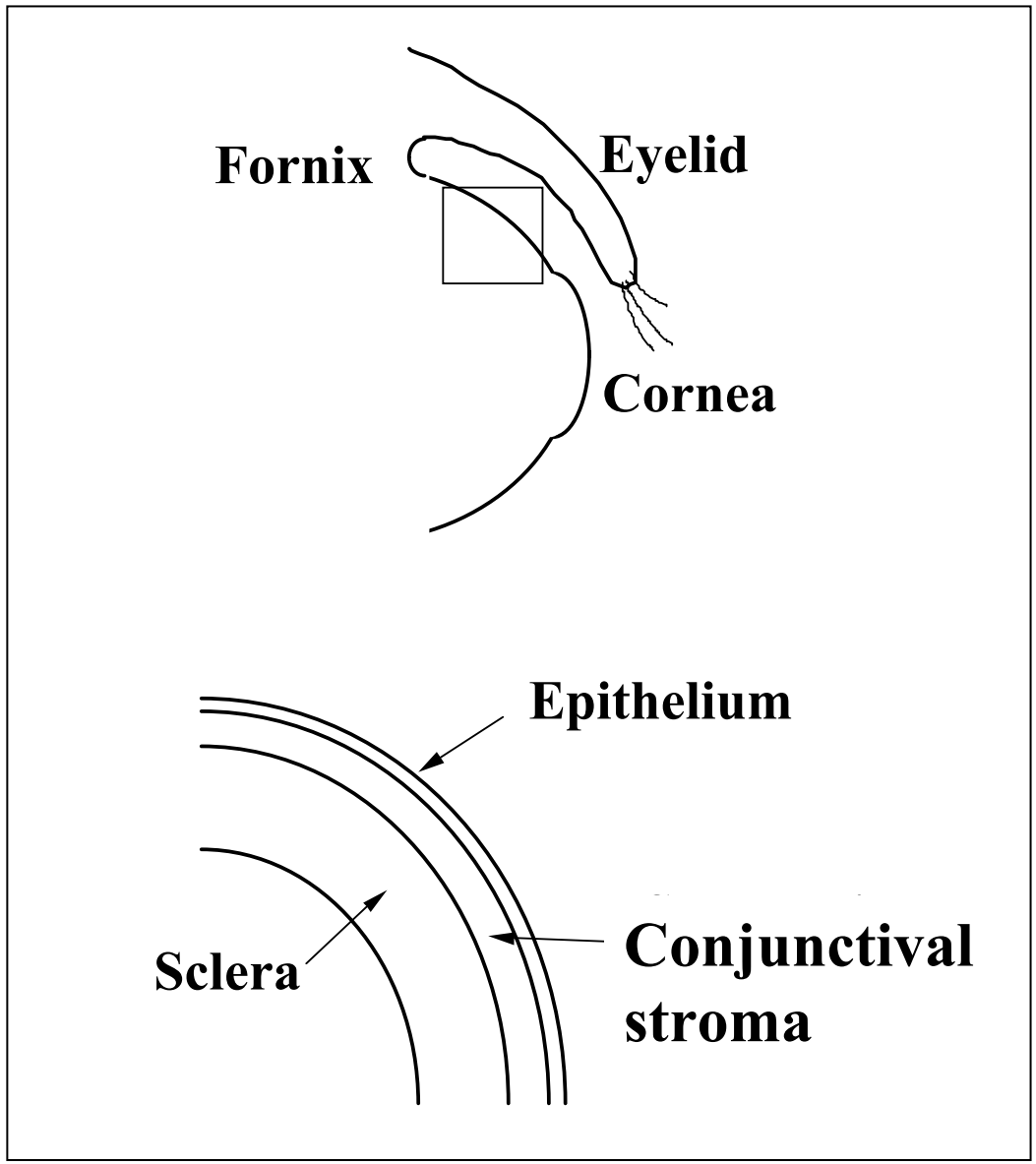
The epithelial tissue is stratified and contains goblet cells in the surface layers. Goblet cells synthesize and secrete mucus that contributes to the protective and lubricating layer on the exposed surface of the eye.

Underneath the epithelial tissue is the conjunctival stroma, a loose vascular supportive tissue.

2. Clinical effects of conjunctival scarring

- **Spontaneous healing of deep wounds in the conjunctiva occurs by contraction, scar formation and re-epithelialization.**
- **Conjunctival scarring is common endpoint for several opthalmic disorders, resulting from infection (trachoma), traumatic (chemical burns) and surgical (pterygium) causes.**

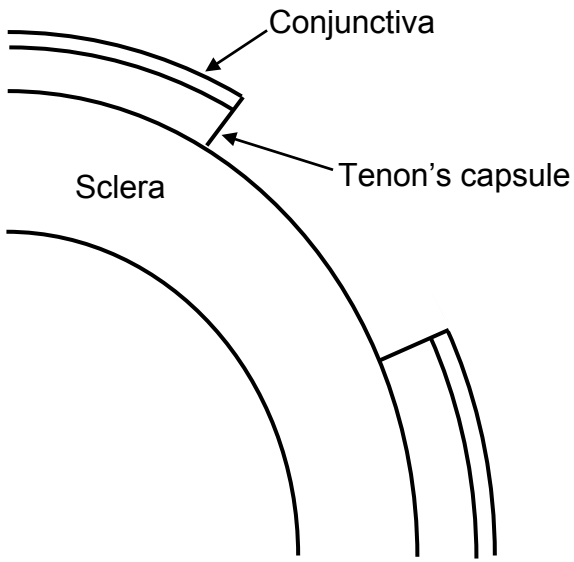
Anatomy of the conjunctiva

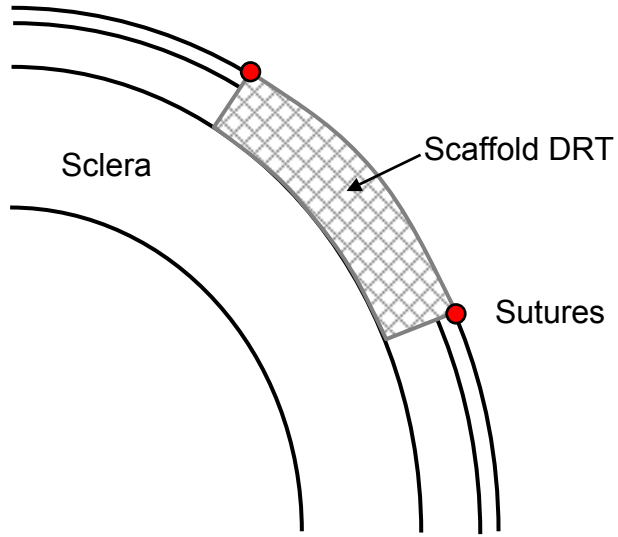


3. Anatomically well-defined defect

Defect: Excise complete epithelia and stroma.
Spontaneous healing: Full-thickness defect in conjunctiva heals by contraction and scar synthesis.

Conjunctiva wound model





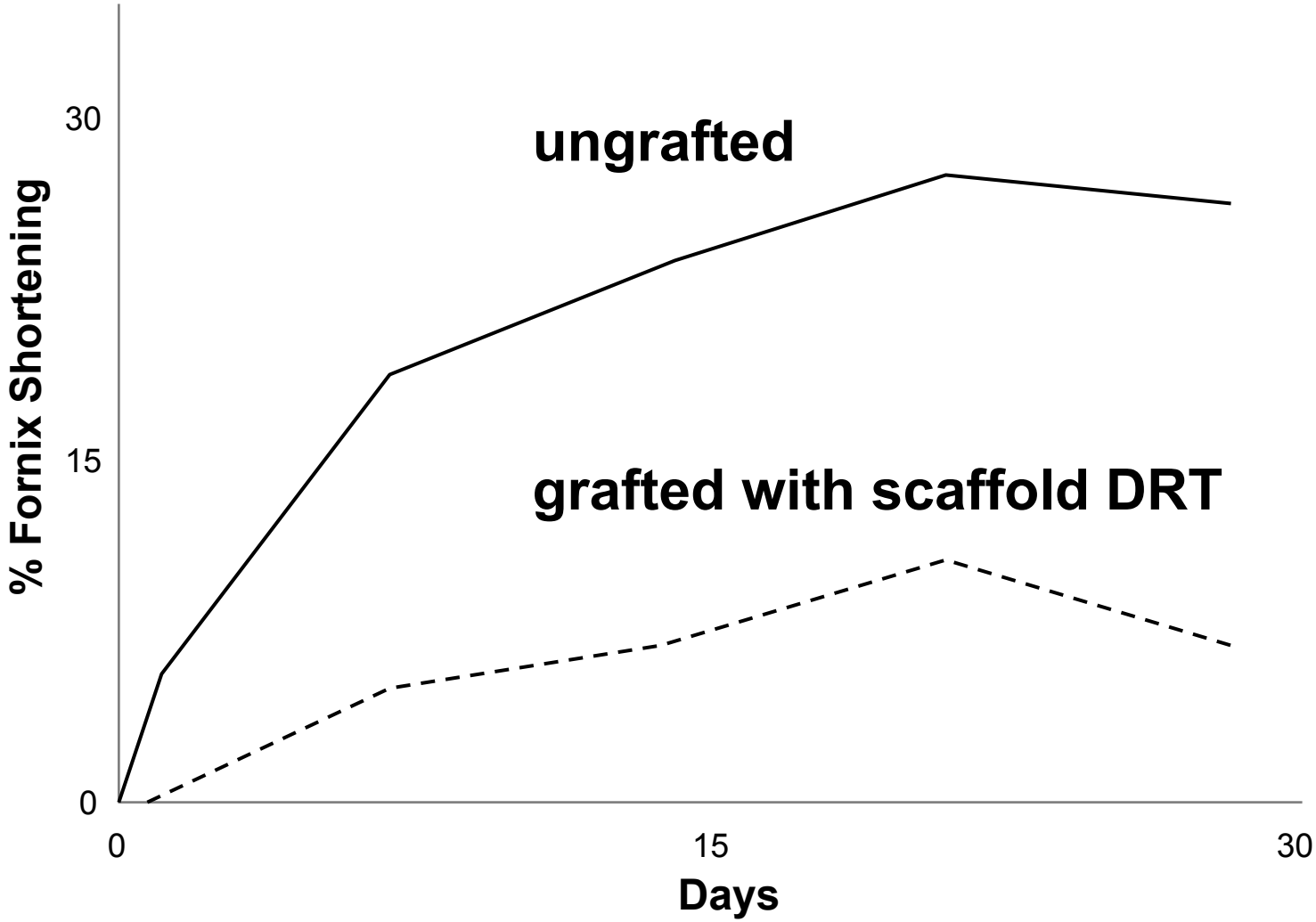
4. Synthesis of conjunctival stroma, followed by re-epithelialization

Sequential synthesis (as for skin):

Synthesize stroma using dermis regeneration template (DRT).

Epithelial tissue spontaneously synthesized over new stroma.

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.



Hsu et al., 2000

14 days

ungrafted

epithelialization (red) of scar (blue)

Image removed due to copyright considerations.
See Figure 6 in [Hsu 2000]

grafted with DRT

epithelialization (red) of incipient new dermis (blue)

Test of synthesis of conjunctival stroma (use microscope polarizing stage to study orientation of collagen fibers)

Images removed due to copyright considerations.
See [Hsu 2000]

conjunctival scar

synthesized conjunctival stroma

normal conjunctiva

C. Nerve synthesis in vivo (regeneration) (Ch. 6)

1. Structure of peripheral nerve.
2. Experimental parameters for study of induced regeneration.
3. Synthesis of myelinated axons and BM (nerve fibers)
4. Evidence (?) of synthesis of an endoneurium.
5. Synthesis of a nerve trunk (including summary of kinetics of synthesis).
6. Comparative regenerative activity of various reactants.

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

Image removed due to copyright considerations.

Nervous System

Image removed due to copyright considerations.

CNS **PNS**

Chamberlain et al., 1998

1. Structure of a peripheral nerve. I

Nerve fibers comprise axons wrapped in a myelin sheath, itself surrounded by BM (diam. 10-30 μm in rat sciatic nerve).

Axons are extensions (long processes) of neurons located in spinal cord. They comprise endoplasmic reticulum and microtubules.

1. Structure of a peripheral nerve. II

Myelinated axons (diam. 1-15 μm) are wrapped in a myelin sheath; nonmyelinated axons also exist. They are the elementary units for conduction of electric signals in the body. Myelin formed by wrapping a Schwann cell membrane many times around axon perimeter. No ECM inside nerve fibers.

Myelin sheath is a wrapping of Schwann cell membranes around certain axons.

1. Structure of a peripheral nerve. III

Nonmyelinated axons (diam. <1 μm) function in small pain nerves. Although surrounded by Schwann cells, they lack myelin sheath; Schwann cells are around them but have retained their cytoplasm.

Basement membrane (tubular) encases the myelin sheath. Structure similar to that of skin BM.

1. Structure of a peripheral nerve. IV

Nerve fibers are embedded in endoneurium: a delicate packing of loose vascular supporting tissue that is rich in collagen fibers. Definitely ECM!

Many nerve fibers with their associated endoneurium are packed in a collagenous layer, the perineurium. This forms a fascicle.

Multifascicular nerves encased in a collagenous layer, the epineurium.

Cylindrical symmetry of peripheral nerve structure

Summary of nerve trunk structure proceeding radially from the center out:

[axon — myelin sheath — BM] — endoneurium — perineurium — epineurium.

[….] = “nerve fiber”

Rat sciatic nerve (nerve trunk).
One fascicle.
Several thousand axons.

Image removed due to copyright considerations.
See Figure 10.7 (upper left) in Yannas, I. V. *Tissue and Organ Regeneration in Adults*. New York: Springer-Verlag, 2001.

(idealized)

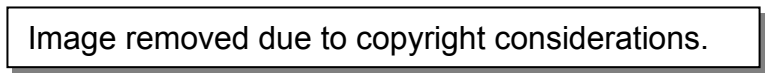
Rat Sciatic Nerve Cross Section

nerve trunk

Individual Axon

nerve fiber

Longitudinal view of nerve fiber



Chamberlain, 1998

2. Experimental parameters for study of regeneration

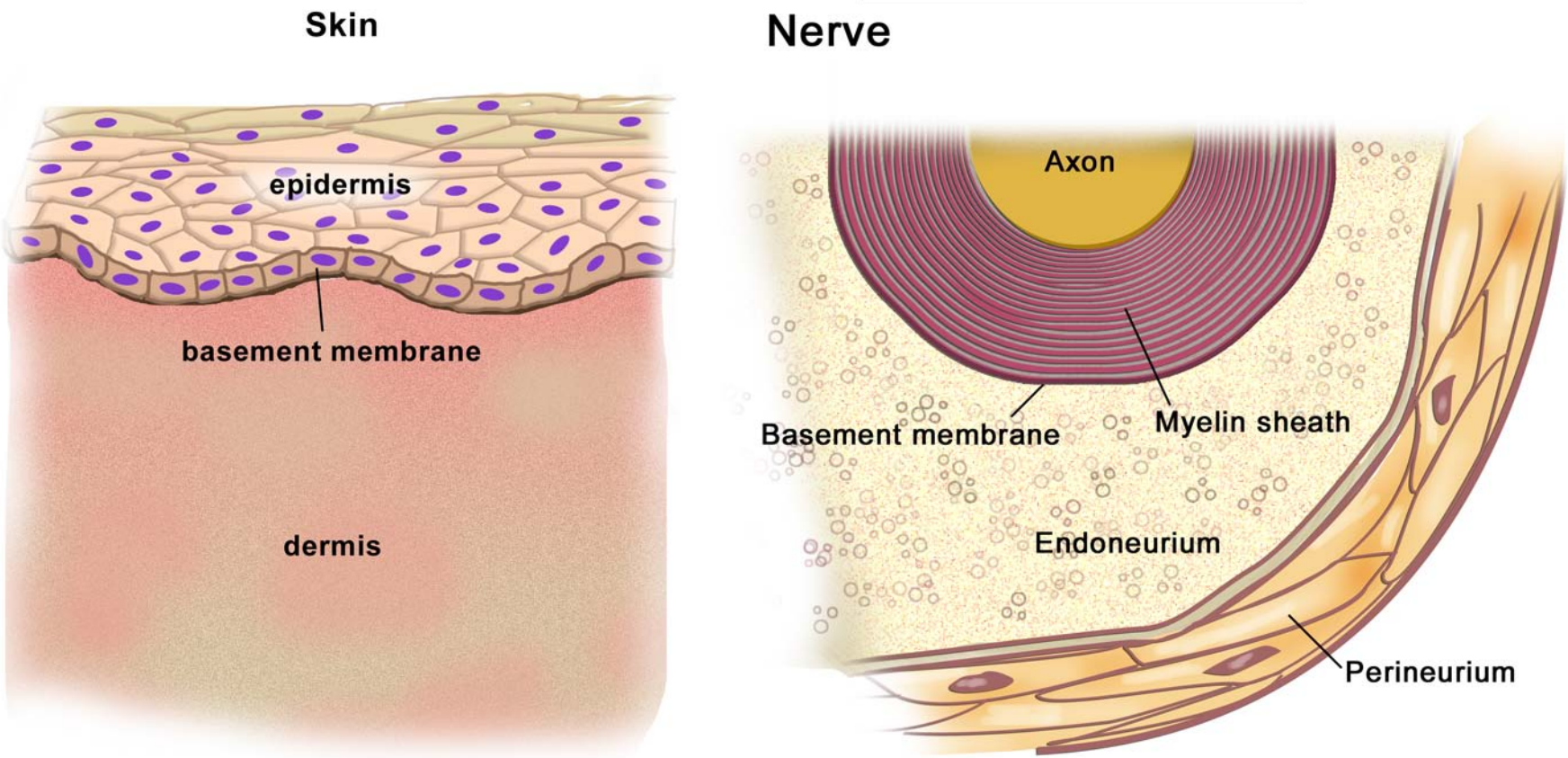
A. Anatomically well-defined defect

- Designate experimental volume
- Delete nonregenerative tissue(s)
- Anatomical bounds
- Containment of exudate

B. Timescale of observations

- Short-term (<20 wk) and long-term (>20 wk) assays

The tissue triad in skin and nerves

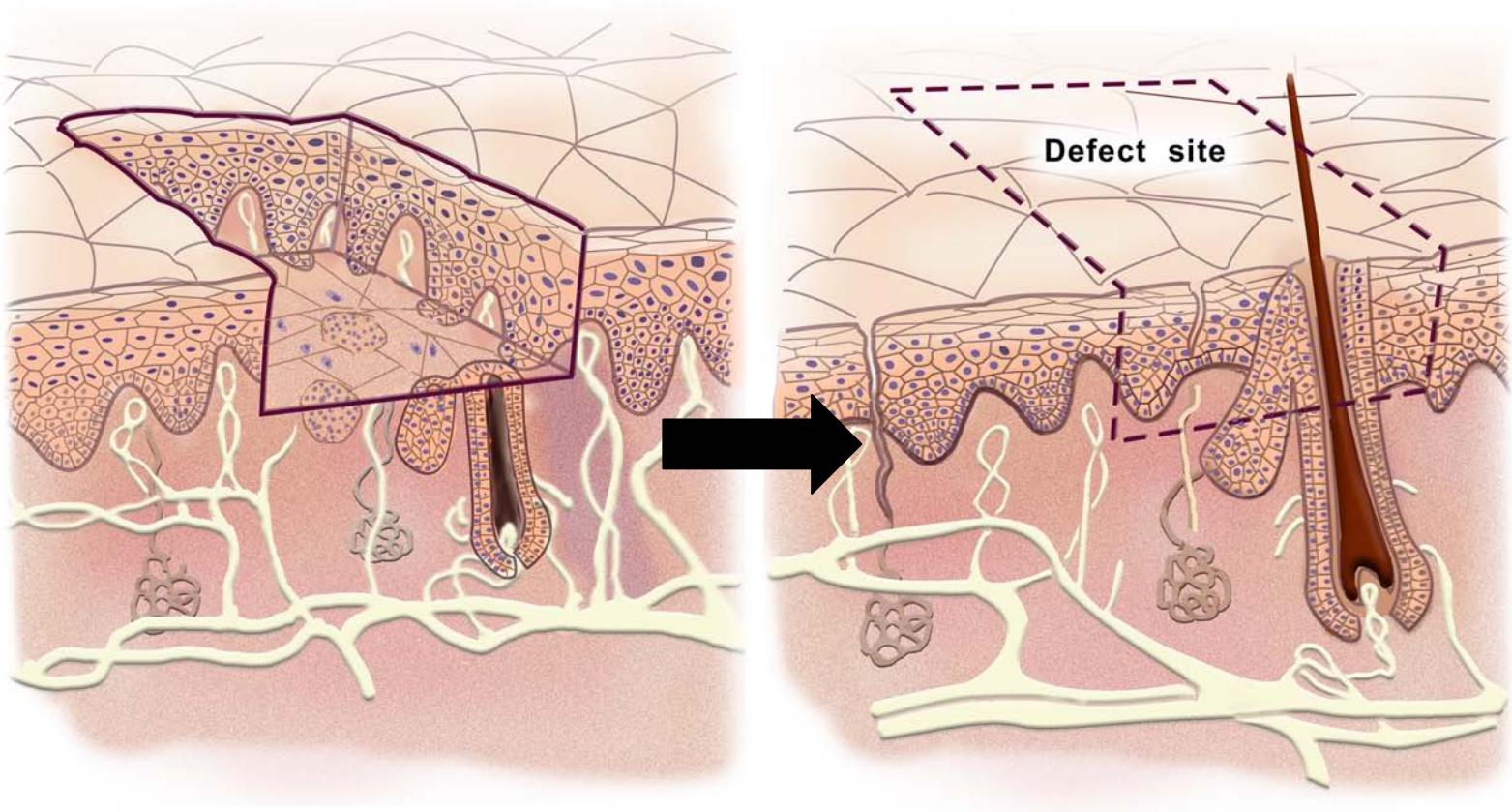


Regenerative similarity of tissues in skin and nerves

TABLE 2.1. Regeneratively similar tissues in skin and peripheral nerves.

Skin	Peripheral nerves
A. Regenerative tissues	
epidermis	myelin sheath
basement membrane	basement membrane (perineurium, in part only)
B. Nonregenerative tissues	
dermis	endoneurial stroma

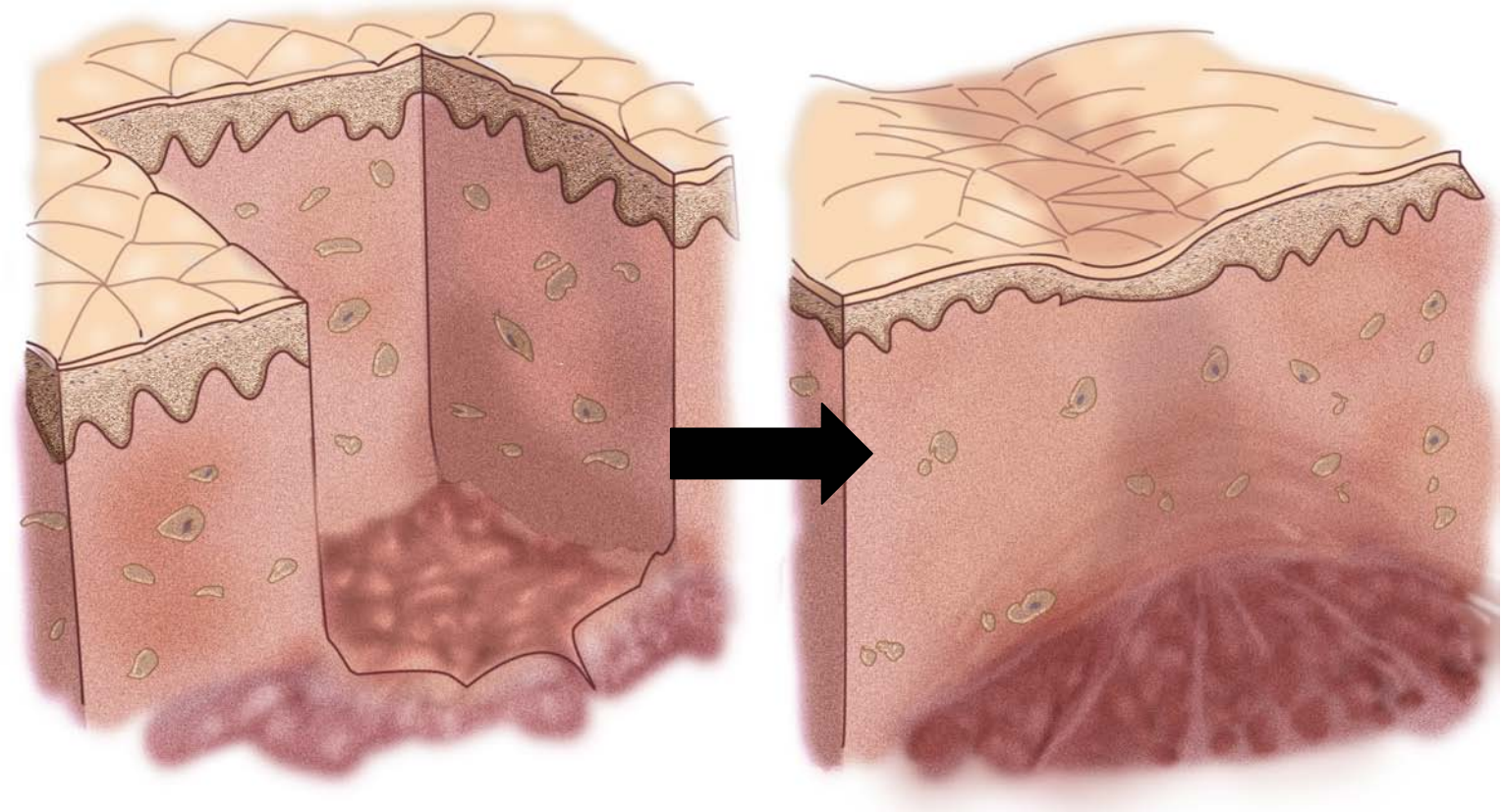
Skin



The epidermis is a regenerative tissue. After excision, it regenerates spontaneously. Reversible injury. No contraction.

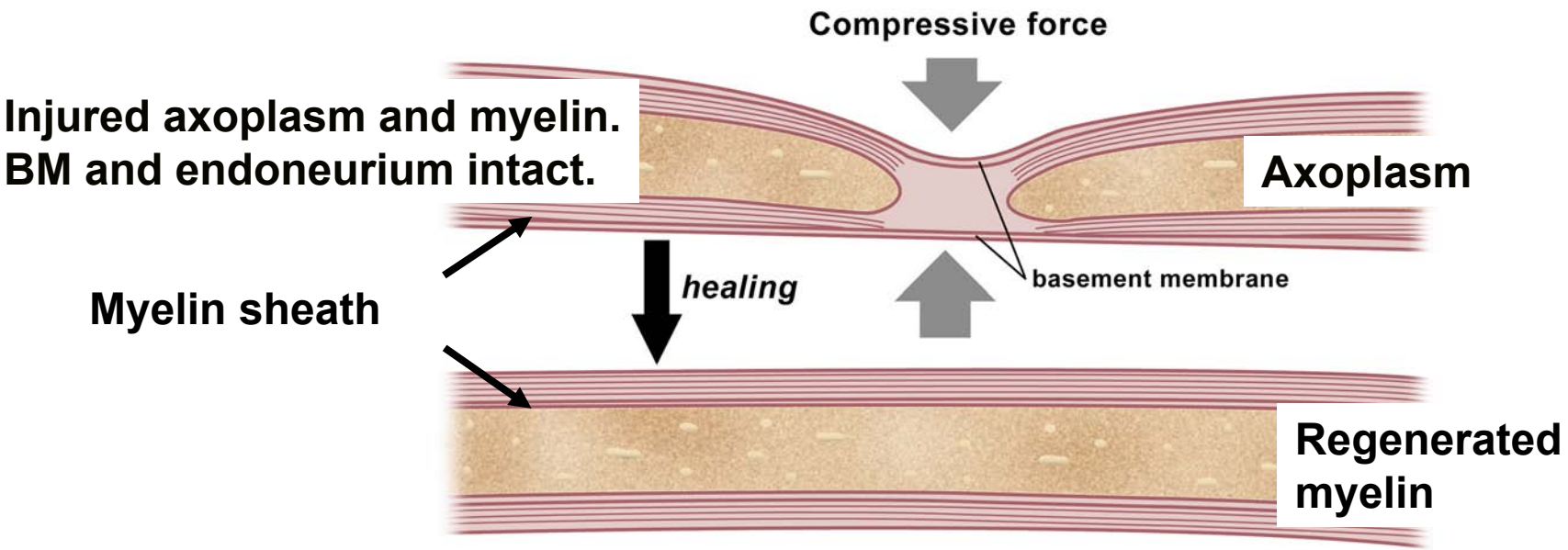
Skin

spontaneous healing of full-thickness skin excision by contraction and scar formation

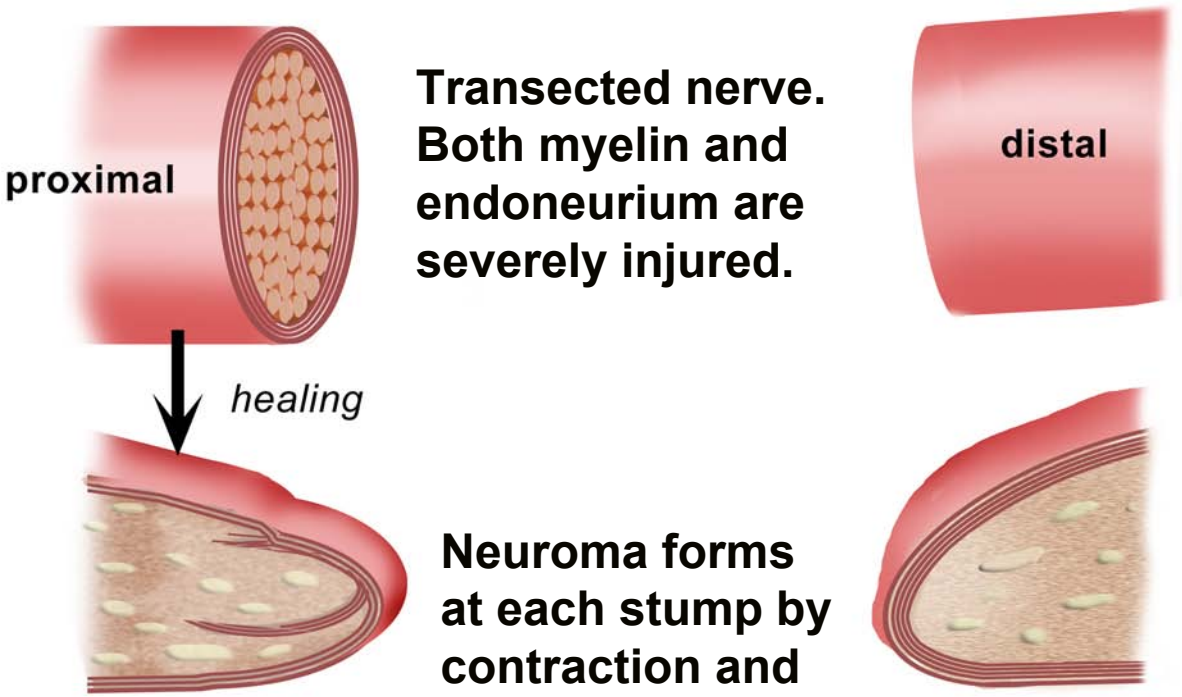


The dermis is a nonregenerative tissue in the adult. After excision, it does not regenerate spontaneously. Irreversible injury. Contraction occurs with scar formation.

The injured myelin sheath regenerates spontaneously



Neuroma formation. The endoneurium does not regenerate.



Transected nerve. Both myelin and endoneurium are severely injured.

Neuroma forms at each stump by contraction and scar formation.

Intact nerve fiber

healing

Spontaneously healed nerve fiber

Image removed due to copyright considerations.
See Figure 2.5 in [Yannas].

2. Experimental parameters (cont.)

C. Assays of configuration

- Correction for experimental gap length.
- Correction for animal species.
- Critical axon elongation, L_c. Relation to defect closure rule.
- Shift length, ΔL. Characterization of devices.
- Long-term: fidelity of regeneration.

C. Assays of configuration (cont.)

Use corrected values of frequency of reinnervation (%N) across tubulated gaps. This correction allows comparison of %N data from studies with different gap lengths and different species.

Critical axon elongation, L_c, the gap length above which %N drops below 50% (or the gap length where the odds of reinnervation are even). Data from several investigators have shown that $L_c = 9.7 \pm 1.8$ mm for the rat sciatic nerve and 5.4 ± 1.0 mm for the mouse sciatic nerve.

Characteristic curve defines critical axon elongation, L_c, at %N = 50%

$L_c = 9.7 \pm 1.8$ mm for the rat sciatic nerve and 5.4 ± 1.0 mm for the mouse sciatic nerve

Image removed due to copyright considerations.
See Figure 6.1 in [Yannas].

Data from rat and mouse superpose when plotted vs. reduced length, L/L_c

Use single data point to determine L_c for unknown device

Image removed due to copyright considerations.
See Figure A.1 in [Yannas].

Relation between L_c, ΔL and C, S, R terms in defect closure rule

Image removed due to copyright considerations.
See Table 6.2 in [Yannas].

2A. Synthesis of myelinated axons

[NB: Neuron in culture provides spontaneous outgrowth of axons that serve as "substrate" for synthesis of myelin and BM. Schwann cells also obtained in culture from a neuron.]

A myelin sheath around axons has been synthesized in vitro in the presence of Schwann cells, with or without presence of an ECM component.

2B. Synthesis of nerve BM

A BM has been synthesized in vitro in presence of neurons and Schwann cells.

However, neurons were not required to be present when fibroblasts were cultured with Schwann cells.

Even fibroblasts not required when laminin added to neuron-free Schwann cell culture.

3. Evidence (?) for synthesis of an endoneurium

Structure. Endoneurial microenvironment surrounding each nerve fiber comprises blood vessels coursing through space filled with fluid and thin collagen fibers (51-56 nm diam.). Fluid outside blood vessels is maintained under small, positive hydrostatic pressure. Endoneurial blood vessels comprise cells that are bound by tight junctions and constitute a permeability barrier.

Function. Endoneurial environment protects nerve fibers from changes in ionic strength and from pathogens in blood vessels that might modify conductivity ("blood-nerve barrier").

Endoneurium

Image removed due to copyright considerations.
See Figure 6.2 (top) in [Yannas].

Evidence (?) for synthesis of endoneurium (cont.)

In vitro. No evidence for synthesis of endoneurial stroma.

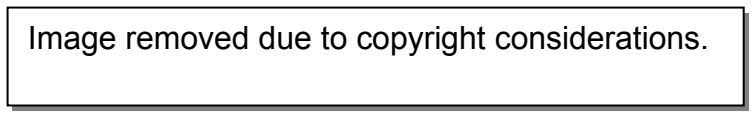
In vivo. Nerve trunks have been synthesized but endoneurial structures have rarely been studied. Is endoneurium present? Using silicone tube to bridge 10 mm gap between stumps did not yield a functional endoneurium. Occasionally, collagen fibers, 25-35 nm, or even 40 nm, reported outside BM, were smaller than endoneurial collagen fibers.

5. Synthesis of a nerve trunk (including kinetics)

Structure. A nerve trunk comprises one or more fascicles. Each fascicle comprises several thousand nerve fibers. If monofascicular, it is covered by perineurium; if multifascicular, it is covered by epineurium. A fascicle comprises the perineurium with its bundle of thousands of nerve fibers. Some nerves comprise many fascicles, each with its own perineurial sheath; these fascicles are wrapped in a collagenous tissue, the epineurium.

Function. Conducts strong nerve signals (about 10 mV) at 70 m/s.

Tubulation model.
Gap length variable.



Tubulation model.
Gap length variable.

Image removed due to copyright considerations. Diagram of implant configuration.

A look inside the gap

Image removed due to copyright considerations.
See Figure 10.6 in [Yannas].

sequence:
Schwann cells +
Fibroblasts >
Nonmyel.
Axons >
Blood vessels >
Myel. axons

axon elongation→

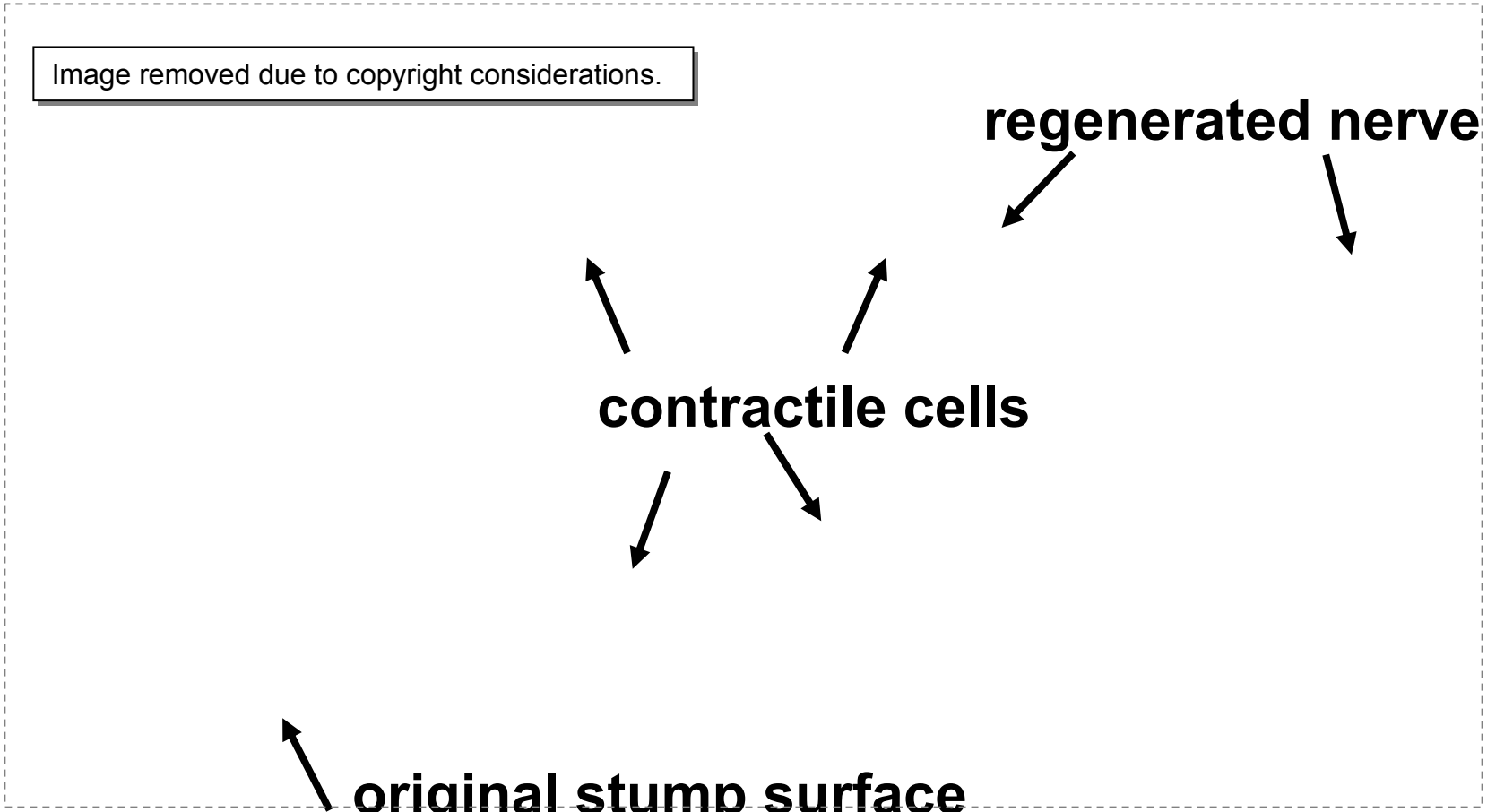
proximal stump

distal stump

Columns of Schwann cells form even in absence of axons

Image removed due to copyright considerations.
See Figure 10.8 in [Yannas].

Contractile cell zone surrounds regenerating nerve



Spilker and Seog, 2000

Cell capsule around regenerated nerves

4-mm gap

Normal rat sciatic nerve

Image removed due to copyright considerations.
See Figure 10.7 in [Yannas].

8-mm gap

Regenerated across 0-mm gap

8 weeks

Regenerated nerves typically comprise several minifascicles

Image removed due to copyright considerations.
See Figure 6.4 in [Yannas].

39 weeks

article by Chamberlain et al. handed out

Chamberlain, L.J. et al. “Collagen-GAG Substrates Enhances the Quality of Nerve Regeneration through Collagen Tubes up to Level of Autograft.” Experimental Neurology 154: 315-329 (1998)

KINETICS OF NERVE SYNTHESIS

30 weeks

Image removed due to copyright considerations.
See Figure 6.5 in [Yannas].

60 weeks

Normal

Electrophysiological behavior of normal (light line) and regenerated nerve (dark line)

Regenerated nerve is weaker (lower peak amplitude) and slower (delayed peaking)

Y-axis: Amplitude (strength) of transmitted electric signal
X-axis: Time following stimulation (at 0 ms)

Image removed due to copyright considerations.
See Figure 6.6 in [Yannas].

6. Comparative Rregenerative activity of various devices
(Table 6.1, pp. 147-8)

What does each of these device features contribute to the quality of regeneration? Compare values of L_c and ΔL.

- **Tubulation**
- **Tube wall composition**
- **Tube wall permeability**
- **Fillings: Schwann cells, solutions of proteins, gels based on ECM components, insoluble substrates**

Device components

Tubulation

Tube wall

Image removed due to copyright considerations.
See Table 6.1 in [Yannas].

Tube wall permeability

Filling: Schwann cells

Filling: protein solutions

Filling: ECM-based gels

Filling: insoluble substrates

PNS regeneration. Length shift, ΔL, measures regenerative advantage of device relative to silicone tube standard. e.g., $\Delta L > 0$ is better than standard.

Filling: insoluble substrates

Image removed due to copyright considerations.
See Table 6.1 in [Yannas].

PNS regeneration (cont.).

Length shift, ΔL, measures regenerative advantage of device relative to silicone tube standard.

e.g., $\Delta L > 0$ is better than standard.

Tubulation. Tube wall composition and permeability

- **Bridging the two stumps with a tube, almost any kind of tube, greatly improves quality of regeneration.**
- **Tube wall composition is critically important. Silicone tubes are greatly inferior to collagen tubes.**
- **Increase of cell (but not protein) permeability improved quality.**

Silicone tube

Partly regenerated rat sciatic nerve. Tubulated in silicone tube.

Image removed due to copyright considerations.
See Figure 4.5 in [Yannas].

cross-section shows thick sheath of contractile cells

Silicone tube

Contractile cells (brown) ensheathe regenerating stump of transected rat sciatic nerve

Image removed due to copyright considerations.
See Figure 4.6 in [Yannas].

near original proximal stump

near original distal stump

Tube fillings

- **Schwann cells, growth factors (aFGF and bFGF) and several insoluble substrates increased quality of regeneration, sometimes greatly.**
- **NGF had no effect.**
- **Gels based on ECM components (collagen, fibronectin, laminin) had no effective or impeded regeneration.**

Regeneration across a 10 mm gap bridged by a silicone tube

filled

Image removed due to copyright considerations.

See Fig. 10 in Yannas, I. V. "Biologically Active Analogues of the Extracellular Matrix: Artificial Skin and Nerves."
Angew. Chem. Int. Ed. Engl. 29 (1990) 20-35.

unfilled

Effect of degradation rate of tube filling based on a porous ECM analog (NRT)

- **Undegraded ECM analog physically impeded axon elongation.**
- **Optimal quality of regeneration obtained with ECM anlog that degraded at an intermediate rate.**

Effect of pore diameter and degradation rate on inverse conduction velocity (latency)

Image removed due to copyright considerations.
See Figure 10.9 in [Yannas].

Structural features of ECM analogs used as tube fillings in nerve regeneration

1. pore structure (ligand density)

Image removed due to copyright considerations.

Image removed due to copyright considerations.

2. macromolecular structure (ligand duration)

3. chemical composition (ligand identity)

4. orientation of pore channel axes

Long-term electro-physiological properties of various regenerated nerves

Image removed due to copyright considerations.
See Tables 6.1 and 6.3 in [Yannas].

Summary of results

- **Tube presence was essential**
- **Tube wall composition: collagen > degradable synthetic polymer > silicone.**
- **Tube wall permeability: cell-permeable > protein permeable > impermeable.**
- **Tube fillings:**

  --- suspensions of Schwann cells

  --- solution of either aFGF or bFGF (not NGF!)

  --- crosslinked ECM networks > ECM gels

  --- thin polymeric filaments oriented along tube axis

  --- highly porous, insoluble ECM analogs with appropriately small pore diameter, axial orientation of pore channel axes and critically adjusted degradation rate.