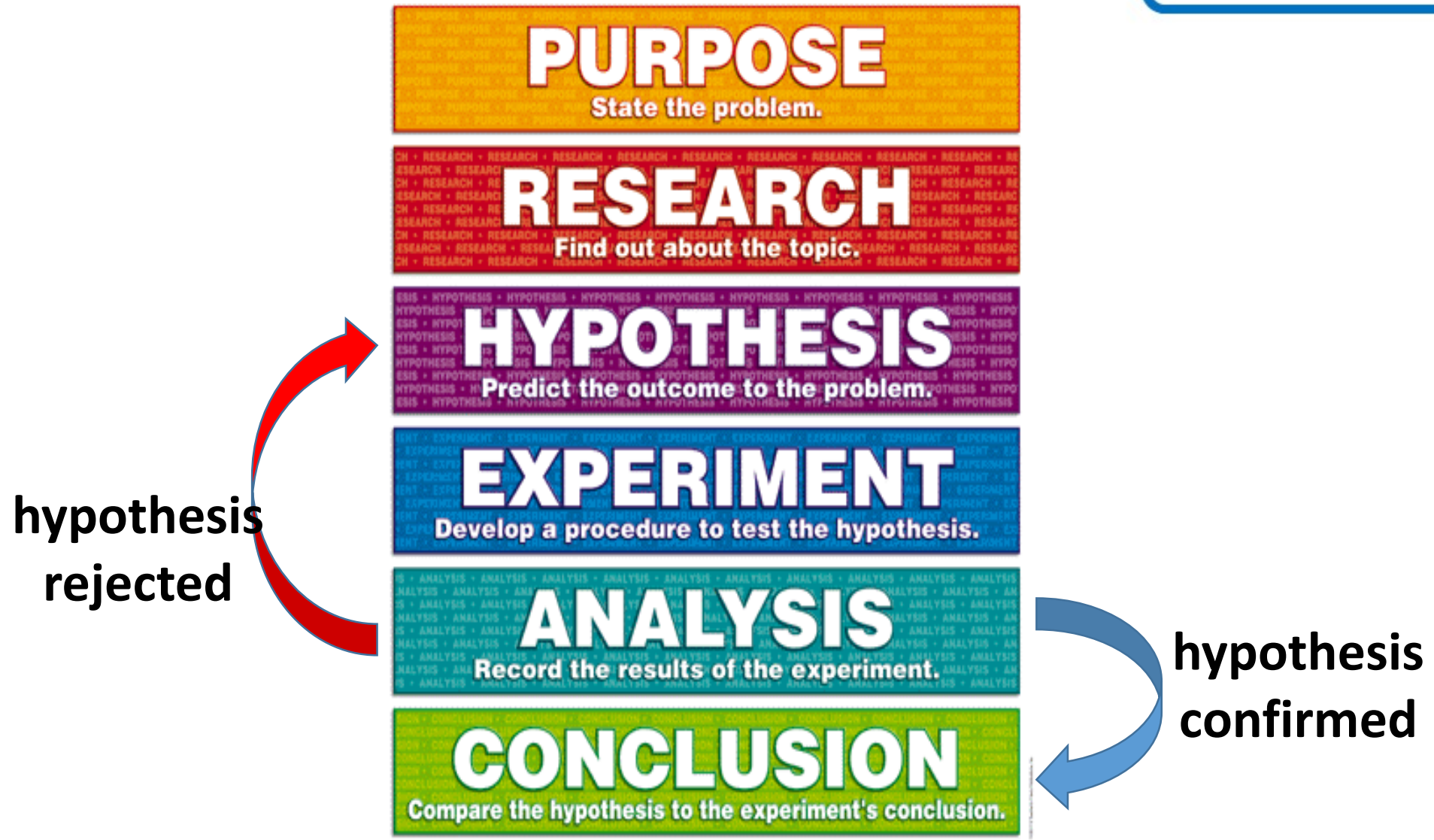
UNIVERSITÀ
DEGLI STUDI DI TRIESTE

Techniques in cellular neurobiology

Lesson 1

Gabriele Baj
gbaj@units.it

SCIENTIFIC METHOD



hypothesis rejected

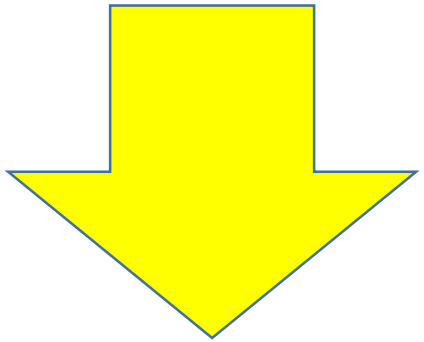
hypothesis confirmed

PURPOSE

State the problem.

UNIVERSITÀ
DEGLI STUDI DI TRIESTE

How it works?



characterization at molecular level

PURPOSE

State the problem.

UNIVERSITÀ
DEGLI STUDI DI TRIESTE

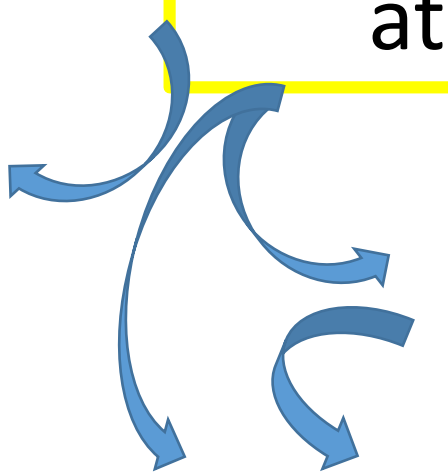
Pathology cure

Therapy

pharmacological target

What's Wrong??
pathology characterization
at molecular level

target identification



Scientific Method

UNIVERSITÀ
DEGLI STUDI DI TRIESTE

Problem/Question

Observation/Research

Formulate a Hypothesis

Experiment

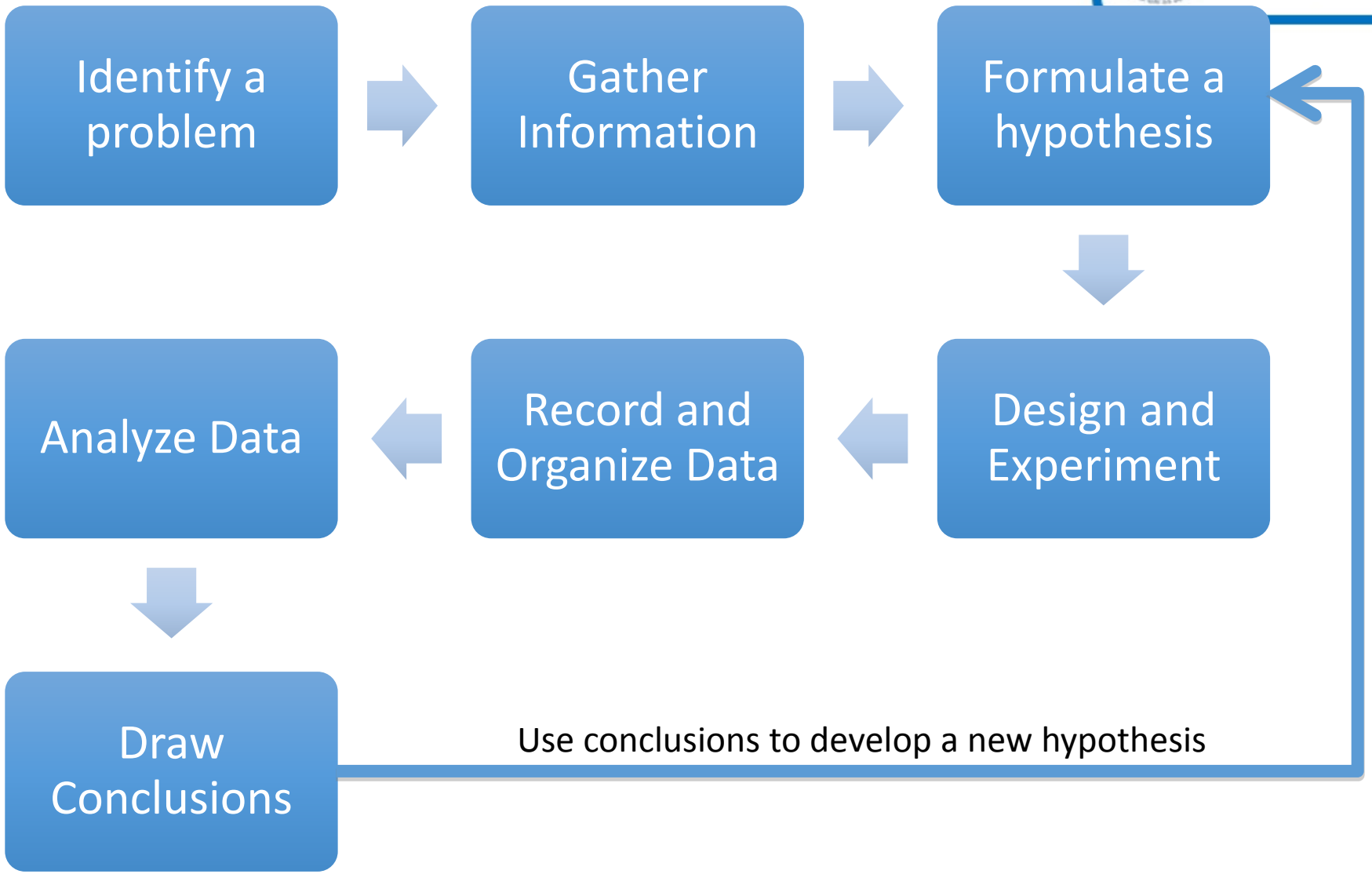
Collect and Analyze Results

Conclusion

Communicate the Results

Introduction to the Scientific Process

UNIVERSITÀ DEGLI STUDI DI TRIESTE



General Layout for an Experimental Design Diagram

Title

The Effect of _____ (Independent Variable)

on _____ (Dependent Variables)

Hypothesis

If _____ (planned change in independent variable),

then _____ (predicted change in dependent variables).

Independent Variable

_____

Levels of Independent Variable and Numbers of Repeated Trials

Level 1 (Control)	Level 2	Level 3	Level 4
Number of trials	Number of trials	Number of trials	Number of trials

Dependent Variable and How Measured

_____

Constants

1.

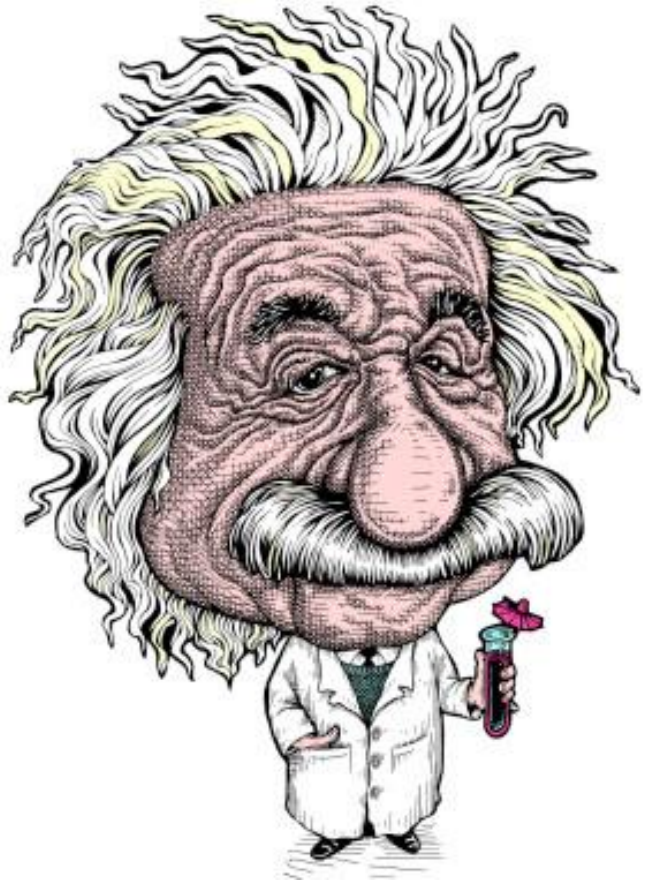
2.

3.

4.

Do you know the difference between the independent and dependent variables?

UNIVERSITÀ
DEGLI STUDI DI TRIESTE



Review

Variable: the one thing you change in an experiment.	Data: the information you get when you test the variable.	Controls: the parts of the experiment that stay the same.
Manipulated variable: you change this yourself.	Responding variable: responds to the change you made.	Controlling variables
Independent variable: on its own.	Dependant variable: depends on the change you made.	Controlling variables

Control Group

In a scientific experiment, the control is the group that serves as the standard of comparison.

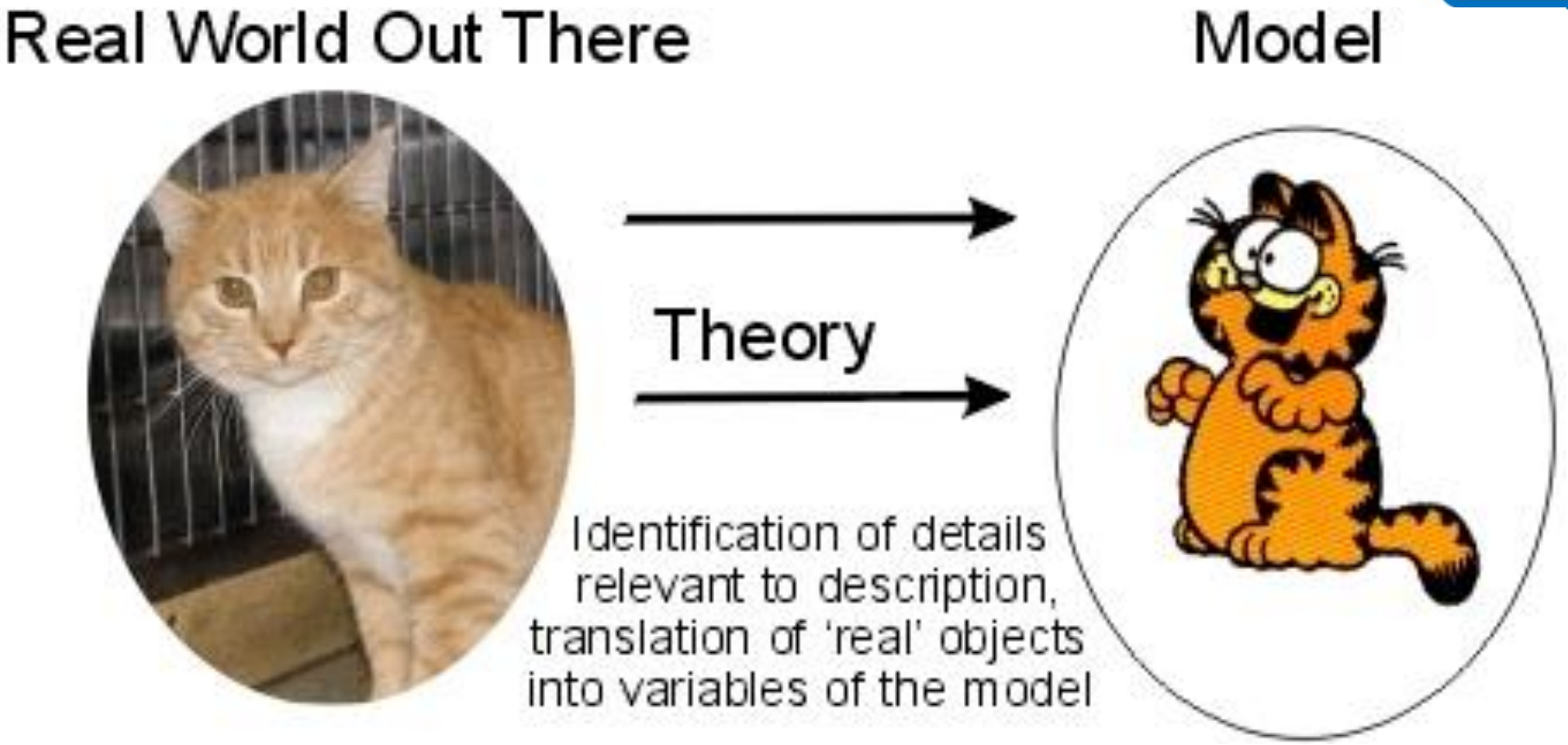
The control group may be a “no treatment" or an “experimenter selected” group.

Control Group

The control group is exposed to the same conditions as the experimental group, except for the variable being tested.

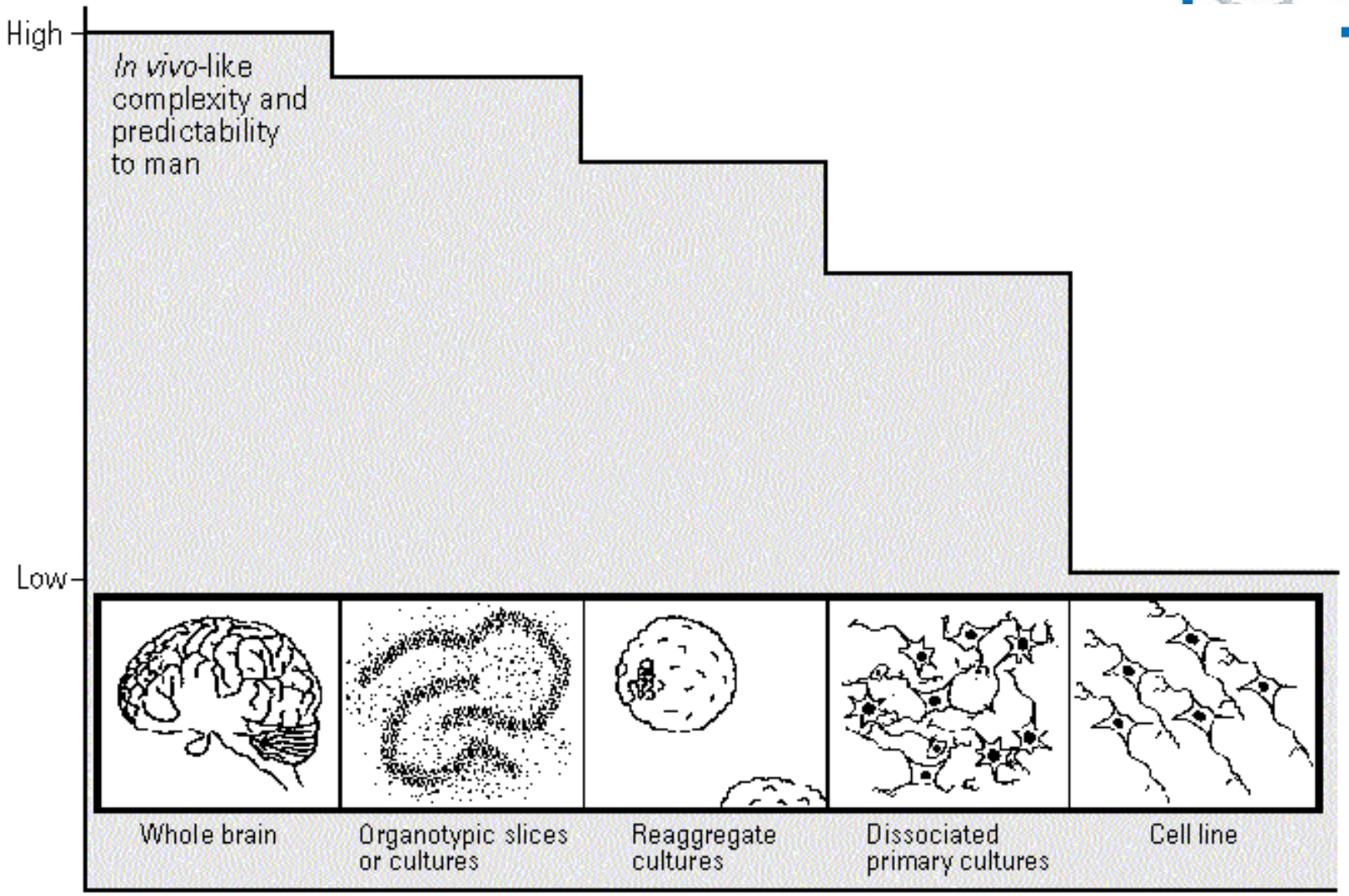
All experiments should have a control group.

Scientific MODEL



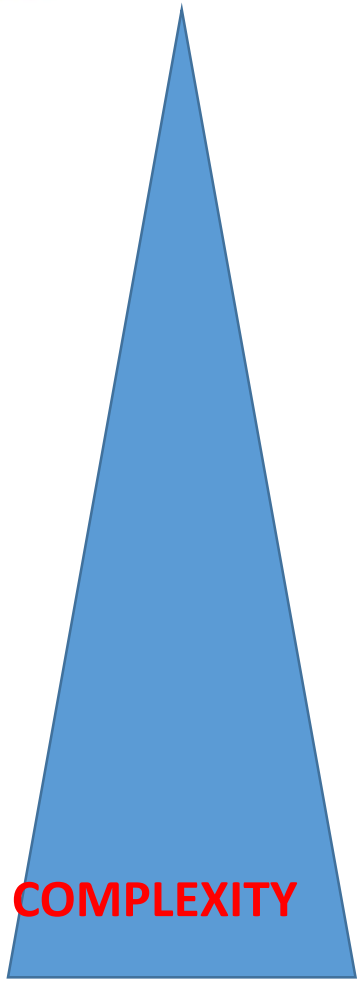
The model is a simplified version of the real world out there, simplified in the sense that it deals only with a limited amount of details.

MODELs IN NEUROBIOLOGY



Classification of tissue cultures based on the origin of the cells

- Established (transformed) cell lines
- Extended culture (multipassage culture) – cell strain
- Primary culture (directly from animal tissue)
- Organotypic cultures
- Animal Model



Classification of tissue cultures based on the origin of the cells

Cultured Slice

Cut slices → Place in culture medium → Feed → Run experiment

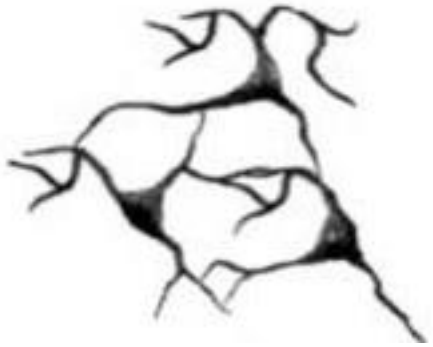
Acutely Dissociated Neurons

Dissect tissue → Dissociate tissue → Run experiment

Dissociated Cultured Neurons

Dissect tissue → Dissociate tissue → Place in culture medium → Feed → Run experiment

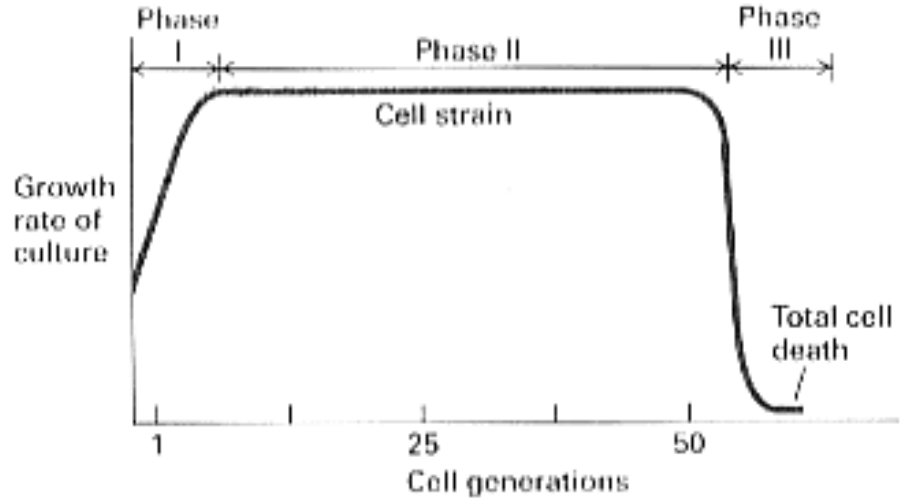
0 — 1 hour — 1 day — 1–4 weeks



Primary tissue culture

- A culture derived directly from a tissue

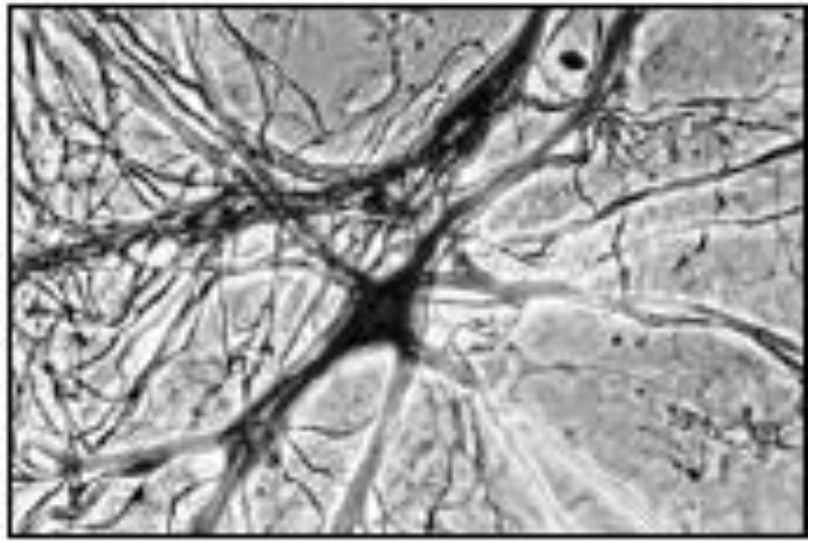
– A stage from cell isolation to first subculturing



- Best resembling natural tissue
- Limited growth potential
- Limited life span
- May give rise to a cell strain or be immortalized
- Strain – a lineage of cells originating from one primary culture

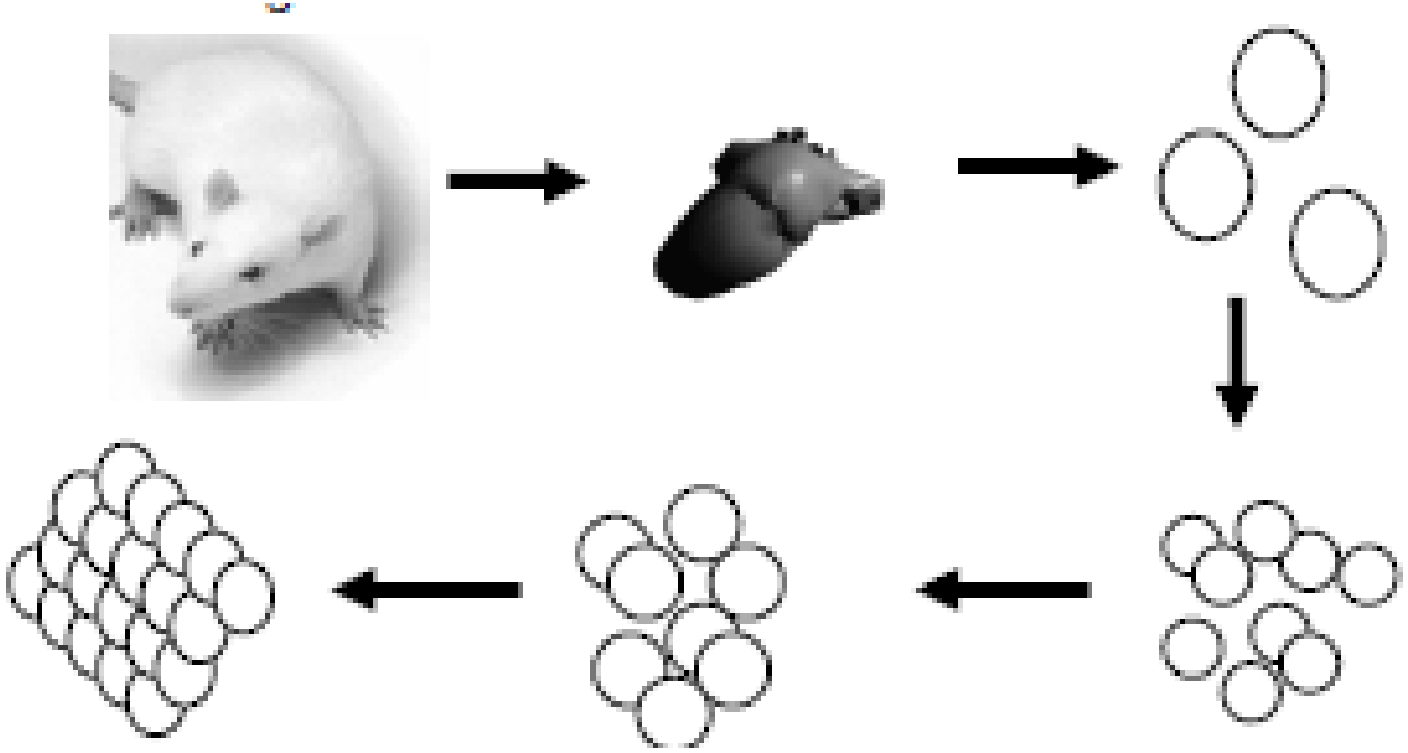
Survival times of primary neuronal cells in culture

- Spinal cord
- Cortex
- Olfactory bulb
- Cerebellum
- Hippocampus

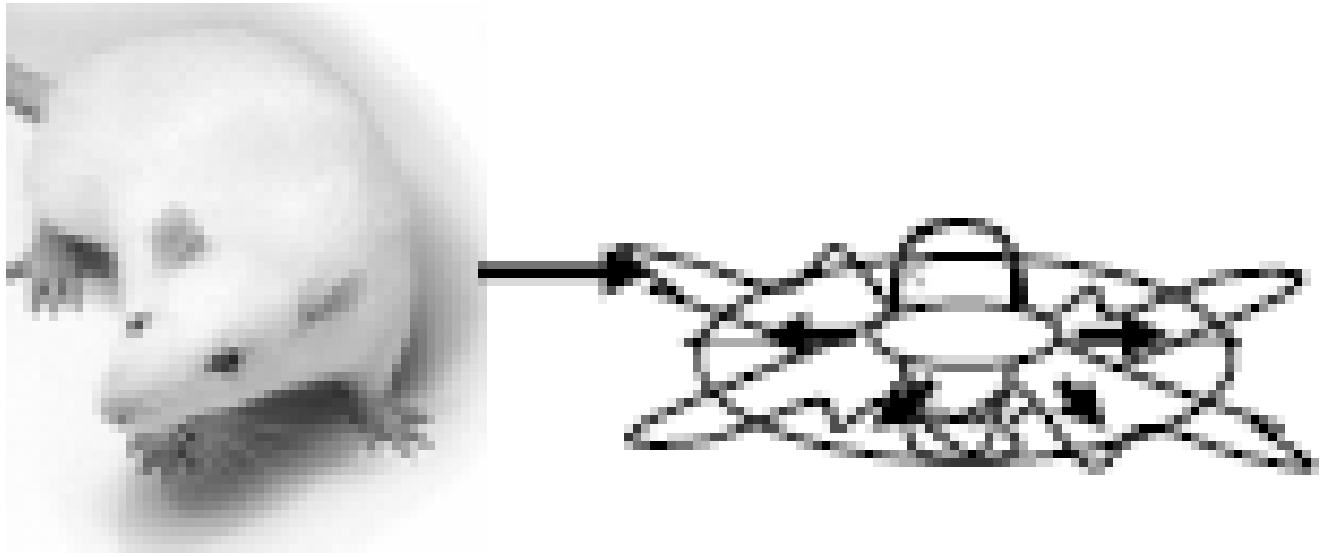


Steps in primary tissue culture

- Isolation of tissue
- Disaggregation of cells – initiation of culture
- Incubation and growth



Explant culture



Explant culture

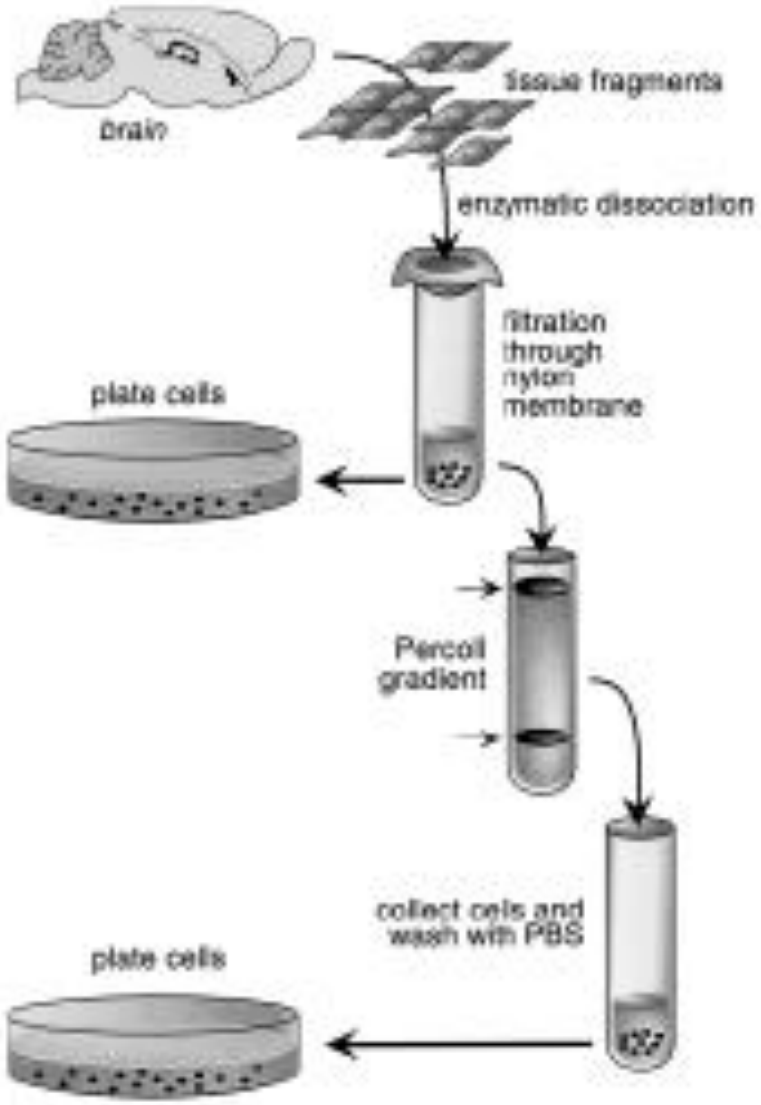


Fig. 1. Schematic diagram of the protocol for isolation of stem cells from adult brain. Tissue pieces from brains are subjected to enzymatic digestion, debris removed and the cell suspension is plated. Alternatively, stem cells can be partially purified by density gradient centrifugation and then plated.

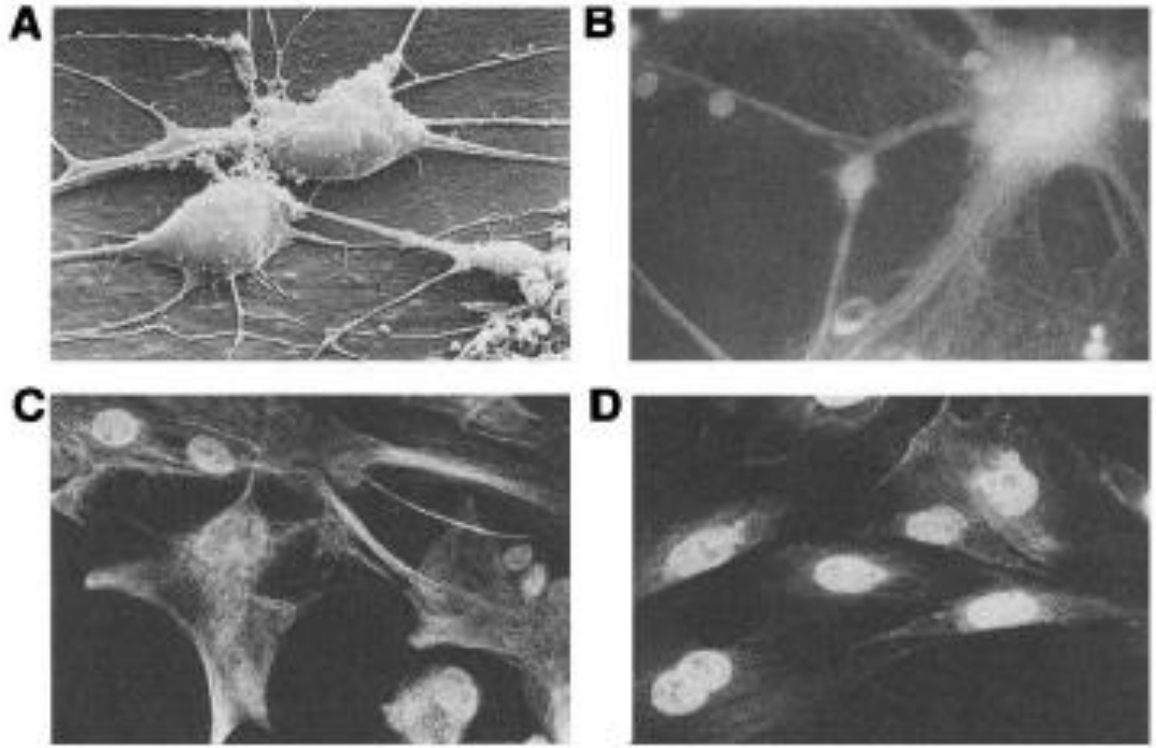
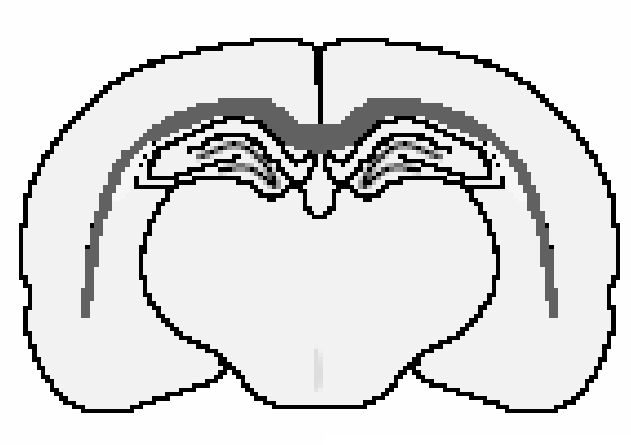


Fig. 11. Photomicrographs of neuronal and glial cells in primary culture from rat brain. **A.** Scanning electromicrograph of neuronal cells in culture. **B.** Immunofluorescent staining of neuronal cultures with antibody against neurofilament (DA_2B1). **C.** Immunofluorescent staining of glial culture from 1-d-old rat brain with antiglial fibrillary acidic protein (GFAP). **D.** Immunofluorescent staining of glial cultures from 21-d-old rat brain with anti-GFAP.

METHODS

UNIVERSITÀ
DEGLI STUDI DI TRIESTE

- Hippcamapl primary neurons from rats embrio 18 days old. A culture model of dendritic development starting from purified neurons



- Organotipyc cultures (400μm thick),Hippocampal slice from P7 rats are chosen as a good *ex-vivo* model to study *the in-vivo* dendritic re-organisation

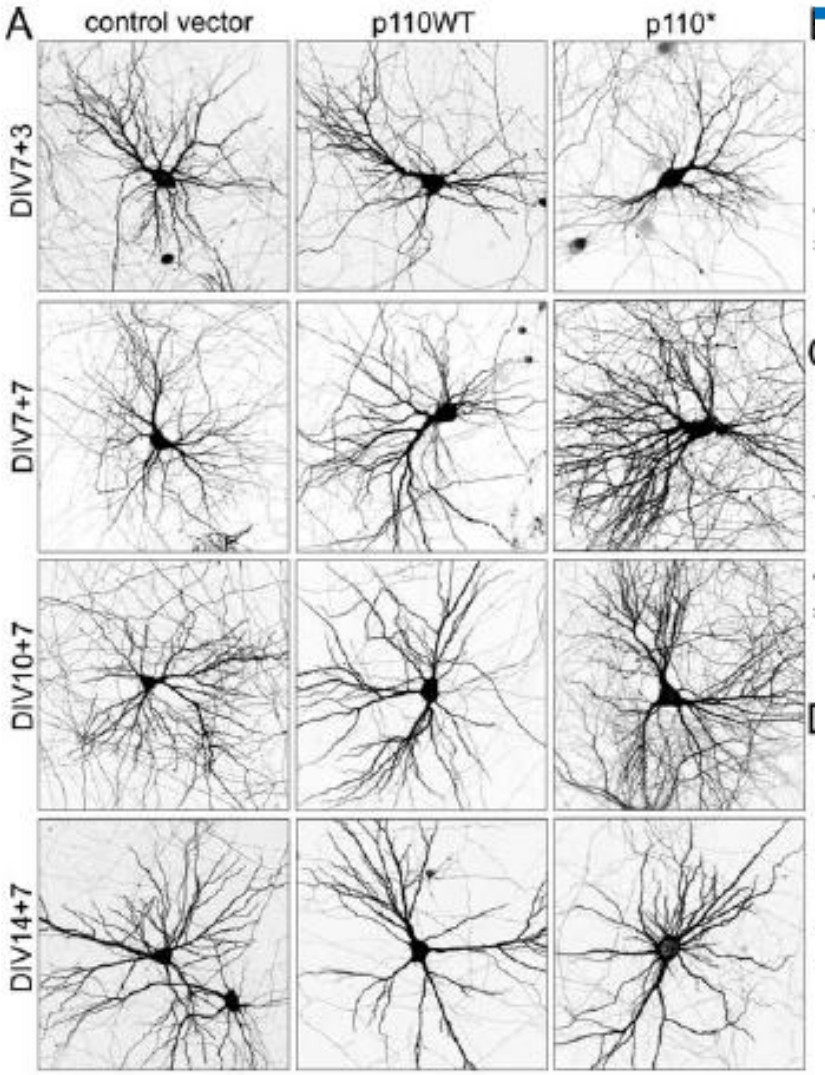
RESULTS

Activation of PI3K/AKT pathway

Representative micrographs of hippocampal neurons transfected with control vector or wild-type p110 or constitutively active p110*.

Neuron morphology was visualized by cotransfected GFP. Age and duration of transfection are indicated (e.g., DIV 7 + 3 indicates transfected at 7 DIV and expressed for 3 d).

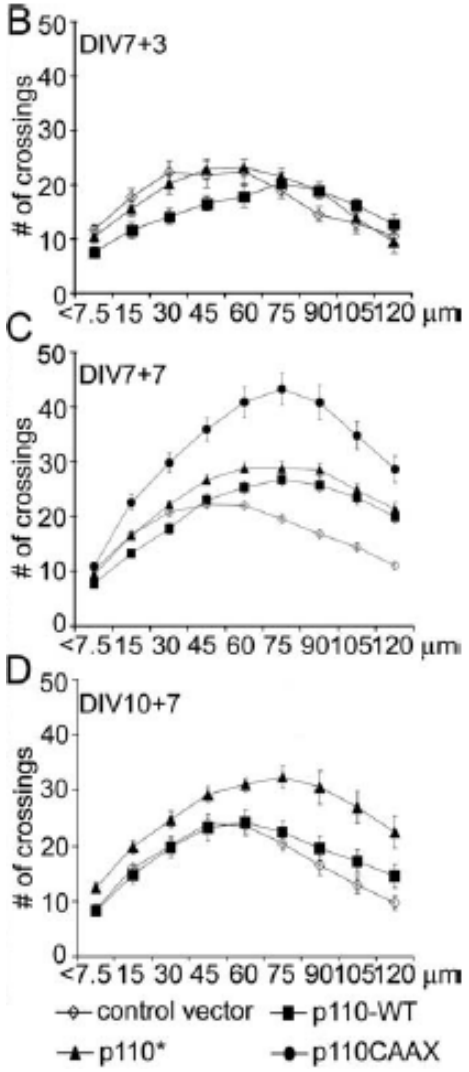
P110* and CAAX ras-IP3-dep. constitutively active



RESULTS

UNIVERSITÀ
DEGLI STUDI DI TRIESTE

Activation of PI3K/AKT pathway

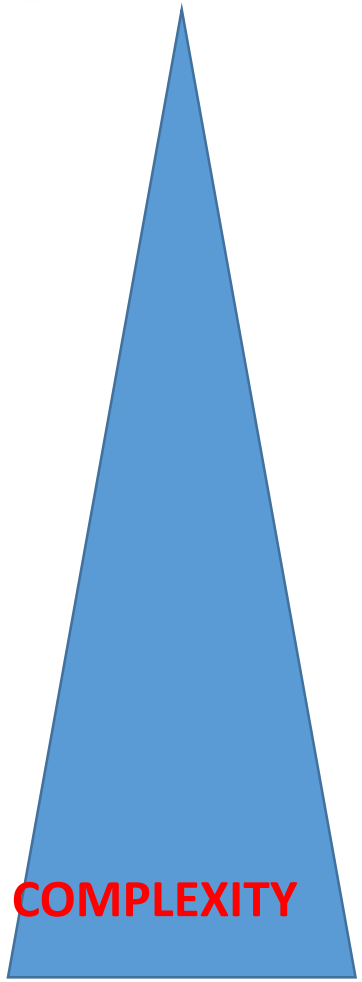


Sholl analysis of neurons transfected with wild-type or active p110 mutants or control vector at various DIV as indicated.

The horizontal axis of the Sholl plot indicates the distance from the cell soma.

Classification of tissue cultures based on the origin of the cells

- Established (transformed) cell lines
- Extended culture (multipassage culture) – cell strain
- Primary culture (directly from animal tissue)
- Organotypic cultures
- Animal Model



Cell strain

(i) the scientific method,

(ii) signaling processes involved in cellular differentiation, and

(iii) the use of pharmacological agents to manipulate a cell culture system.

Cell strains

Advantages	Disadvantages
Controlled physiochemical environment (pH, temperature, oxygen, carbon dioxide, osmotic pressure, etc.)	Expertise is needed
Controlled and defined physiological conditions (constitution of medium, etc.)	10 times more cheap for the same quantity of animal tissue
Homogeneity of cell types (achieved through serial passages)	Unstable aneuploid chromosome constitution
Economical, since smaller quantities of reagents are needed than in vitro	Requirement of controlled and defined physiological conditions

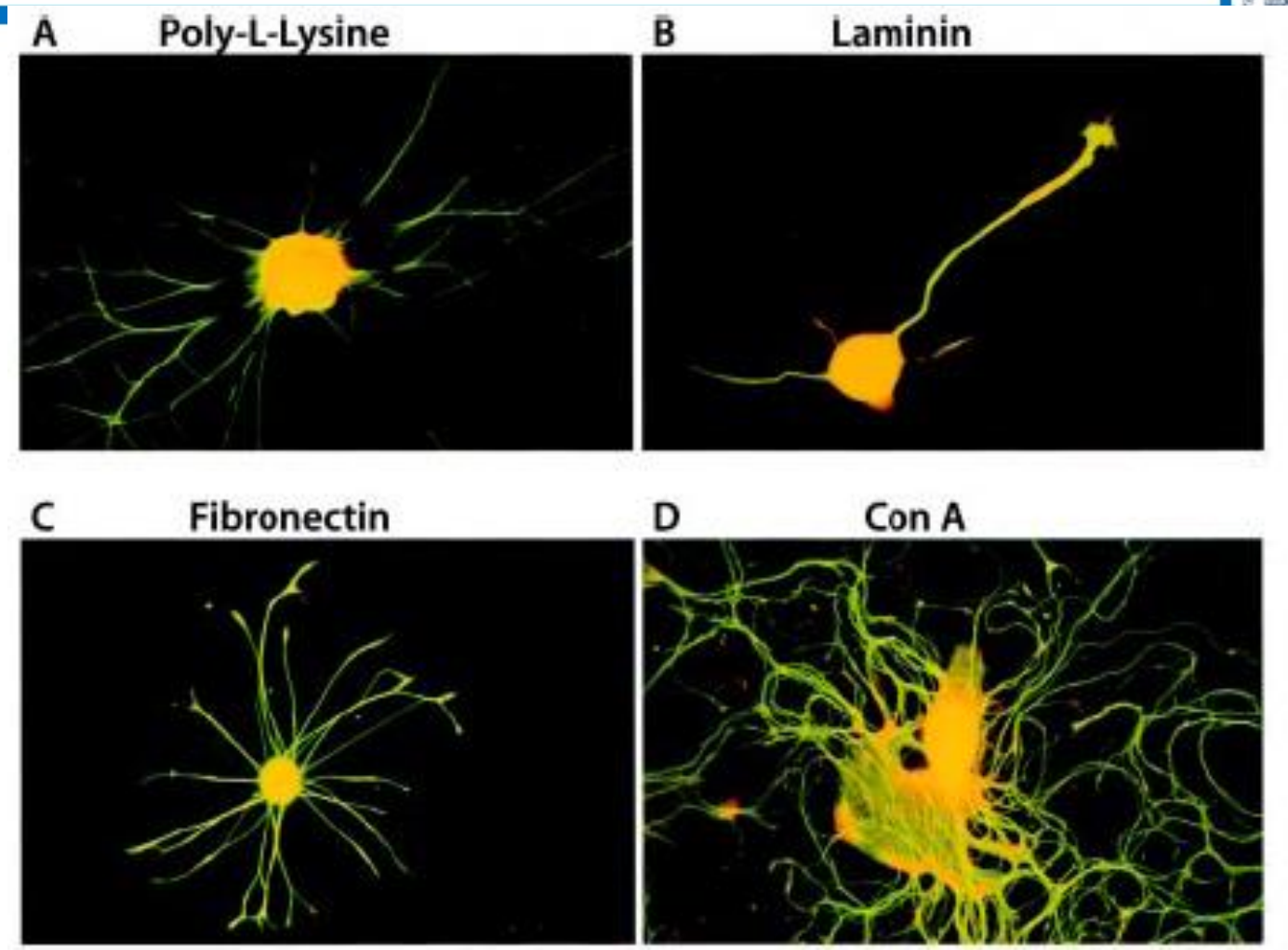
Advantages and Disadvantages of Primary cells and cell Lines

UNIVERSITÀ DEGLI STUDI DI TRIESTE

Features	Primary cells	Cell Lines
1. Ploidy	Diploid Euploid	Heteroploid Aneuploid
2. Transformation	Normal	Transformed
3. Tomourigenicity	Non-tumourigenic	No Tumourigenic
4. Anchorage Dependence	Yes	No
5. Contact Inhibition	Yes	No
6. Density Limitation of Growth	Yes	No

Features	Primary cells	Cell lines
7. Mode of Growth	Monolayer	Monolayer of Suspension.
8. Maintenance	Cyclic	Steady State Possible.
9. Serum Requirement	High	Low
10. Cloning Efficiency	Low	High
11. Markers	May be tissue specific	Chromosomal, enzymic
12.Special Functions	May be retained	Often lost
13. Growth Rate	Slow	Rapid
14. Yield	Low	High
15. Control Features.	Generation Number in vivo markers	Strain Characteristics

Primary cells



Primary cells

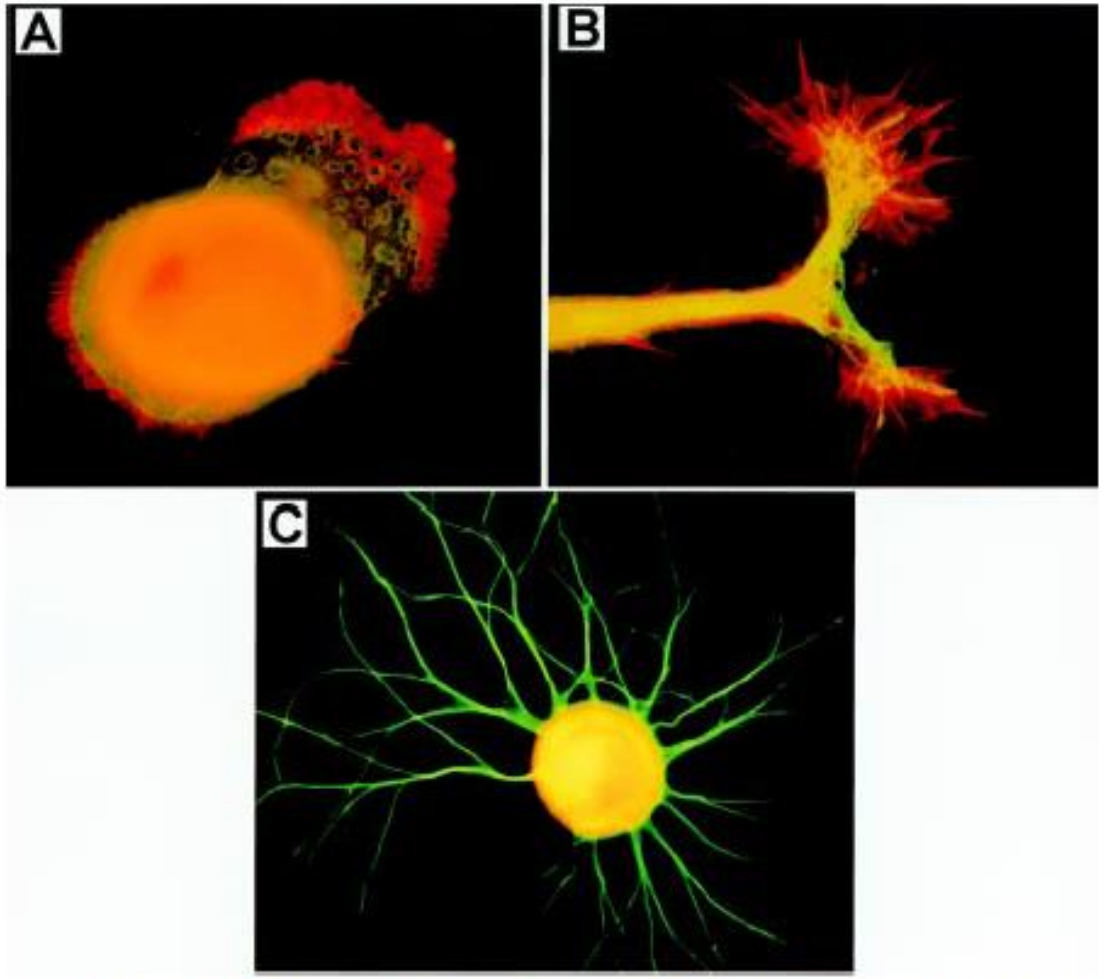


Fig. 4. Actin and microtubular organization at various stages of neurite outgrowth. A) During early sprouting, actin (*red*) is most prominent at the peripheral domain. Note that at this time point (1–2hrs of culture), microtubules (*green*) are not well organized. B) Actin dominates the peripheral domain of the growth cones, whereas microtubules are now well organized within the neurite (*green/yellow* – 12–18hrs *in vitro*). C) After 24hrs in cell culture, microtubules make up the core of cytoskeletal elements.

Primary cells

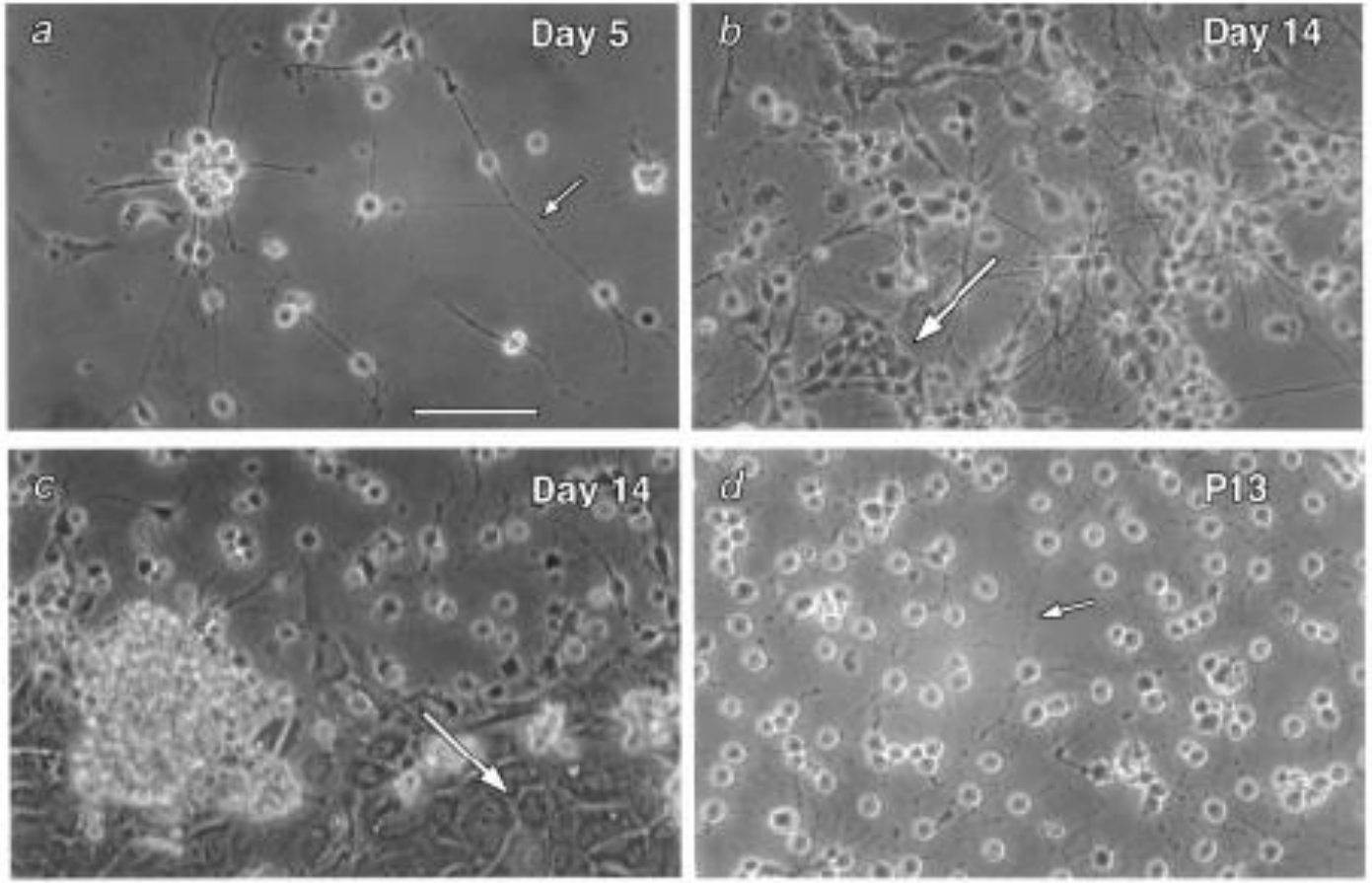


Fig. 2. Morphology of adult rat neural stem cells cultured in serum-free N2 medium containing FGF-2. (*a*) Proliferating cells can be seen by 3–5 days in vitro (DIV). Stem cells have small phase bright cell bodies and two or more long processes (small arrows in *a*, *d*). (*b*) By 14 DIV, a large number of stem cells are present. (*c*) The cultures also contain flat cells (indicated by long arrows) which do not stain for any stem or precursor cell markers. Small phase bright cells seem to generate on top of these flat cells. (*d*) Mostly stem cells are present in the passaged cultures. Scale bar: 100μm

Neural stem cells

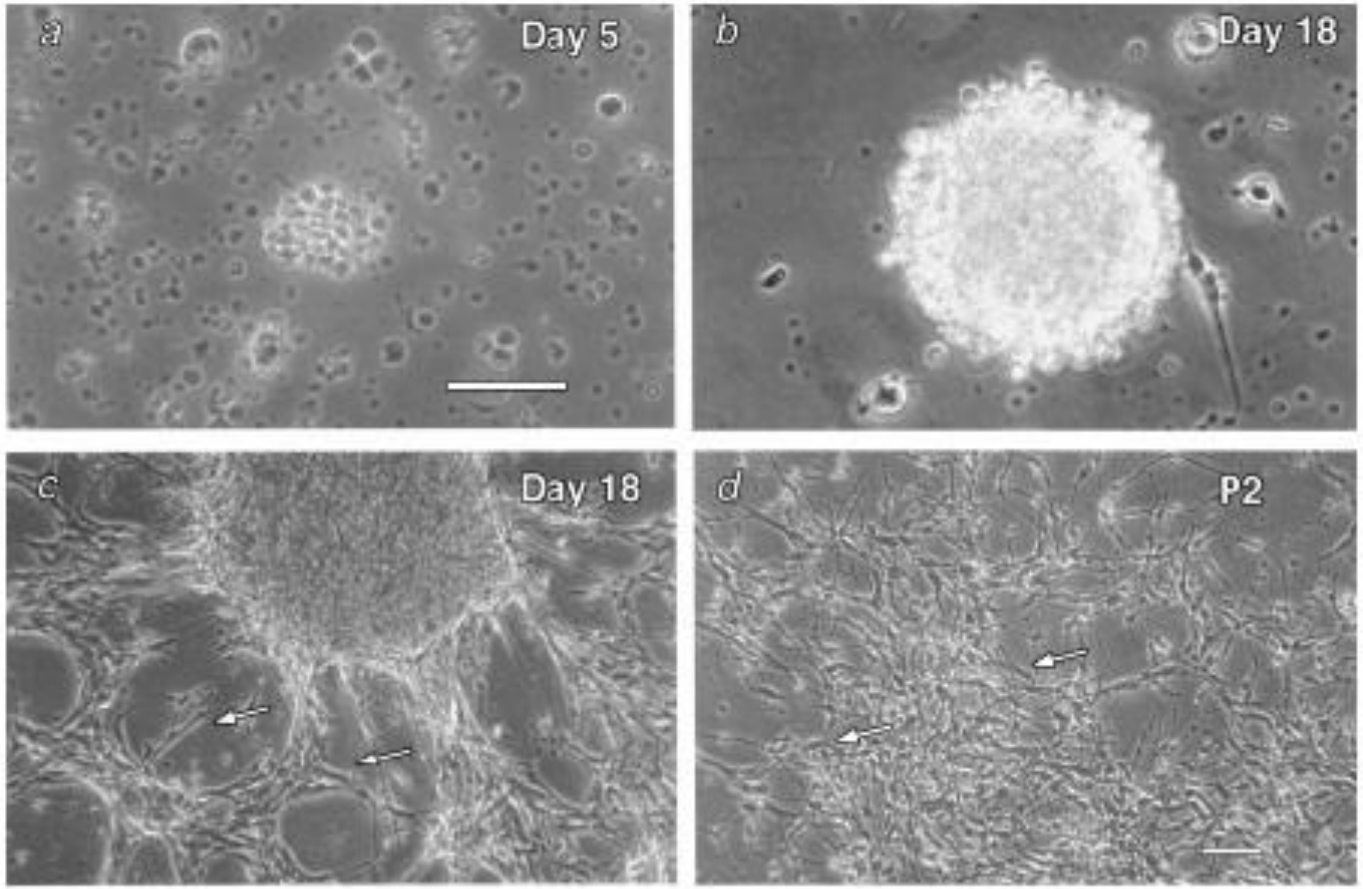


Fig. 3. Morphology of adult mouse neural stem cells cultured in serum-free N2 medium containing EGF, FGF-2 and heparin. (*a*) Neurospheres are visible by 5 DIV. With time in culture neurospheres increase in size (*b*) and some of the spheres attach to the substratum (*c*). Cells stream out of the spheres and grow as monolayer. (*d*) Upon passage attached cells grow as monolayers. Mouse stem cells have more elongated cell bodies (arrows in *c*, *d*) and smaller processes than rat stem cells. Scale bar: 100 µm

Organotypic slice cultures

PREPARATION

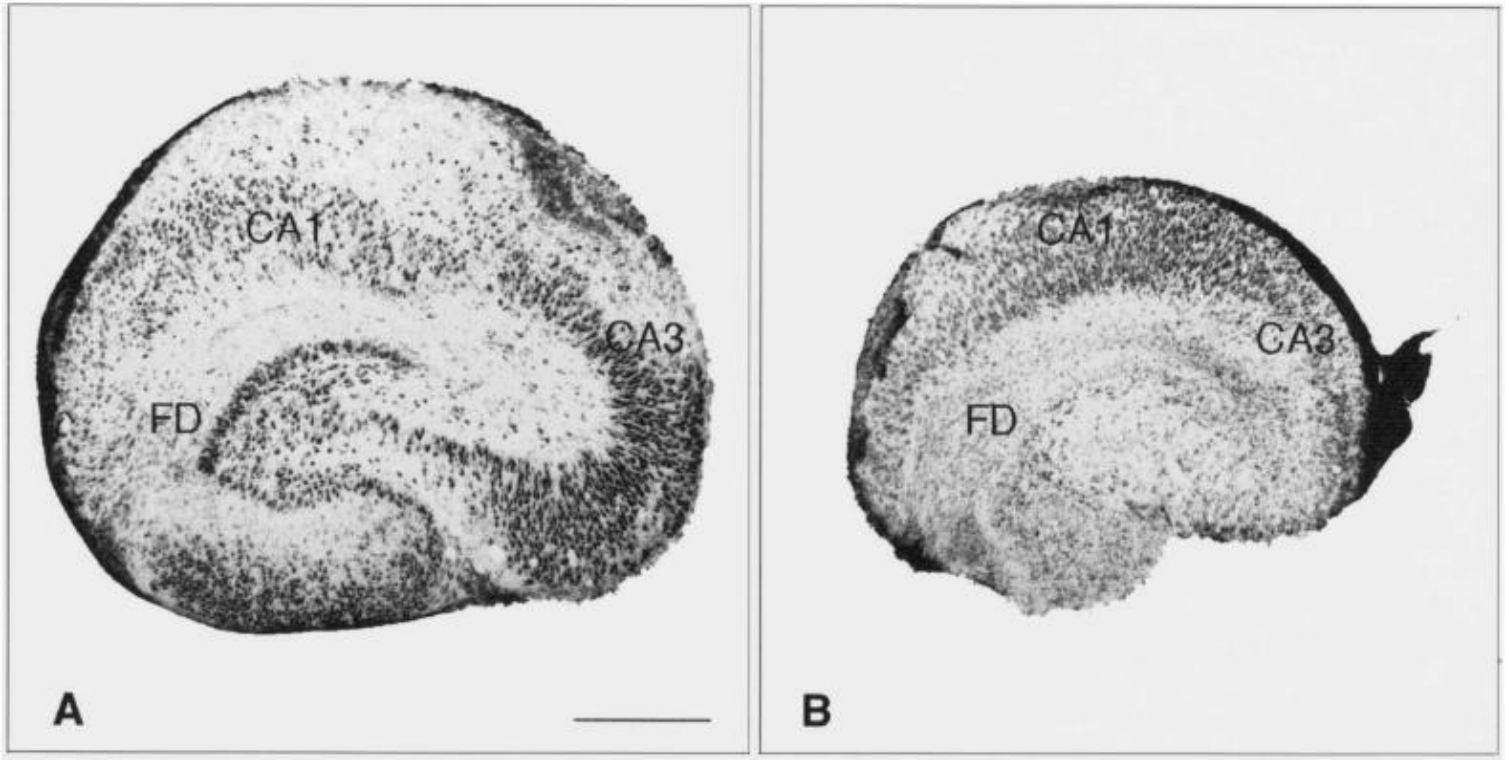
The tissue explant may be a thin slice, a chunk, or the complete organ, as is the case for the sympathetic ganglia.

Ex : The hippocampi is dissected and their dorsal halves sectioned transversely at 400 μm by a chopper or a vibratome

Why organotypic slice culture ?

UNIVERSITÀ
DEGLI STUDI DI TRIESTE

The goal of this culture technique is to obtain a preparation with a high degree of cellular maturation and differentiation and with an organotypic organization with the ability to assess individual neurons.



Why organotypic slice culture ?

- Receptor activity has been studied in various organotypic slice cultures

- Pharmacological comparison of these receptors *in vivo* and in slice preparations show identical characteristics not only in activity but also in cellular distribution.

EXPLANT/SLICE CULTURES

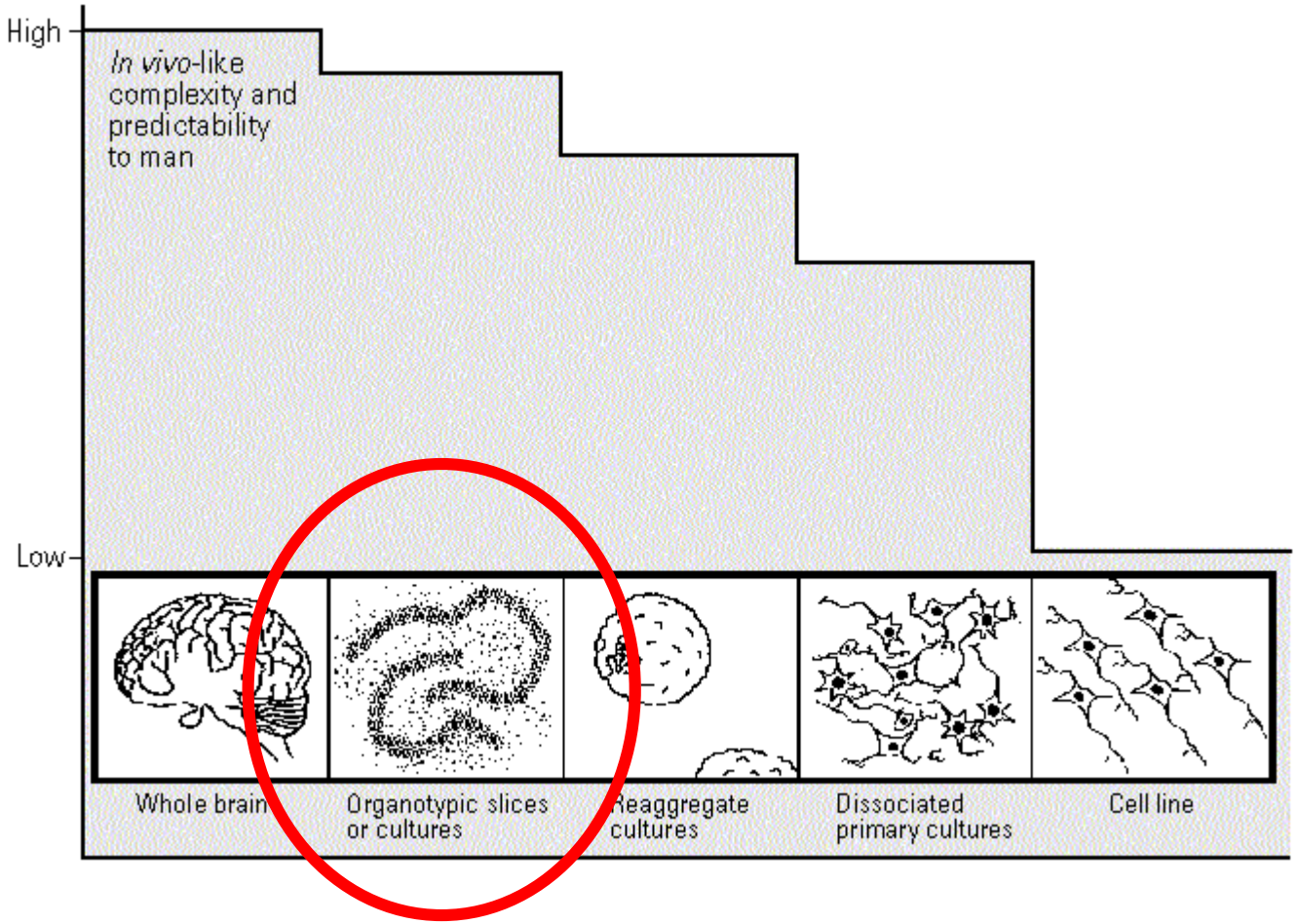
Organotypic cultures are derived from explants of relatively undifferentiated embryonic brain, spinal cord, or sensory organs and develop into integrated neuronal and glial populations.

For some regions of the nervous system, the age of animals used in explant cultures can vary from embryonic to postnatal periods but must remain constant across comparisons.

ADVANTAGEs

- The main advantage of this system is the presence of a three-dimensional organization in which some of the organ's structural and functional characteristics are retained.
- The organotypic explants/slices can survive in culture for a week to months, depending on the culture conditions
- These cultures have been helpful in solving several problems that could not be addressed in animal experiments

ADVANTAGEs


High
In vivo-like complexity and predictability to man
Low
Whole brain
Organotypic slices or cultures
Reaggregate cultures
Dissociated primary cultures
Cell line

DISADVANTAGEs

- The difficulty in quantifying small changes in differentiation or viability, depending on the parameters measured.

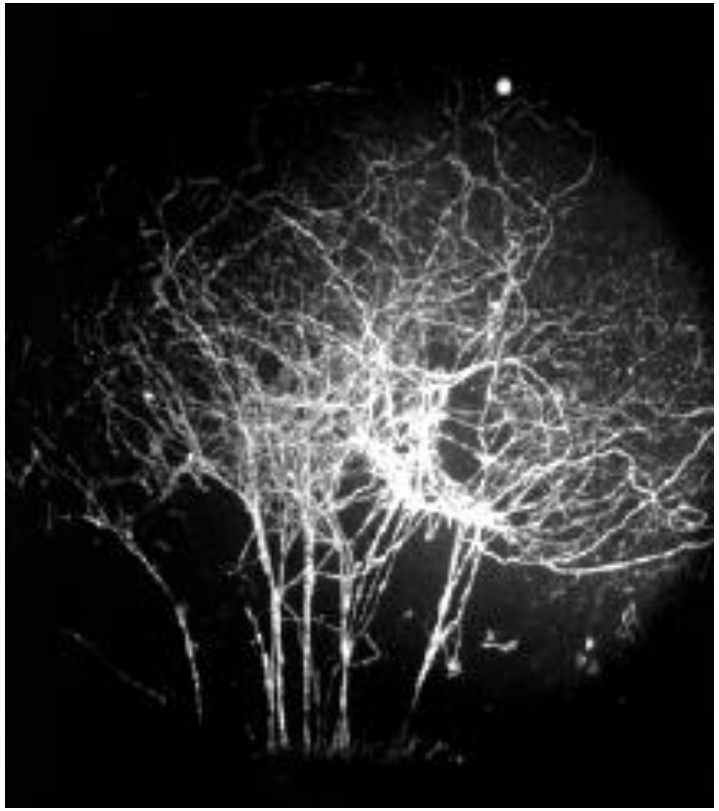
- The nutrient supply (e.g., oxygen, media compounds) available to the cells within the slice/explant is dependent on the diffusion rate and is governed by the thickness of the slice/explant.

- Often, depending on the thickness of the slices after prolonged culture periods, a hypoxia in the center of the tissue resulting in necrosis cannot be precluded.

DISADVANTAGEs

- Only in a small layer of the tissue is it possible to maintain *in vivo*-like physiological conditions

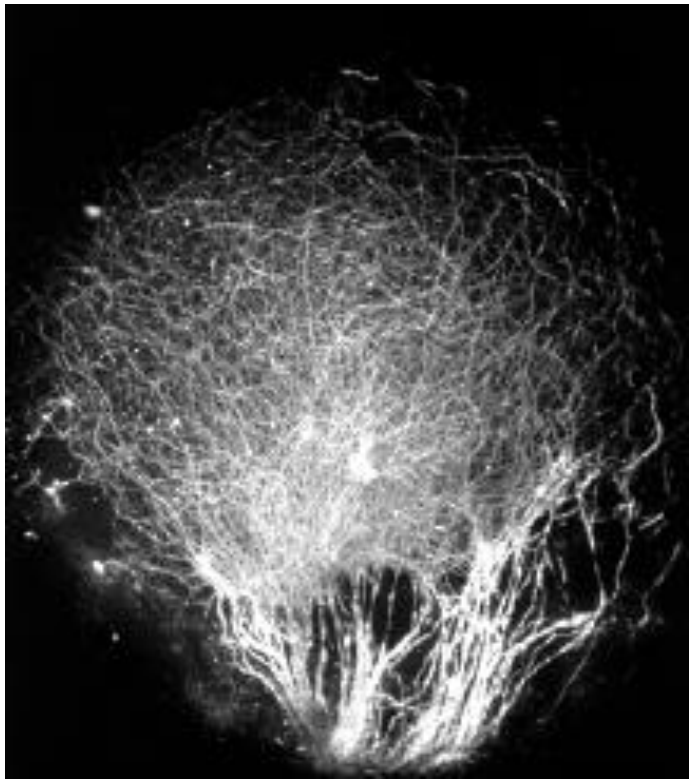
- Given the massive denervation and cell death caused by the explantation procedures and the novel influences of the tissue culture environments, it cannot be assumed that the properties of organotypic cultures are identical to their *in vivo* counterparts.



SLICE CULTURES

- The age of the donor is a critical parameter with regard to the degree of organotypic organization achieved in the final preparation (Usually, tissue is obtained from rats within the first week of life)

- Within about 2 to 3 weeks in culture, an original 400-µm thick slice will thin to form a pseudomonolayer of cells and with contrast enhancing optic, the individual neurons can be observed in the living state



SLICE CULTURES

- Microscopic examination of individual cells is limited by the thickness of the explant. When using slice culture techniques, the degree of maturation and differentiation must be determined under the culture conditions used.

- There is a high likelihood of synaptic rearrangement which must be examined using histological techniques. Morphological differentiation can be evaluated by cellular injections with dyes such as Lucifer Yellow or horseradish peroxidase.

SLICE CULTURES

- The organotypic culture provides direct access to the neurons and allows placement of stimulating and recording electrodes.
- A number of features of the slice culture make it attractive for evaluating the electrophysiological activity of individual cells and determining the effects of various pharmacological agents.
- The presence of functional synapses needs to be assessed by electrophysiological recording techniques.

USING SLICE CULTURES

- Explant/slice cultures originate from animals; although a number of slices can be obtained from any one animal, statistical concern requires slices generated from numerous animals.
- The various maturation times for each brain region, limit the likelihood of collecting various brain regions from any one animal.
- Additionally, survival time must be considered i.e., the slices must be processed into culture within a defined period of time, usually within 45 min

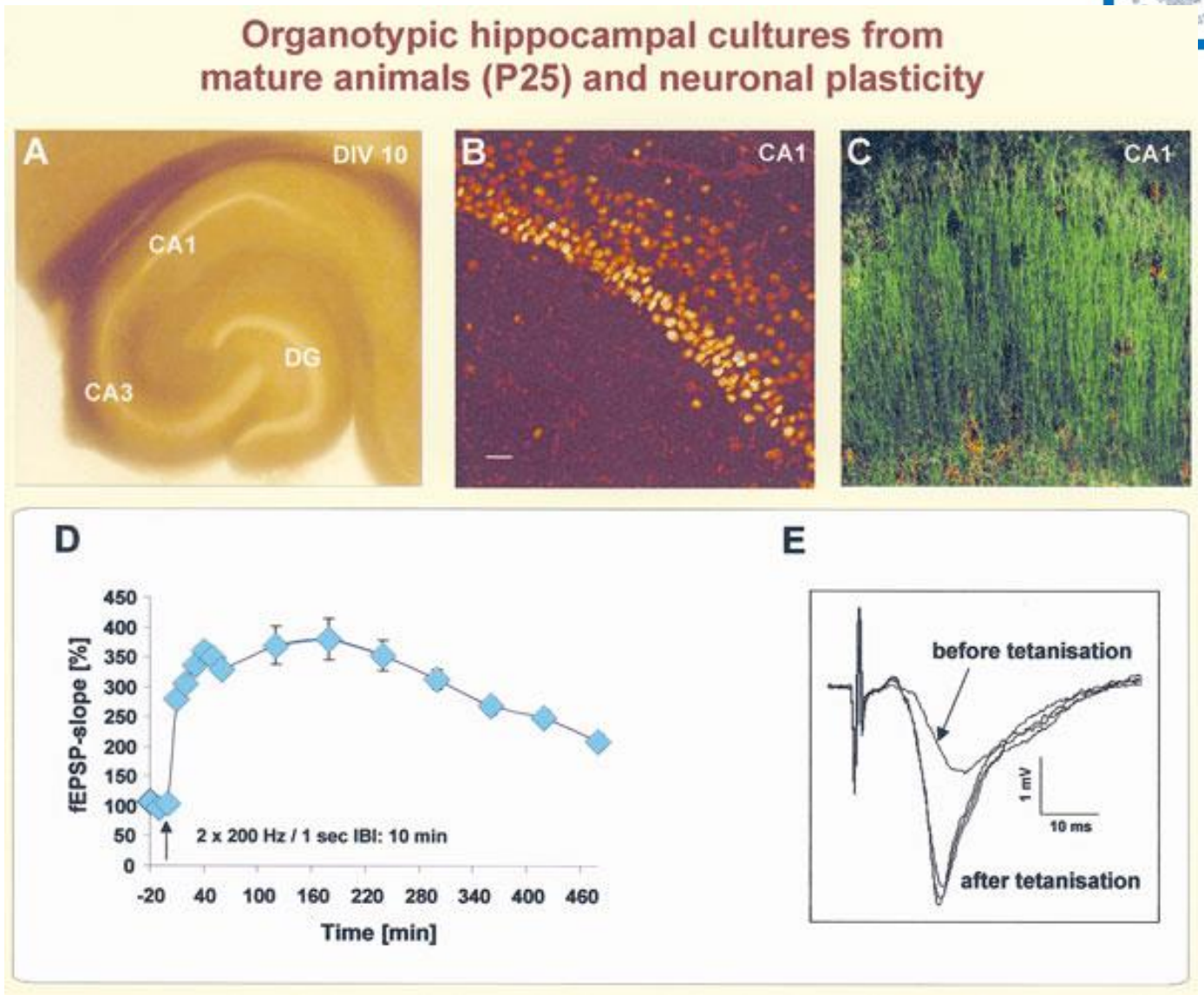
HIPPOCAMPAL SLICE CULTURES

UNIVERSITÀ
DEGLI STUDI DI TRIESTE

- Preparations are obtained from the brains of 5- to 7-day-old rodents. A 400-micron slice is placed on a specially prepared slide and cultured in roller drums.

- An estimated number of 10 slices can be obtained from each animal. After several weeks *in vitro*, the thick slices will flatten to virtual monolayer thickness thus allowing observation of individual neurons under phase contrast microscopy.

Example


Organotypic hippocampal cultures from
mature animals (P25) and neuronal plasticity
A
DIV 10
CA1
DG
CA3
B
CA1
C
CA1
D
fEPSP-slope [%]
450
400
350
300
250
200
150
100
50
0
2 x 200 Hz / 1 sec IBI: 10 min
-20
40
100
160
220
280
340
400
460
Time [min]
E
before tetanisation
1 mV
10 ms
after tetanisation

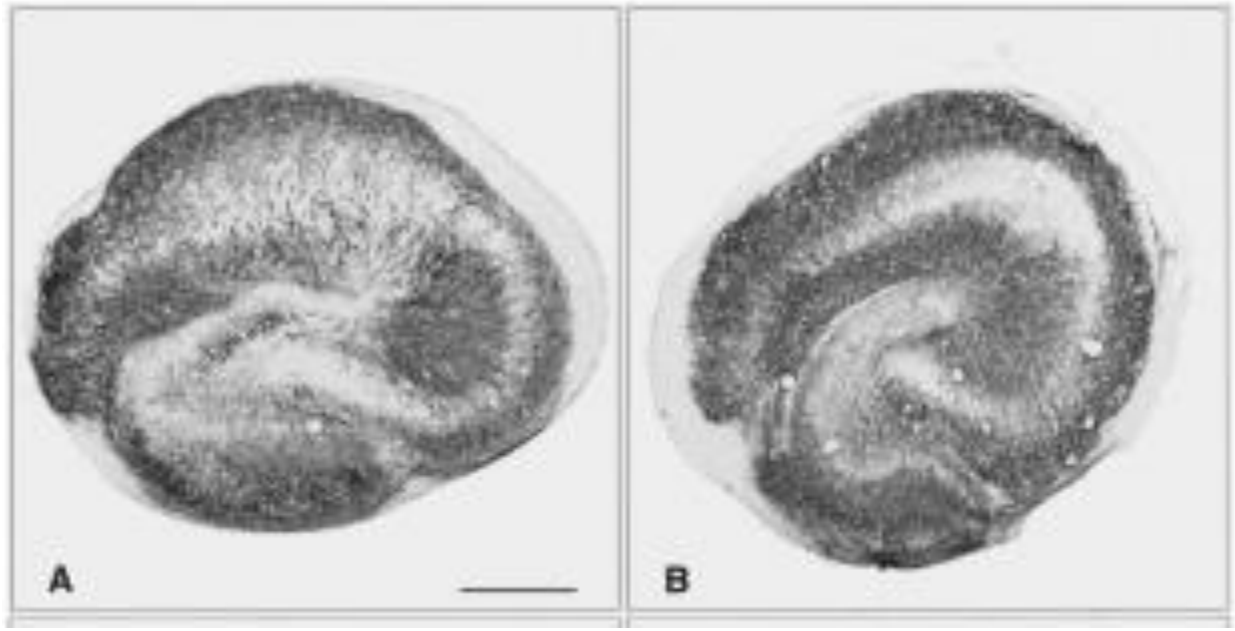
HIPPOCAMPAL SLICE CULTURES

- With the hippocampal slice, the reorganized three-dimensional structure retains the original hippocampal cytoarchitectural relations.
- Synaptic organization can also be maintained; however, aberrant supragranular projections are often reported.

- This flattening is due to a lateral migration of nerve cells that are located in the deeper layered structures. This reorganization is proposed to be due to a limited oxygen supply at the deeper layers. Additionally, any structures adjacent to the cut surface or the severed afferent and efferent fibers degenerate

HIPPOCAMPAL SLICE CULTURES

Morphological differentiation can be maintained similar to in situ conditions for both the pyramidal and granule cells. Techniques using Golgi impregnation and dye injections allow evaluation of morphological differentiation and dendritic arborization.



J. Noraberg and J. Zimmer

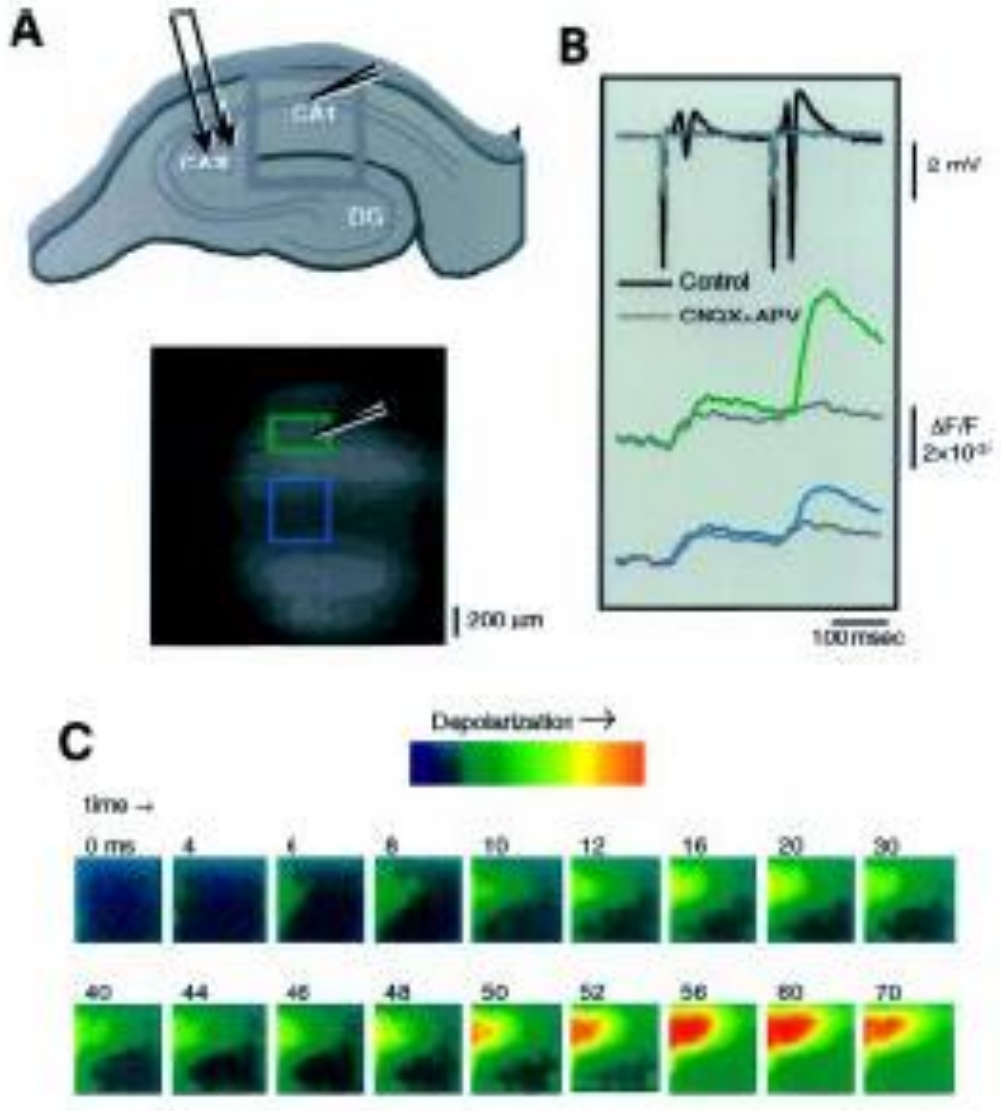
HIPPOCAMPAL SLICE CULTURES

- In slice culture, neurons and associated cells can be maintained for relatively long periods of time, thus allowing for extending periods of exposure to assess toxicological effects.

- Some researchers claim that slice cultures can be viable for up to a year; however, the cellular phenotype will change with age.

- Brain slices, especially the hippocampal slice, have been used extensively for neurobiological studies and the approaches have been adapted for utilization in neurotoxicity evaluation.

Example


A
CA1
CA3
DG
200 µm
B
2 mV
Control
CNQX+APV
ΔF/F
100 msec
C
Depolarization
time →
0 ms
4
6
8
10
12
16
20
30
40
44
46
48
50
52
56
60
70