Experimental Biology

Cell Culture & Transfection

Zilong Qiu (仇子龙), Ph.D
Institute of Neuroscience
Chinese Academy of Sciences

Lecture outline

- Cell culture

  dissociated from tissue

  maintenance

- Transfection

  Reporter system

  Physical: biolistics, electroporation, injection

  Chemical: liposome, Ca_2PO4

  Biological: viral-mediated

- Crash course--Dummies book for gene function

in vitro culture

- **Cell culture**
- **Tissue culture**
- **Organ culture**

Why need culture?

- **Simplify the environment**
- **Apply the experimental factors**
- **Easy observation**

Adherent cells:

Most cells derived from tissues are anchorage-dependent.

Suspension cells:

Can survive and proliferate without being attached to a substratum. Hematopoietic cells (derived from blood, spleen, or bone marrow) and cells derived from malignant tumors can be grown in suspension.

1. 原代培养(primary culture)：
从动物机体取出的进行培养的细胞群。原代培养的细胞生长比较缓慢，而且繁殖一定的代数后（一般10代以内）停止生长。

2. 细胞株(cell strain)：
从原代培养细胞群中筛选出的具有特定性质或标志的细胞群，能够繁殖50代左右，在培养过程中其特征始终保持。

3. 细胞系(cell line)：
从肿瘤组织培养建立的细胞群或培养过程中发生突变或转化的细胞，在培养条件下可无限繁殖。

4. 克隆(clone)：
亦称无性繁殖系或简称无性系。对细胞来说，克隆是指由同一个祖先细胞通过有丝分裂产生的遗传性状一致的细胞群。

Safety

Disinfection

Work surfaces, culture waste and equipment must be kept clean to prevent contamination

Hypochlorides (bleach)

- Good general purpose disinfectant
- Corrosive against metals (i.e., centrifuges)
- Use at 1000 ppm for general surface use

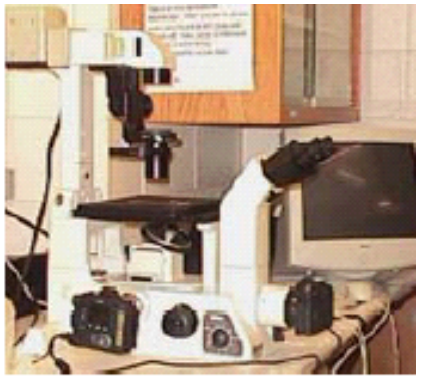
Alcohol

- 70% ethanol or 60-70% for isopropanol

Waste disposal

Dispose regularly, do not allow to accumulate tissue culture waste (media, pipettes, flasks, etc.)

Cell culture

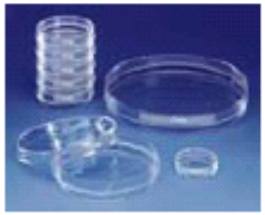


Equipment in cell culture room

- Laminar flow hood
- CO_2 incubator
- Mechanical pipetter
- Inverted microscope (equipped with camera)
- Vacuum pump flask
- Refrigerator dedicated to storage of cell culture solutions

Materials

- Complete cell culture medium, appropriate for the cell line
- Tissue culture flasks and dishes of appropriate sizes
- Individually-wrapped sterile pipets
- Multichannel pipet and sterile tips
- 70% alcohol



Cells/tissues grown in culture long period of time

BASIC STERILE TECHNIQUE

- Work environment and surface
- Plasticware and glassware
- Handling techniques
- Sterilization of solutions for maintenance growth or treatment

WORK ENVIRONMENT AND SURFACE

Laminar flow hood

- ✓ Relatively enclosed space
- ✓ Little traffic flowing past work space
- ✓ Confined space can be easily cleaned and maintained
- ✓ Must have annual checkup of HEPA filters
- ✓ Should have internal outlets for electricity, vacuum and gas
- ✓ Frequently outfitted with UV light
- ✓ Prevent contamination by daily scrub with 70% ethanol
- ✓ Use closed flask attached to vacuum for spent medium

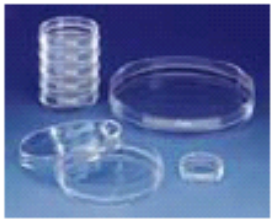
Separate sterile room

- ✓ All incoming air circulated through HEPA filters

PLASTICWARE AND GLASSWARE

Pipettes, bottles, flasks, petri dishes

- Plastic
  - -- better attachment and cell growth of monolayer cell culture
  - -- more expensive than reusable glass
  - -- suitable for storage at 4°C



- Glass
  - -- Can withstand temperatures <0°C use for maintaining frozen stocks
  - -- Steam sterilize by autoclaving
    - * relies on steam pressure and high heat
    - * use fast-dry cycle to dry condensate
    - * use "wet" cycle for salt solutions to prevent evaporation
    - * loosely place caps on bottles

Handling techniques

- ✓ Wipe work area and hands with 70% ethanol before starting. Wear disposable latex gloves
- ✓ Keep sterile flasks, bottles, petri dishes, etc covered until ready to use. Return cover as soon as finished
- ✓ Keep sterile pipettes in their wrapper until ready to use Dispose used pipettes in containers to be autoclaved
- ✓ After removing cap from bottle do not place the open end upright. Hold the container at a tilted angle
- ✓ Use a separate pipette when drawing medium etc. from a different bottle. Use sterile pipette for each bottle
- ✓ Perform techniques as quickly as possible to avoid contamination

Handling techniques (continued)

- ✓ Handle cell lines individually in the hood
  maintain cultures with two bottles of medium
- ✓ Keep medium bottles closed when not in use
- ✓ Bring medium to 37°C before adding to cells
- ✓ Maintain cell lines in medium in which cells originally isolated or to which cells have adapted
- ✓ Humidified incubators—common source of contamination. Periodically clean including tubing, water trays, shelves, etc.
- ✓ Frequently examine cells under the microscope for growth and evidence of contamination

Medium formulation

Composition of medium

- Complement of amino acids, vitamins, organic salts, glucose and serum (source of growth factors, hormones and attachment factors), phenol red and antibiotics
- Serum added to growth medium as supplement., usually as 10% fetal bovine serum or horse serum

  if add only 1% serum, cells go into cell cycle arrest

pH

- Should range between 7.2 and 7.4
- Most are buffered by $NaHCO_3$
- Phenol red (indicator dye) goes to yellow when cells depleted of glucose (lactic acid and CO_2 produced)
- Level of CO_2 must be balanced against level of $NaHCO_3$ (i.e., if 5% CO_2, 1.97 g of $NaHCO_2$/liter added to medium, 3.95 g of $NaHCO_3$ required if CO_2 is 10%)

Medium formulation (continued)

Forms

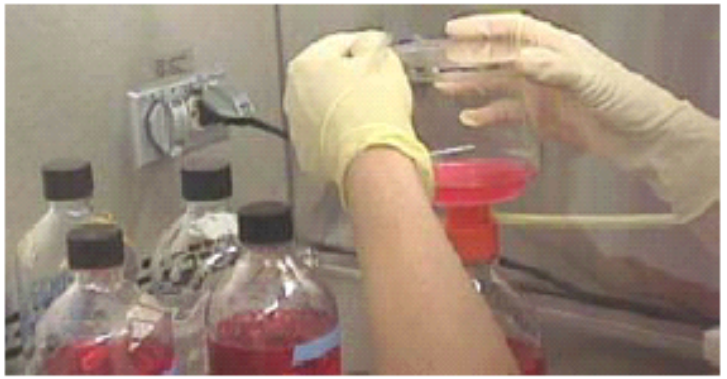
- Powdered—prepare and sterilize before using
  - -- Least expensive
  - -- Requires pump and sterilizing disposable filters (pore size 22 μm required for complete sterilization)
  - -- Do not vacuum filter—foaming denatures proteins
- Concentrated—dilute with sterile water
- Working solution—use directly, most expensive

Storage

- Store medium at 4°C
- Store in dark—some components are light sensitive
- Fetal bovine or horse serum can be added sterilely

Sterilization of solutions for maintenance growth or treatment

- ✓ Do not autoclave nutrient medium—heat will destroy some of the compounds in the medium
- ✓ Pass through sterile filter with small pore size (22 μm) use sterile techniques



Inoculation and passaging of cell lines in culture

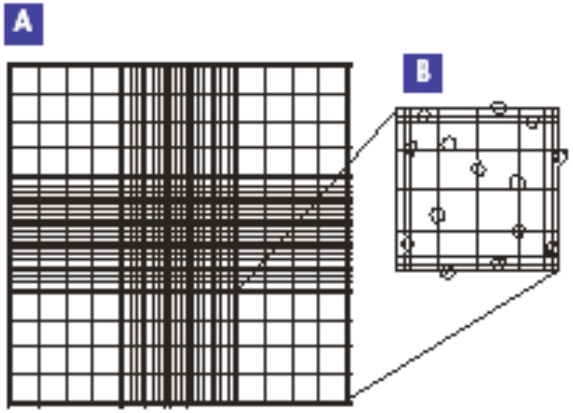
- Suspension cultures

✓ Dilution

✓ Total replacement of medium
  -- Triturate gently to disrupt cell clumps
  -- Remove 1 ml for cell counting
  -- Dilute with growth medium to 2 x 10^5 to 4 x 10^5/ml
  -- Determine cell number by counting, and viability by trypan blue exclusion

- Monolayer cultures
  -- Trypinize cells
  -- Resuspend cells in growth medium
  -- Replate an aliquot of cell suspension in new flask containing growth medium
  -- Disperse cells on flask bottom by slight rotation (cells usually attach within 8-10 h)

Cell counting using a hemocytometer



--Dilute your cell sample in Trypan Blue dye exclusion medium.
Dead cells will be blue. Suggested dilution 1:20 (5µl cell suspension in 95 µl Trypan Blue).

--Carefully fill the hemacytometer chamber.

--Leave undisturbed for 1-2 min.

--Count under the microscope four 1x1x0.1 mm areas.

Ideally you should count more than 200 cells and less than 500 per chamber.
Cell concentration= [cell number(in four areas)/4x10*4] x dilution

Viability staining

1. **Harvest the cells, either by trypsinization (adherent cell cultures) or by centrifugation at 200 x *g* for 5 min (suspension cell cultures). Resuspend the cells in an appropriate volume of prewarmed growth medium to give a cell density of at least 10*6 cells/ml.**

2. **Add 0.5 ml 0.4% (w/v) trypan blue and 0.3 ml PBS or HBSS to 0.1 ml of the cell suspension, mix thoroughly, and let stand for 1–2 min.**

3. **Count the stained and unstained cells using a hemocytometer.**
   Blue-stained cells are nonviable and unstained cells are viable.
   No. of viable cells/Total no. of cells = % viability.

Cryopreservation of cells

- Cells can be stored for years in liquid nitrogen at temperatures between -135°C -175°C or in -80°C freezer for several months
- ✓ Serve as backup for loss from laboratory accidents
- ✓ Cells have not undergone genetic drift

  Nontransformed cells not undergo senescence

Cell Thawing/Freezing Protocol

Freezing Cells:

--Cells should be growing well or known to be in log phase;
--Count, collect and pellet cells in a 15mL test tube;
--Resuspend in freezing media so that the concentration is no more than 5x10*6 cells/ml of cold freezing media;
--Transfer 1mL of cells to appropriately labelled cryovials and maintain on ice for approximately 30 minutes;
--Transfer vials to -80ºC freezer for 24hrs;
--Transfer to liquid nitrogen drawar or -140 ℃ freezer for long-term storage.

Freezing media:

10% DMSO
90% FCS
you'll need 1mL per 5x10*6 cells

Thawing Cells:

Remove vial from Liquid Nitrogen or -140℃ freezer and immediately transfer to 37ºC water bath;

While holding the tip of the vial, gently agitate the vial, being careful not to allow water to penetrate the cap or seal;

When completely thawed, transfer contents of vial to 15mL test tube; Slowly add 10mL warm medium and spin at 1000g for 5min;

Decant media and resuspend pellet in a volume of complete media appropriate for flask or macrowell;

Transfer cells to flask or 24 well plate and incubate at 37ºC and 5%CO2; Cells can be checked visually or counted, beginning at approximately 1hr, for an estimate of viability.

Decontaminate tissue culture

- First, determine if the contamination is bacteria, fungus, mycoplasma, or yeast.
- Isolate the contaminated culture from other cell lines. Clean incubators and laminar flow hoods with a laboratory disinfectant, and check HEPA filters.
- If the cell line or culture is not one-of-a-kind, it is best to throw it away and start over with new cells.
- Culture the cells for two to three passages using the antibiotic at a concentration one to two-fold lower than the toxic concentration(4).
- Culture the cells for one passage in antibiotic-free media (5).
- Repeat (4) and (5) once.
- Culture the cells in antibiotic-free media for four to six passages to determine if the contamination has been eliminated.

Table 10. Characteristic features of microbial contamination

Characteristic	Bacteria	Yeast	Fungi
Change in pH	pH drops with most infections	pH changes with heavy infections	pH sometimes increases
Cloudy medium: Under microscope (100–400x)	Shimmering in spaces between cells; rods or cocci may be observed	Round or ovoid particles that bud off smaller particles	Thin filamentous mycelia; sometimes clumps of spores

Table 11. Commonly used antibiotics and fungicides for animal cell culture

Additive	Working concentration	Effective against	Stability at 37°C
Antibiotic			
Penicillin	50–100 U/ml	Gram-positive bacteria	3 days
Streptomycin	50–100 µg/ml	Gram-negative bacteria	3 days
Kanamycin	100 µg/ml	Gram-positive and Gram-negative bacteria; mycoplasma	5 days
Gentamycin	5–50 µg/ml	Gram-positive and Gram-negative bacteria; mycoplasma	5 days
Fungicide			
Nystatin	100 U/ml	Yeasts and molds	3 days
Amphotericin B	0.25–2.5 µg/ml	Yeasts and molds	3 days

Mycoplasmal infection — detection

--Small, slow-growing prokaryotes that lack a cell wall and commonly infect cell cultures;

--Unaffected by the antibiotics commonly used against bacteria and fungi;

--Do not overgrow cell cultures and typically do not cause turbidity;

--Inhibit metabolism and growth, as well as interference with nucleic acid synthesis and cell antigenicity;

--Acute infection causes total deterioration of the cell culture.

Approaches to detect mycoplasma —
Hoechst 33258 staining
Mycoplasma - specific DNA probes (Fisher Scientific),
and PCR-based assays (e.g., the VenorGeM® Mycoplasma Detection Kit from Minerva Biolabs).

Mycoplasmal infection — eradication

Absolutely irreplaceable?
Isolated hood

Mynox® Mycoplasma Elimination Reagent (Minerva Biolabs)

Enrofloxacin (Baytril®)

Combination of tiamulin and minocycline (BM-Cyclin).

Neuron culture

- Primary culture: dissociated cell culture; tissure culture
- Continuous cell line: immortalized or transformed cell line
- Cell strain: derived from single clone

  neuroblastoma; glioma; pheochromocytoma(嗜铬细胞瘤) (PC12,etc.)

Neuron primary culture

- Tissue sources: Rat, Mouse, Chick, Xenopus
- Culture medium: PH, medium, supplements(B27)
- Culture dishes and substrates: PDL, collagen, laminin
- 神物PDL，also works for 293t long-term culture
- 长期培养大法（2-3weeks），无FBS培养，不分裂细胞

water	**water**	**water**	**water**	**water**	**water**
water					water
water					water
water	water	water	water	water	water

How to tell neurons from heterogeneous cultures?

- Morphology
- Electrophysiology
- Immunostaining:

  Neuron: tubulin-βIII, tau1(axon), MAP2(dendrite), GAP43(axon growth cone)

  Astrocyte: GFAP(glial fibrillary acidic protein)

  Fibroblast: fibronectin

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  Reporter system
  Physical: biolistics, electroporation, injection
  Chemical: liposome, Ca_2PO4
  Biological: viral-mediated

Genetic Reporter Systems

- **The first reporter? Transformation of tumor gene 1970s, 课外阅读 《*Natural Obsessions*》**

- 八卦时间到!
- Story 1: Bob Weinberg
- Story 2: Richard Axel (Ca2PO4)
- Story 3: the great era of gene discovery
- Weinberg for Nobel prize?

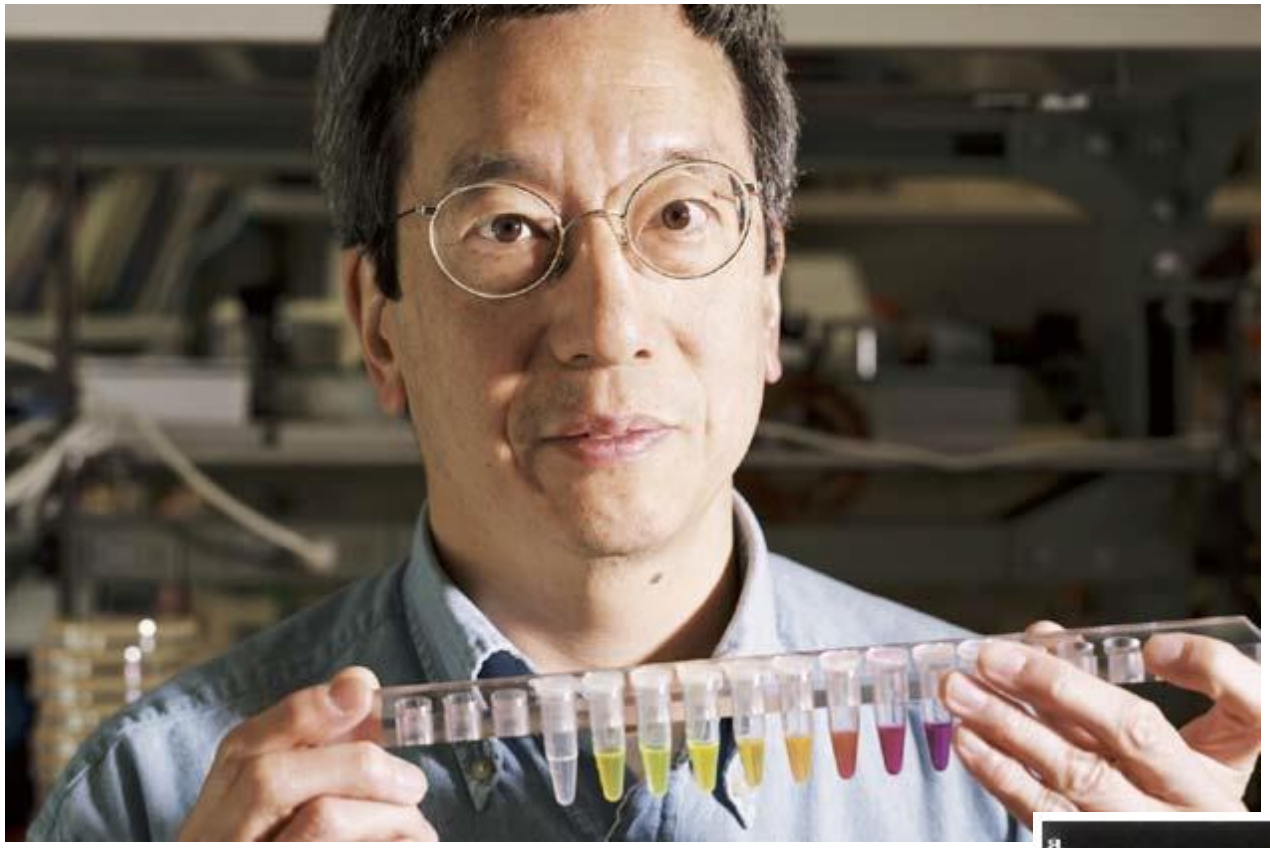
Genetic Reporter Systems

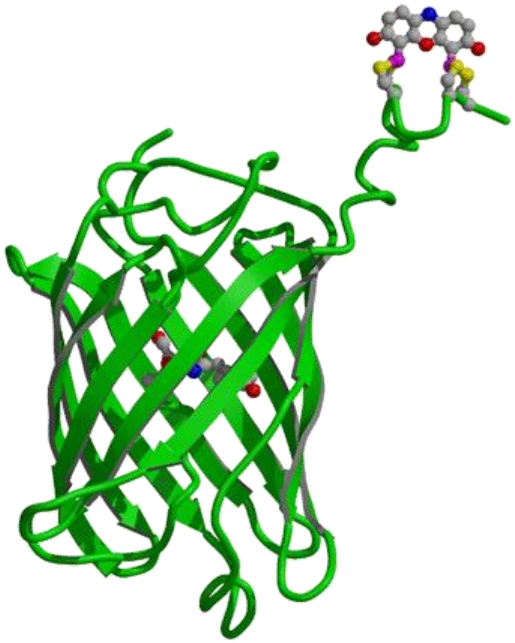
- **The first reporter? Transformation of tumor gene 1970s,** 课外阅读 ***Natural Obsessions***

- **Firefly luciferase:** catalyzes a bioluminescent reaction involving the substrate luciferin, ATP, Mg2+, and molecular oxygen. Light signals are detected using a luminometer or a liquid scintillation counter. Co-transfecting plasmids encoding firefly luciferase (experimental reporter) and Renilla (*Renilla reniformis*) luciferase (control reporter)—promoter assay

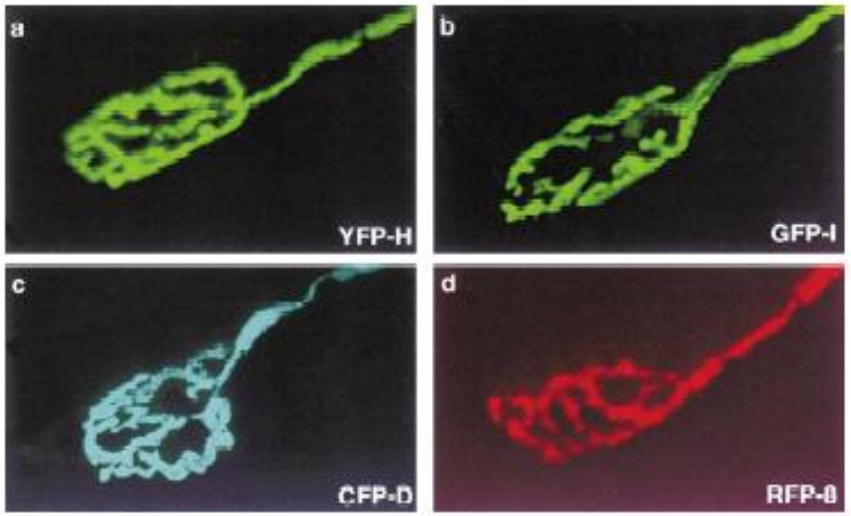
- **β-Galactosidase** *o*-nitro-phenyl-β-D-galactopyranoside (ONPG). yellow-colored product, *o*-nitrophenol, which can be measured photometrically.

- **Green fluorescent protein (GFP)** originally isolated from the jellyfish *Aequorea victoria (1962)*, has the ability to absorb blue light and emit green light. requires no additional substrates or cofactors to emit light; in situ detection of gene expression; GFP variants intensify the fluorescence or shifted emission wave lengths (YFP,CFP,RFP)—by Roger Tsien (钱永建UCSD)

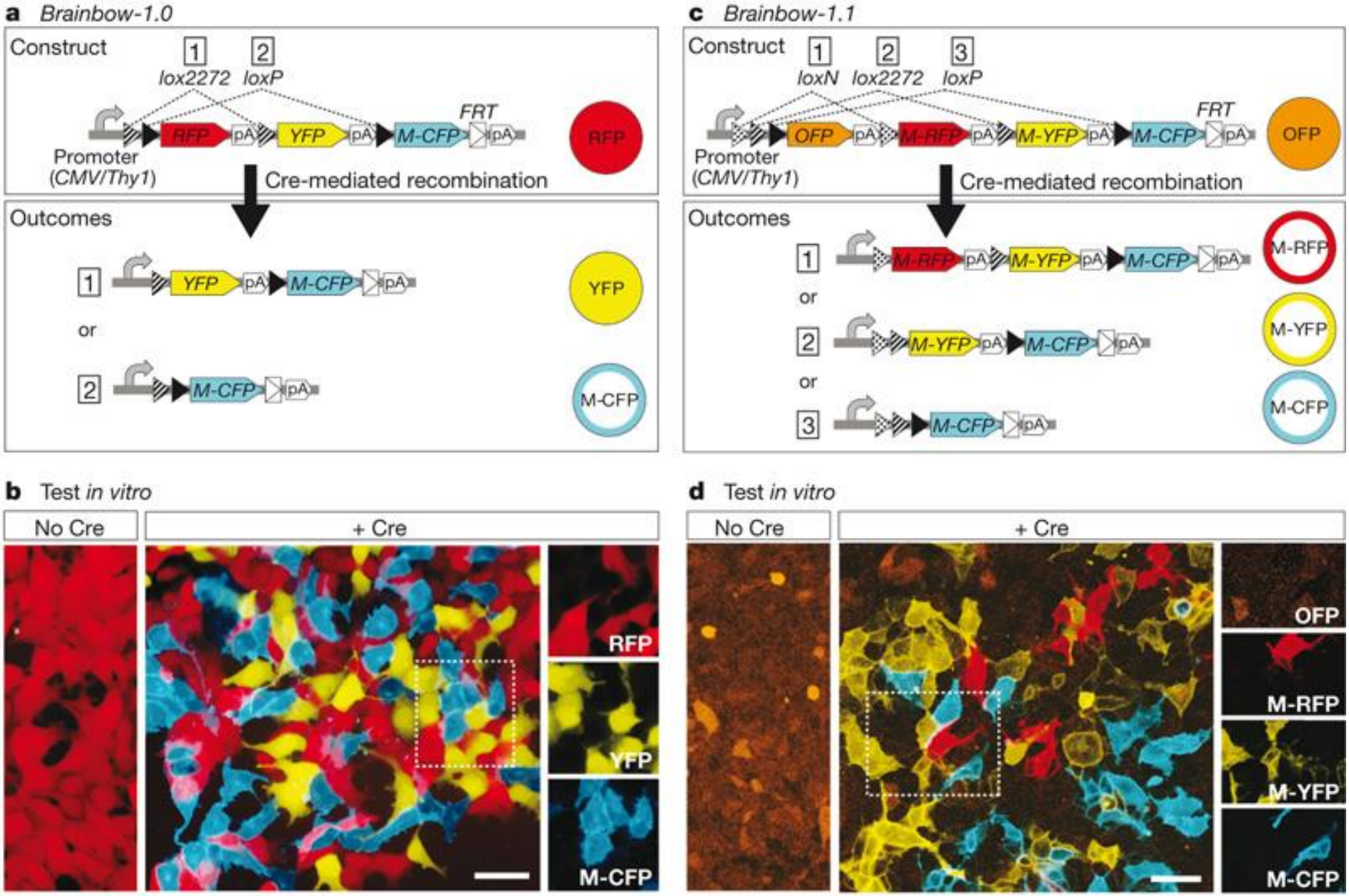




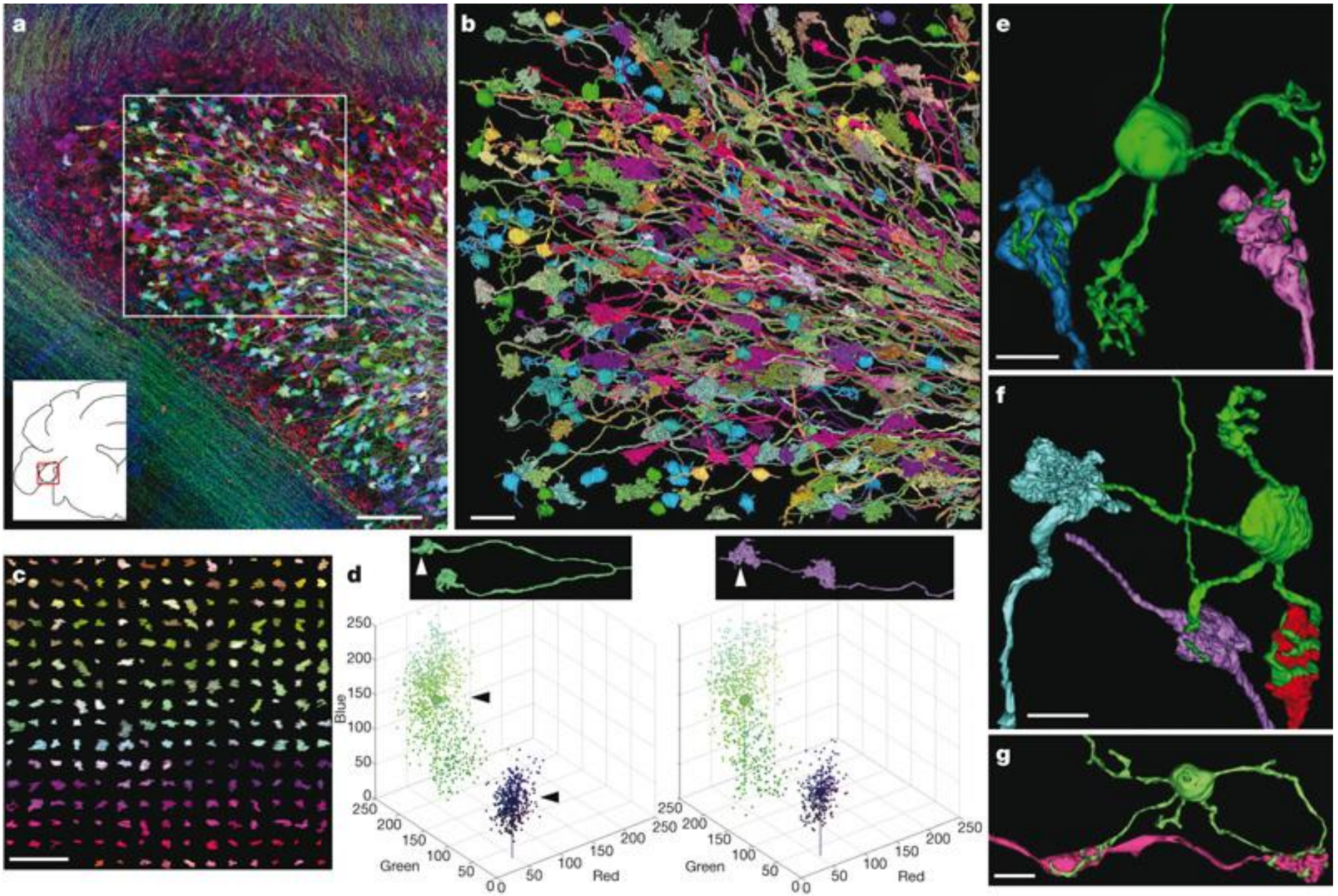
Roger Tsien 钱永健



Feng G,et al 2000 Neuron

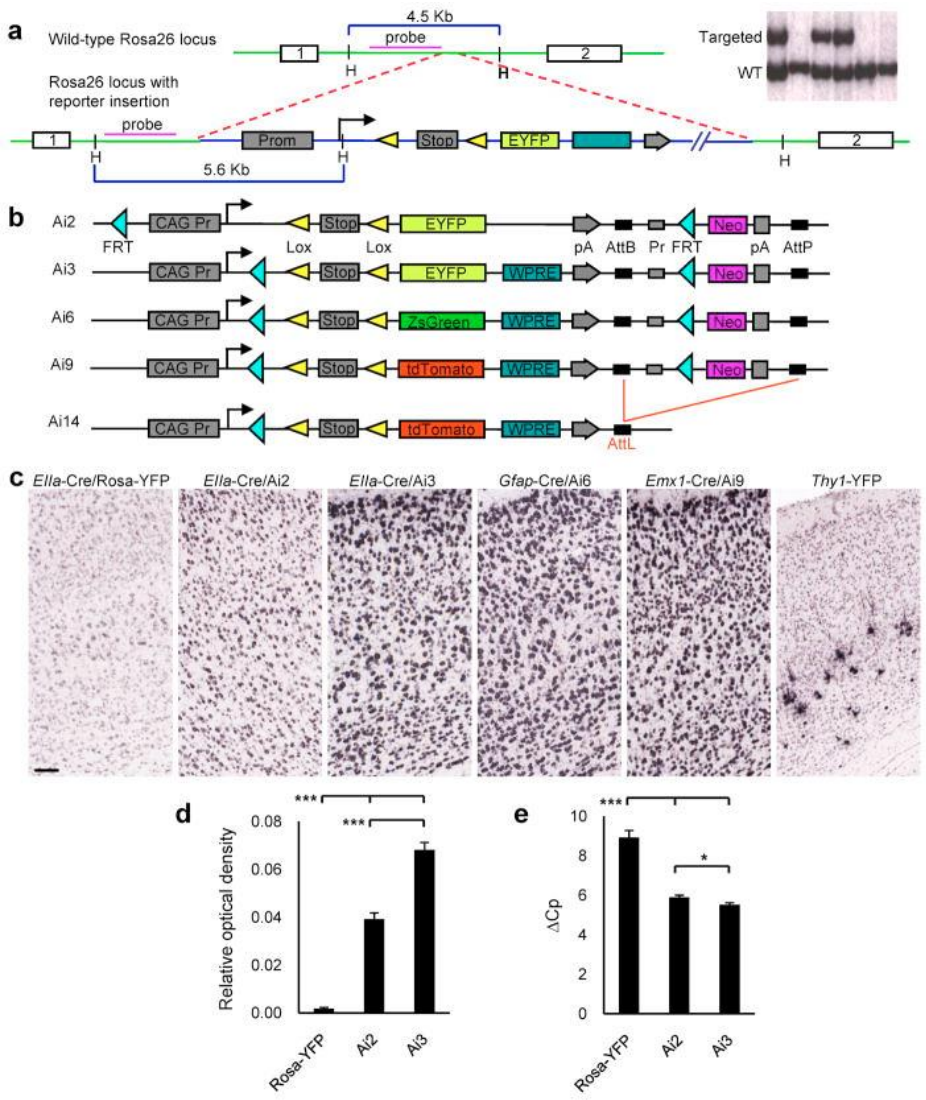


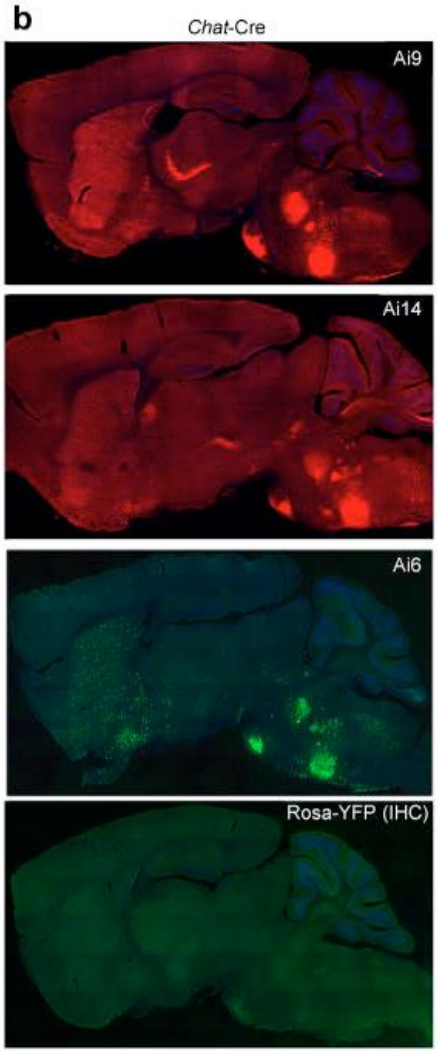
Nature 450, 56-62 (1 November 2007)



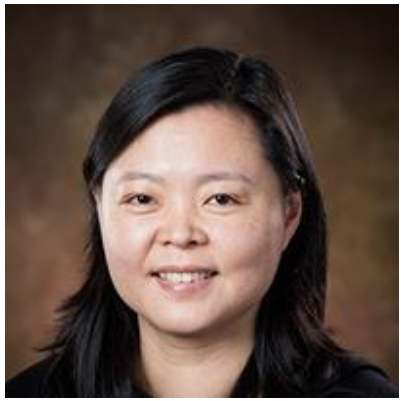
Cerebellar circuit tracing and colour analysis

The new generation of reporter mice from Allen Institute for Brain Science



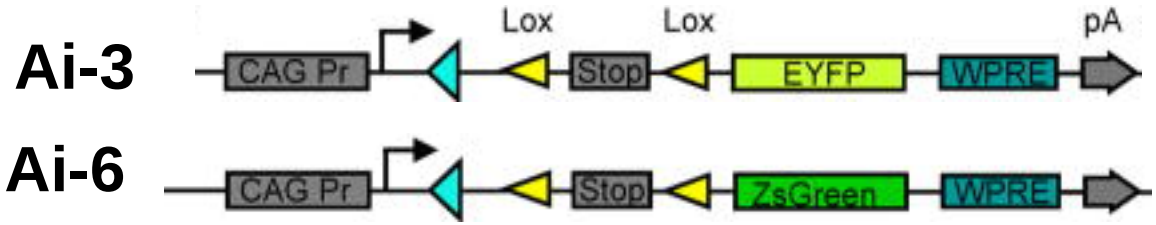


Hongkui Zeng

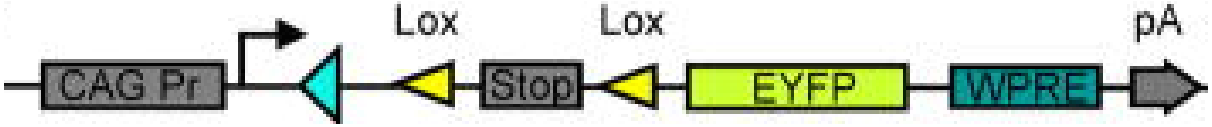


2010 Nature Neuroscience

The next generation GFP reporter mice



too weak!

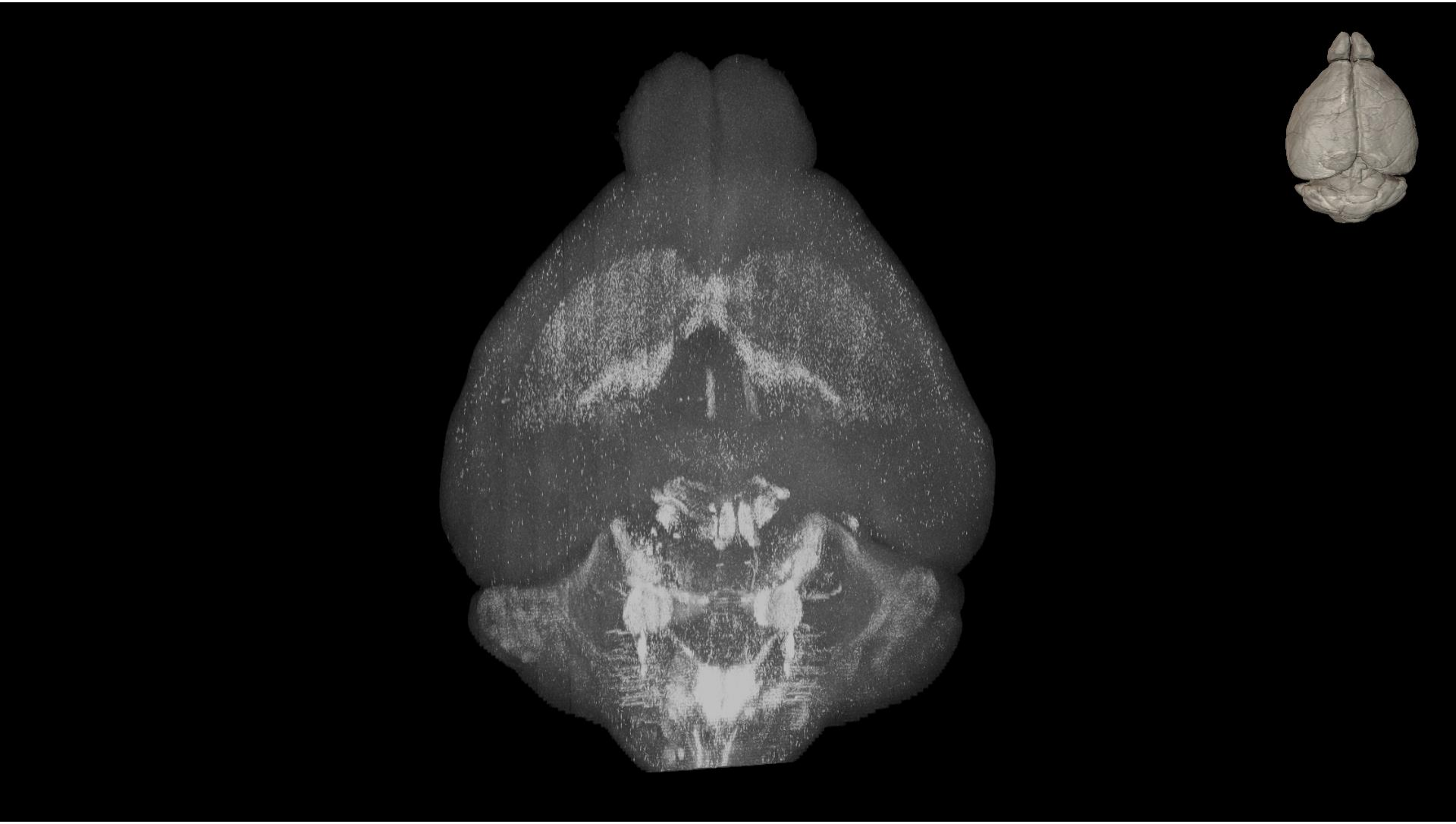


so strong!

3XGFP

Chat-Cre X Ai-47

the whole-brain view for cholinergic neurons



Li Xiang-Ning, Gong Hui

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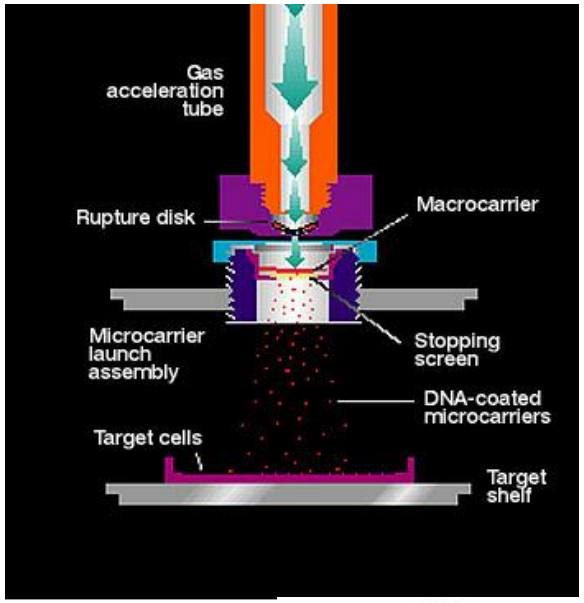
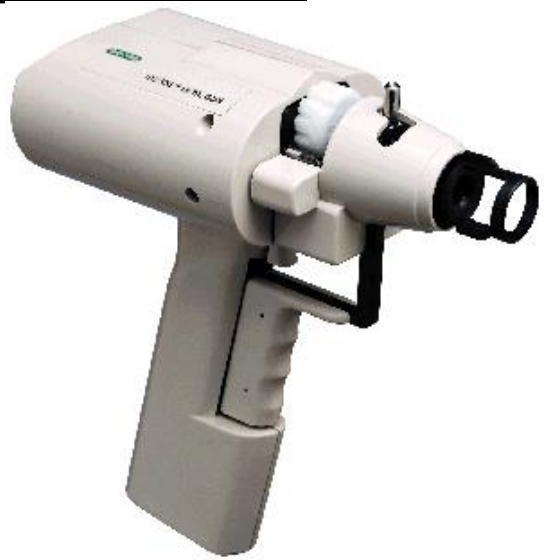
  Reporter system
  Physical: biolistics, electroporation, injection
  Chemical: liposome, CaPO4
  Biological: viral-mediated

Physical: biolistics





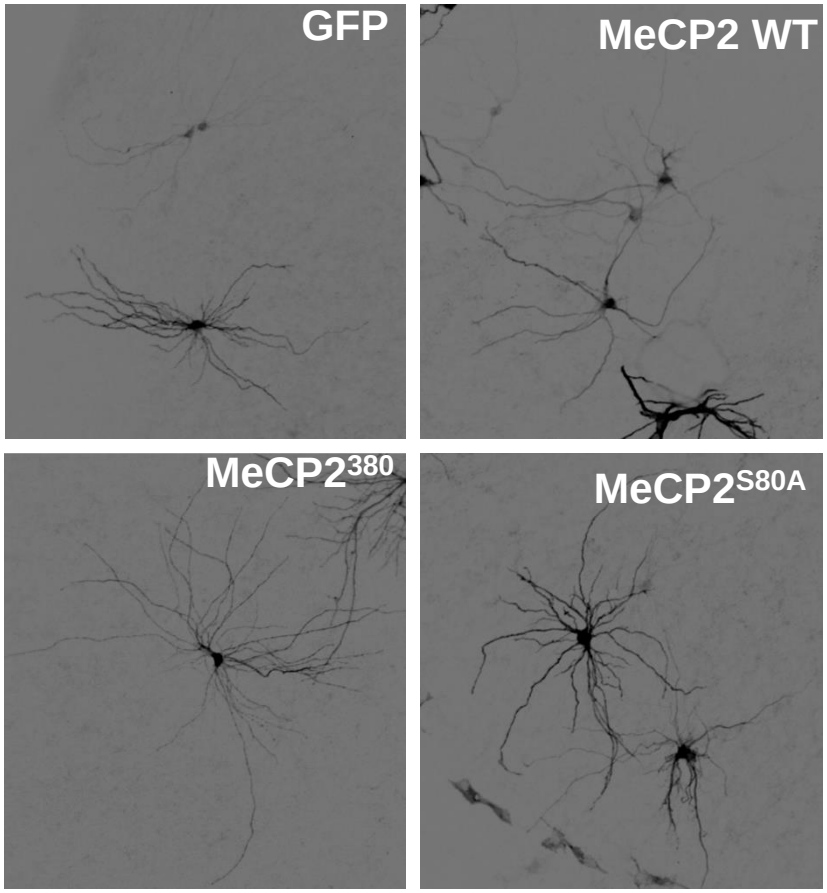
Gene Gun

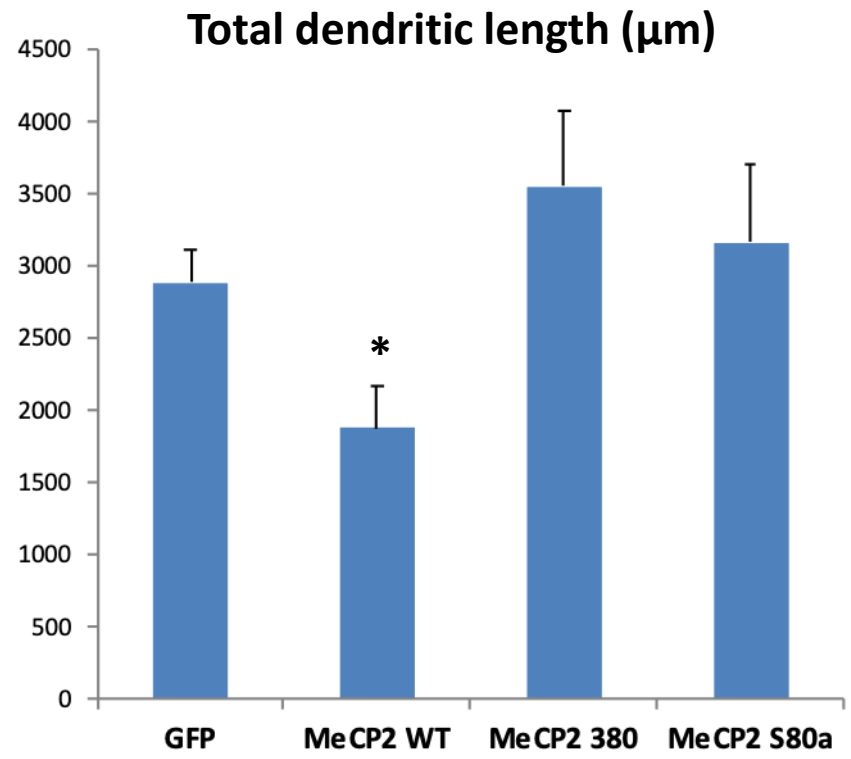
Biolistics—biological ballistics, Bombarding neurons at a high velocity with DNA-coated gold particles

Transfection efficiencies:
low in dissociated cultures (1-5%), higher in cultures slices (several hundred neurons per slice)

Has not been successful in transfecting neurons in vivo

Dendritic growth inhibited by MeCP2

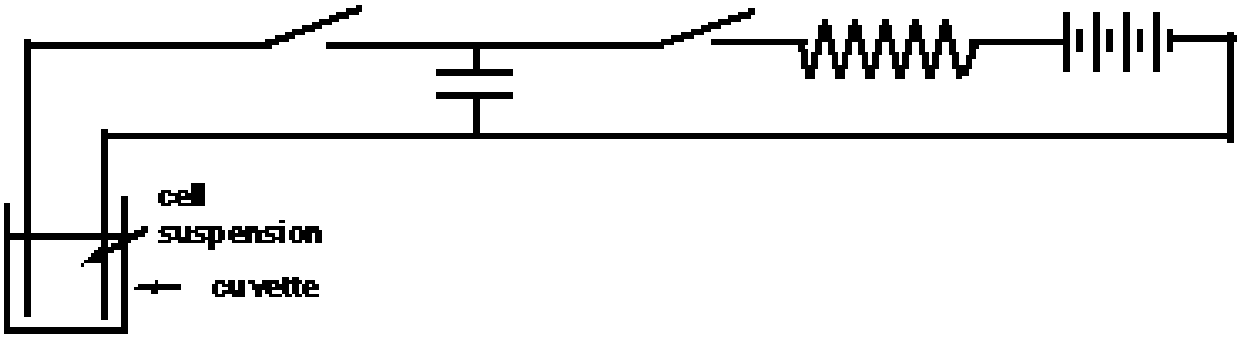




Rat hippocampal slice culture + gene gunning

Cheng et al. *Dev Cell.* 2014

Electroporation—into cell



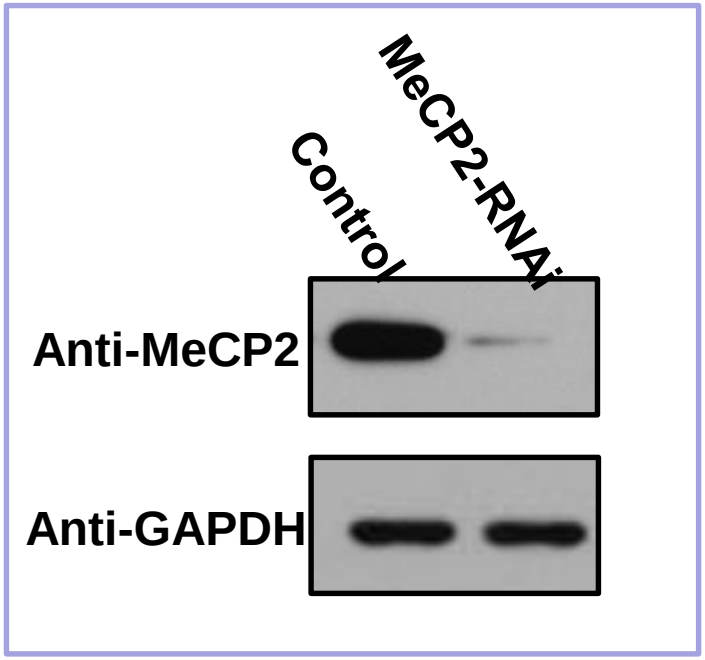
The Nucleofector™ Device

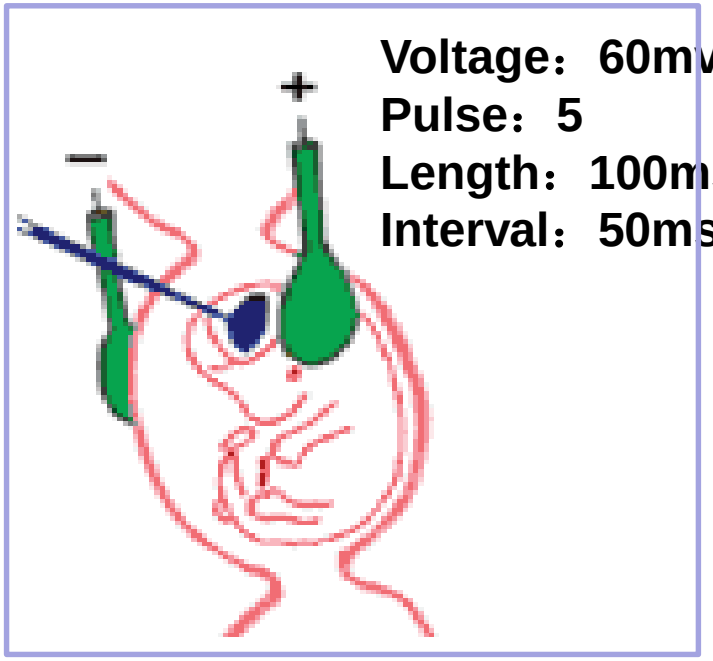
Into tissure

Applications:
In Vivo Gene Delivery

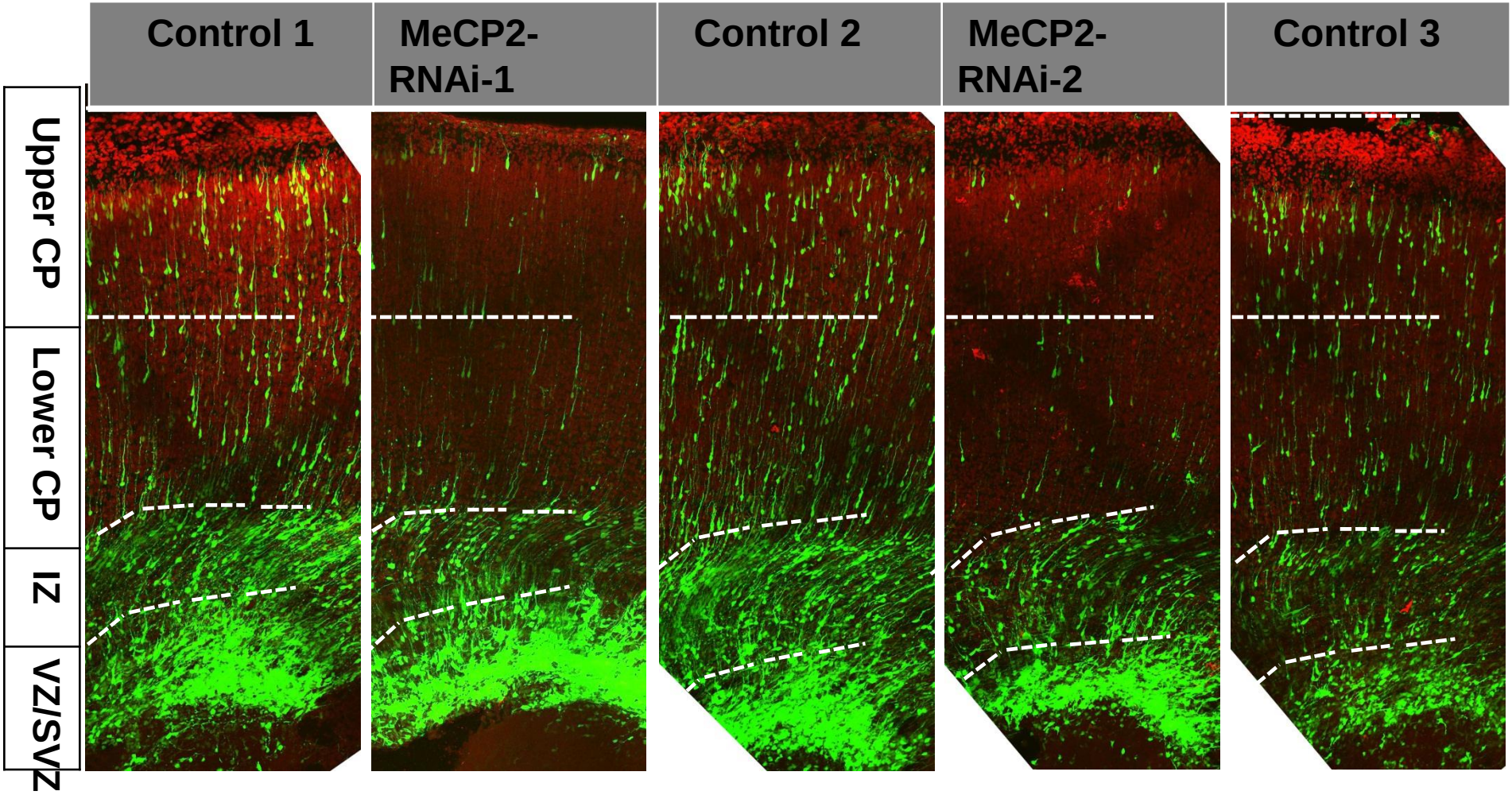
In Utero Electroporation (IUE)

E16 IUE （control/ MeCP2-RNAi）		P0 sacrifice





Deletion of MeCP2 decrease neuronal migration at P0



Microinjection

--Inject plasmid DNA into the nucleus or injecting cRNA into the cytoplasm

--Oocytes(卵母细胞),
Xenopus blastomeres(卵裂球),
Invertebrate neuron

--Mammalian CNS neurons has not become a routine approach

--Labor-intensive

--Small number of neurons at a time

--Single neuron

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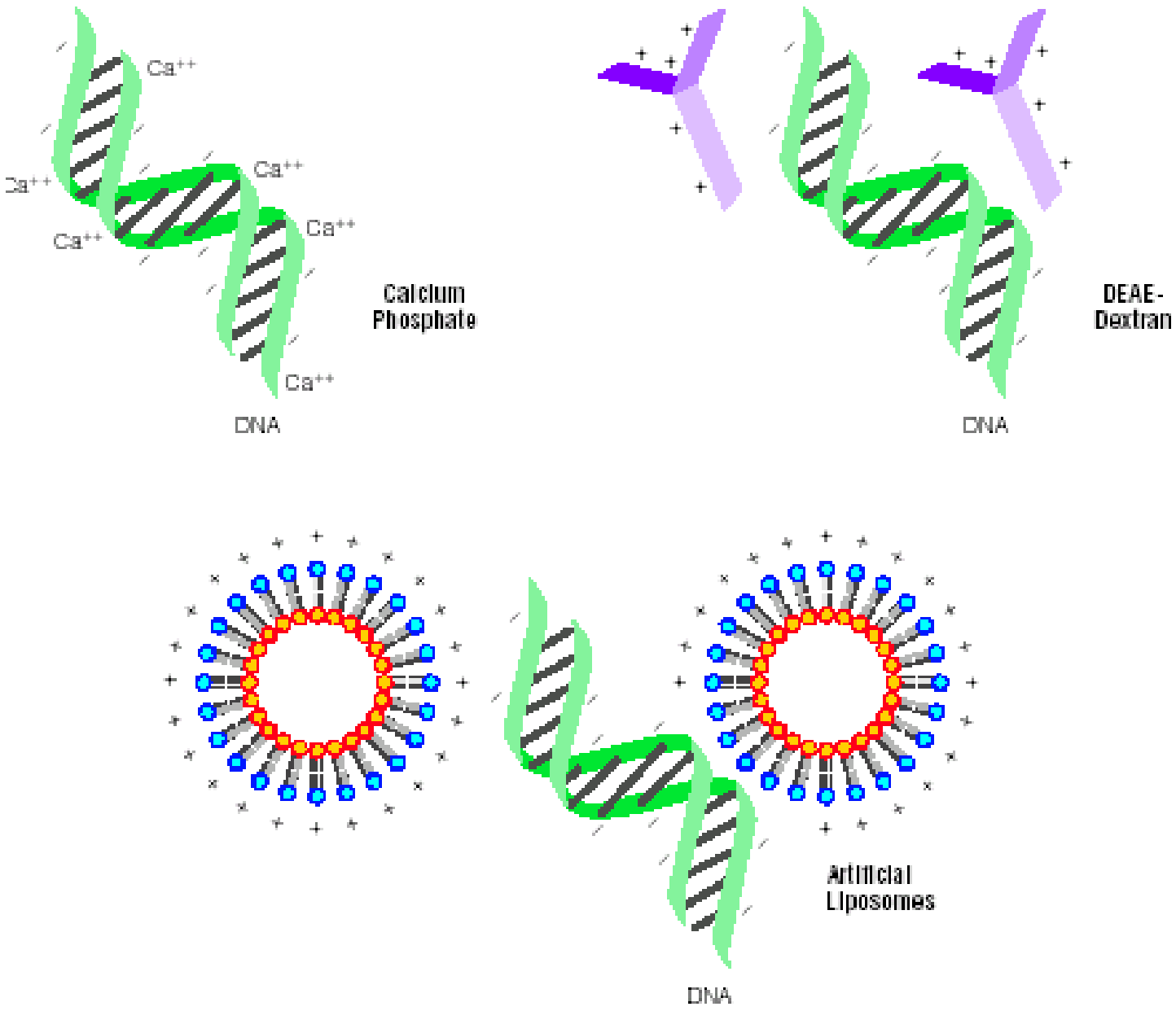
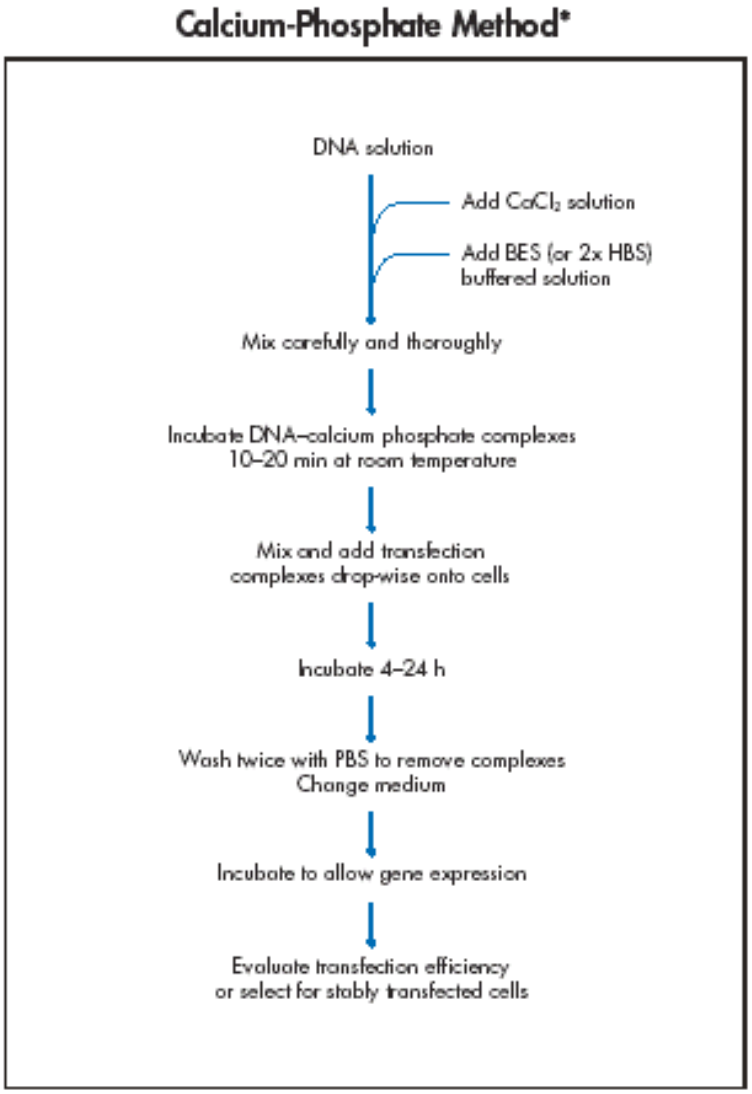
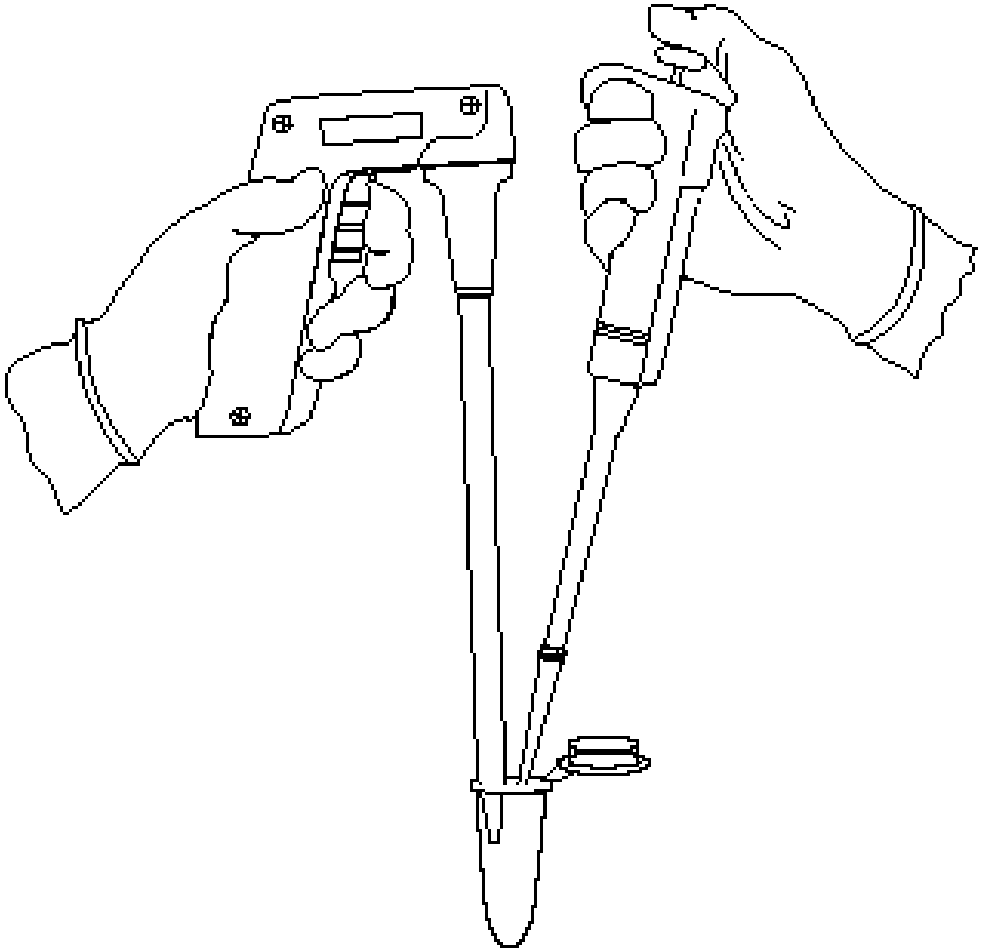


. Schematic representation of various transfection technologies based on chemical reagents.

Transfection--Calcium phosphate

- **Split cells the day before transfection, feed cells with 9.0 ml medium 2-4 hr prior to precipitation**
- **Ethanol precipitate DNA, air dry pellet, resuspend the pellet in 450 ul ddH2O/ and 50 ul 2.5M CaCl2**
- **Place 500 ul of 2XHeBS (PH7.03-7.06) in a 15 ml tube, dropwise mix DNA/CaCl2 mixture with a pasteur pipet, vortex for 5 sec**
- **Sit the precipiate for 20 min at RT**
- **Use a pasteur pipet to distribute the precipitate evenly over the plate for 15 min, then add 9 ml medium**
- **Incubate cells 4-16 hr, remove medium, wash twice with 5 ml 1XPBS, feed with 10 ml fresh medium**
- **Test expression by western-blot or staining**





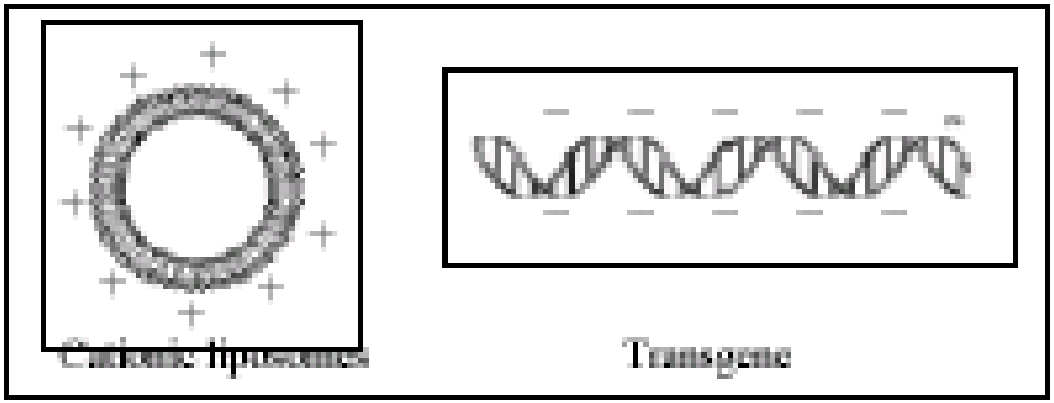
Calcium Phosphate Transfection Technique

Dropwise or not?

Calcium phosphate

- Oldest methods for gene transfer
- Physical basis is unclear, endocytosis ?
- Has not been used to transfect neurons in intact tissue
- Extensively and successfully for dissociated cultures from CNS and peripheral system
- Co-transfection is possible (--100%)
- Drawback: transfection effeciencies are highly variable but consistently low (1-3%); toxic

Liposomes



- Cationic synthetic phospholipid bilayer vesicles.
- Interact spontaneously with DNA to form condensed vesicles.
- Interaction with cell membrane leading to endocytosis.
- Clinical trial for Cystic Fibrosis using liposomes.

Selection of stable transfectants

1. **Using the selected transfection method, cotransfect the cells with a 5:1 molar ratio of plasmid containing the gene of interest to plasmid containing a selectable-marker gene.**

2. **After transfection, allow the cells to divide twice under nonselective conditions.**

3. **Seed the cells at low density in selective medium. Set up at least 5 dishes from each transfected dish to maximize the number colonies that can be isolated and expanded. Replace the selective medium every 4 days (or as needed). After 10 to 12 days, inspect the plates for antibiotic-resistant colonies.**

4. **Select and isolate large (500–1000 cells) healthy colonies (**cloning rings**).**

5. **Fluorescence Activated Cell Sorting**

Targeting to specific cells and tissues

-- Ligands can be added to viruses, liposomes or polycations.
Target specific receptors on the cell surface.
e.g. targets receptors upregulated on tumour cells.

--Tissue specific promoters can control where the gene is expressed.
e.g. muscle specific promoter limits transgene expression to muscle.

--Regulation of gene expression by regulatory promoters.
e.g. Tet on/off system. Gene expression controlled by administering tetracycline.
e.g. Glucose responsive promoters control insulin expression.

Lecture outline

- Cell culture
  dissociated from tissue
  maintenance

- Transfection
  Reporter system
  Physical: biolistics, electroporation, injection
  Chemical: liposome, CaPO4
  Biological: viral-mediated

Non-viral vectors

Advantages

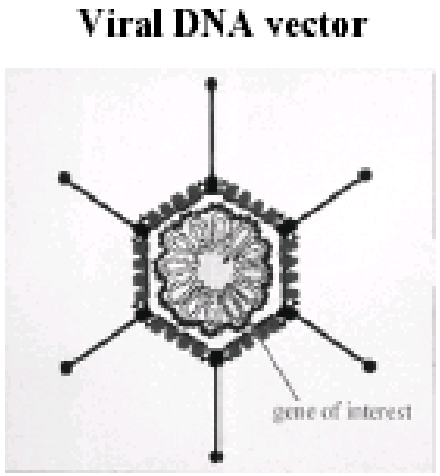
--No viral components
--Safe: non-infectious, no inflammatory or immune response
--No limit to DNA insert size
--Possible to target vectors to specific cells
--Relatively easy to prepare compared to viruses
--Easy to scale-up

Disadvantages

--Inefficient delivery
--Transferred genes do not integrate- short term expression

Virus-based technologies

- Postmitotic neurons, the first case-1988 (Science,241: 1667-1669). A defective HSA1 vector expresses Escherichia coli beta-galactosidase in cultured neurons
- High efficiencies (up to 95% of neurons)
- Replication incompetent, relatively safe
- Locally applied or focally injected into a group of neurons
- Serious limitations: potential toxicity to neurons; limitation on the size of the DNA expression cassette; potential safety hazard to laboratory personnel
- Choose vectors based on infection efficiency, expression level, lag phase, and toxicity for the host cell or animal



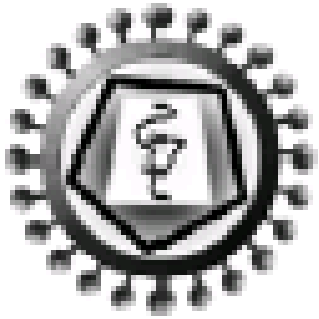
Viruses are natural gene delivery systems.
They have evolved to be highly effective at getting into cells.

Main viral systems

- Lentivirus
- Retrovirus
- Adeno-associated virus

  very low efficiency for culture cells!!!

Retroviruses



Oncoretroviruses (MLV) and lentiviruses (e.g. HIV)

• Enveloped virus containing a ssRNA genome
gag, *pol* and *env* genes responsible for infection and replication .

• *gag*, *pol* and *env* genes removed and produced on separate vectors.
Only vector carrying therapeutic gene has the packaging sequence (Ψ).

• Therapeutic viruses cannot replicate independently and are produced in a packaging cell line.

• These viruses can infect target cell. The ssRNA genome is converted to cDNA and integrates, via Long Terminal Repeats (LTR), at a random point in host genome.

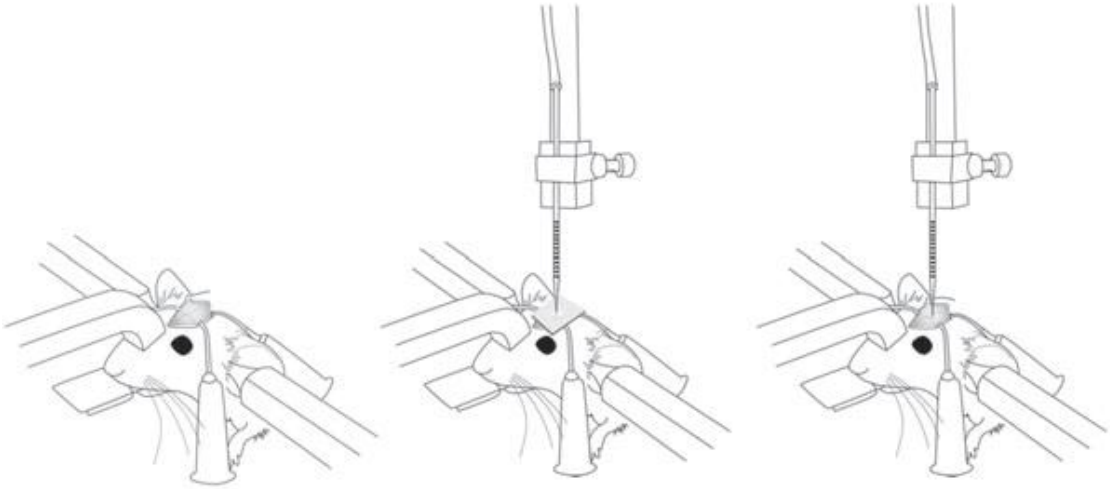
特点，只感染分裂的细胞！

PROTOCOL

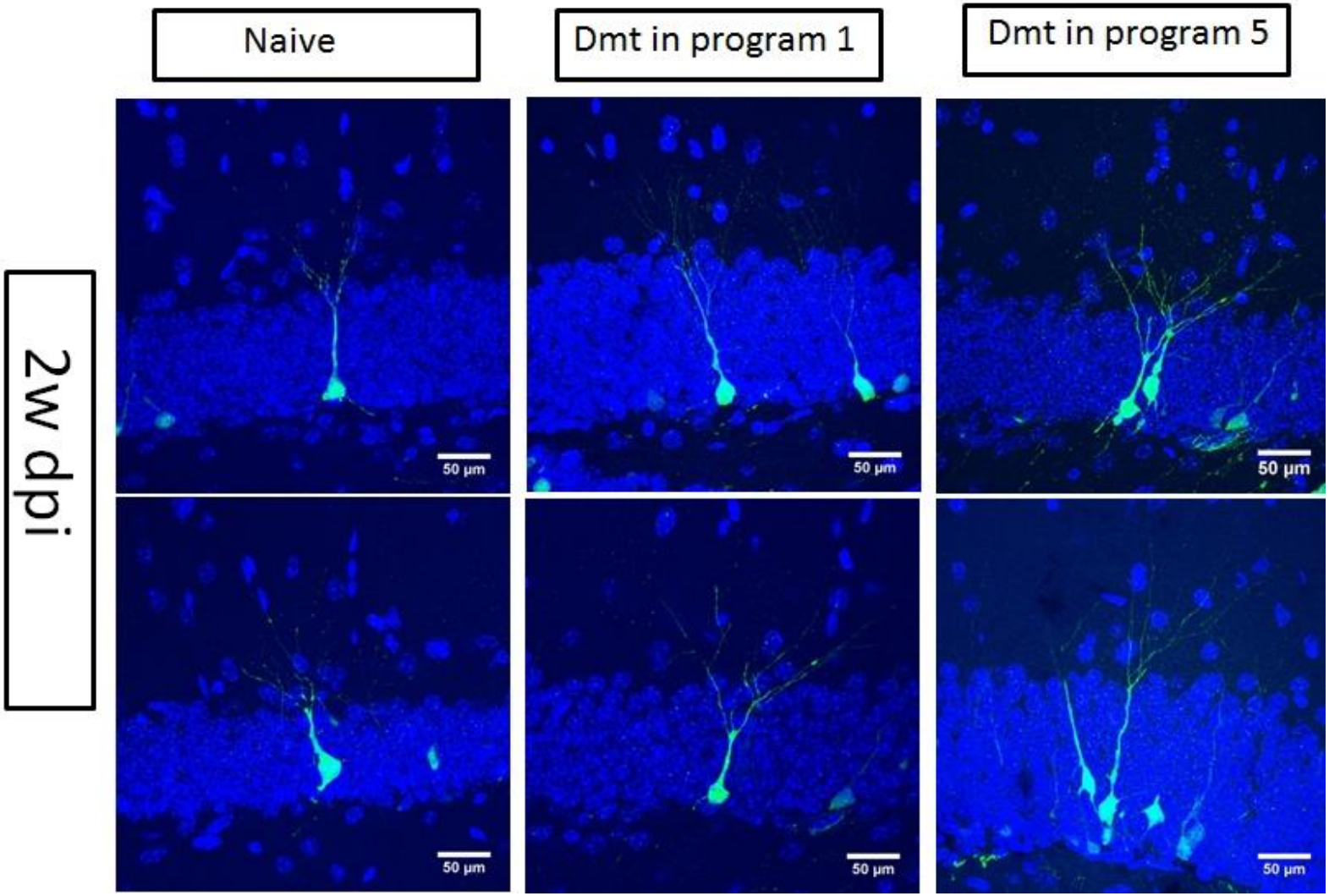
Stereotaxic gene delivery in the rodent brain

Ali Cetin[1], Shoji Komai[2], Marina Eliava[3], Peter H Seeburg[1] & Pavel Osten[3]

[1]Department of Molecular Neurobiology, Max Planck Institute for Medical Research, Jahnstrasse 29, 69120 Heidelberg, Germany. [2]Graduate School of Biological Sciences, Nara Institute of Science and Technology, 8916-5 Takayama, Ikoma, Nara 630-0192, Japan. [3]Department of Physiology, Feinberg School of Medicine, Northwestern University, 303 East Chicago Avenue, Chicago, Illinois 60611, USA. Correspondence should be addressed to P.O. (p-osten@northwestern.edu).



Retro-EF1a-GFP injected into hippocampus DG for new born neurons



Zhang et al. *Mol Brain.* 2014

Retroviruses

Advantages

- Stable long term gene expression
  Integration of therapeutic gene into host genome.

- High efficient of gene delivery especially to dividing cells
  (e.g. rapidly dividing tumour cells).

- Suitable for ex vivo treatment

Retroviruses

Disadvantages

- Maximum insert size 7 - 8 kb

- Oncoretroviruses (e.g. Mouse Leukemia Virus) can only infect dividing cells, although lentiviruses (HIV) can also infect non-dividing cells.

- Safety concerns

  - viral proteins can invoke immune response

  - Recombination may produce replication-competent virus

  - Random integration may lead to oncogenic or tumour suppressor gene activation (insertional mutagenesis)

Example:
SCID clinical trial – 2 boys develop leukemia after retroviral insertion of therapeutic gene activates oncogene

Adeno-Associated viruses (AAV)

Non-pathogenic human parvovirus

- ss DNA genome. Two genes, *rep* (replication) and *cap* (capsid structure) together with terminal repeat containing a promoter.

- Rep and cap genes replaced by transgene. Production dependant on a helper virus, usually adenovirus, and helper cell line to proliferate.

- AAV vector integrates into host genome.

- AAV combine advantages of both retroviruses and adenoviruses.

PROTOCOL

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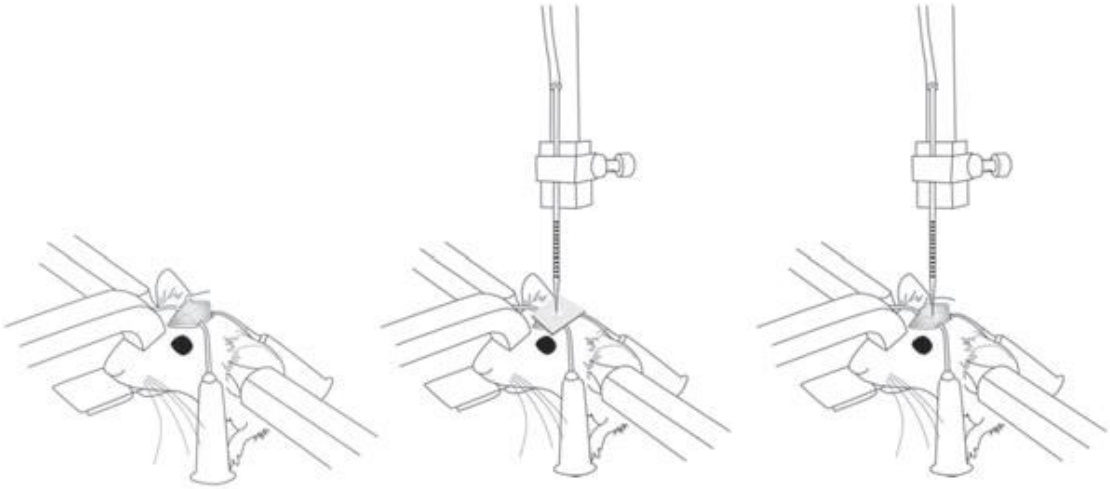
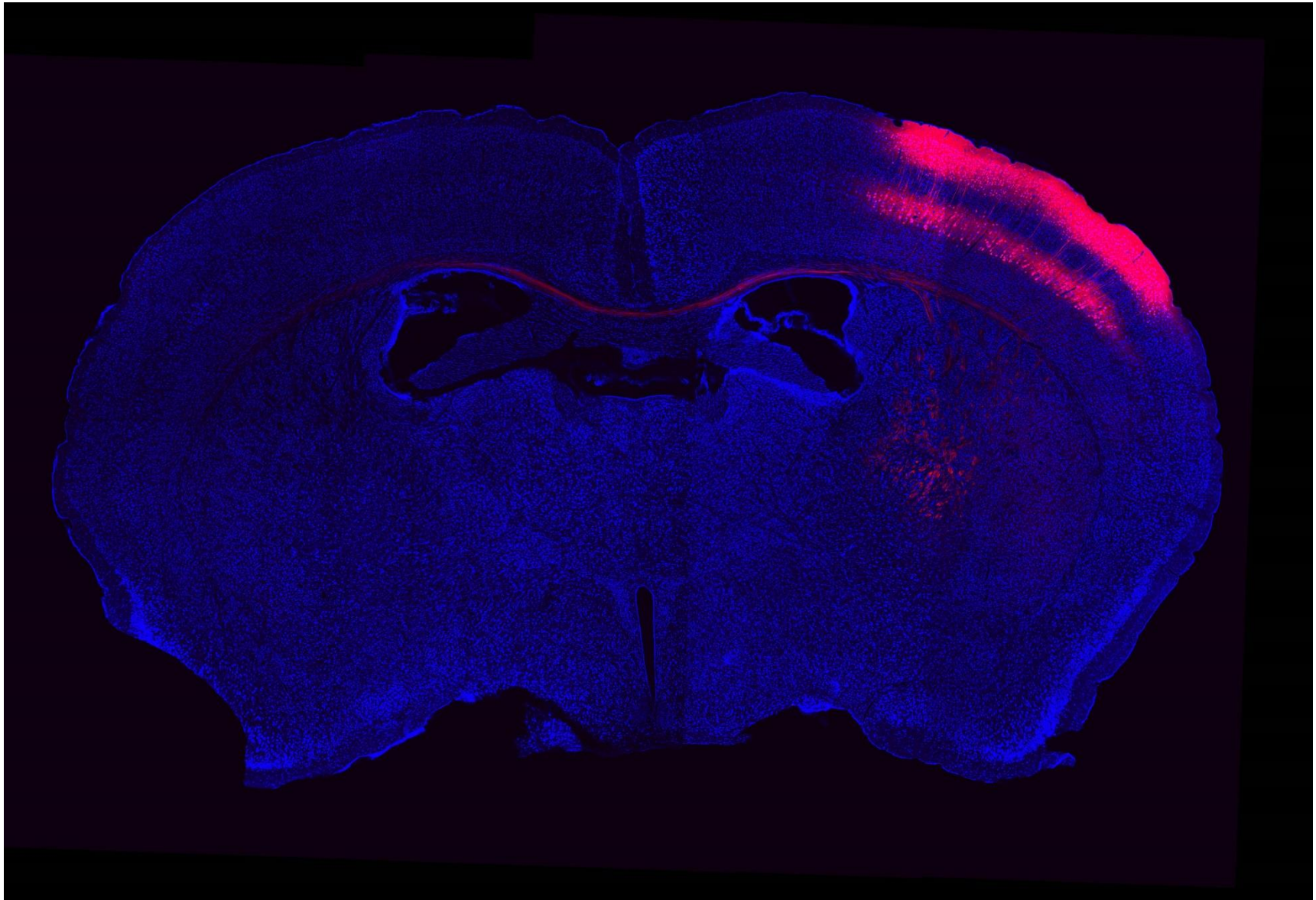


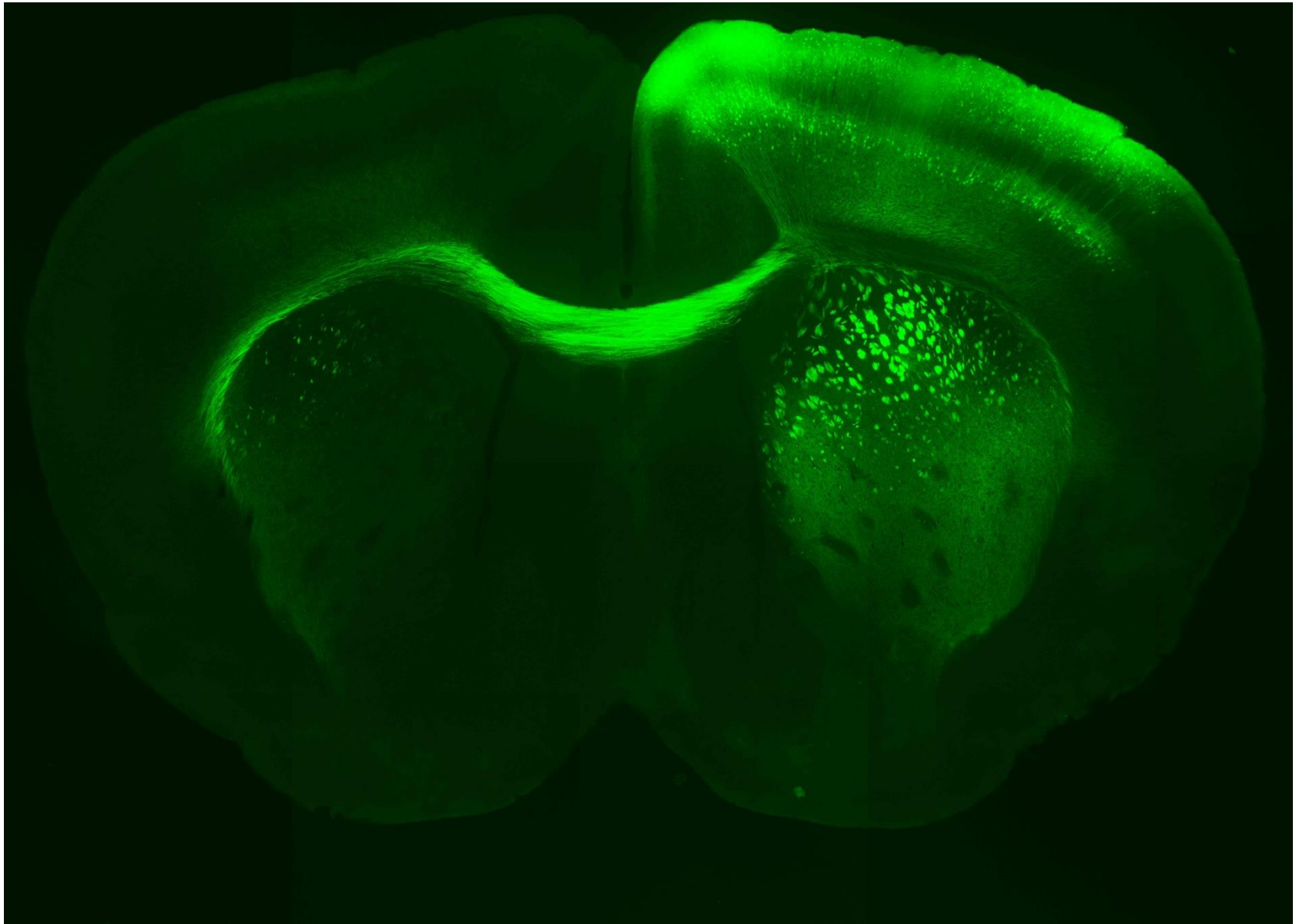
AAV-hSynapsin-GFP， 10~12滴度， S1, L2/3, 10day

李敏寅
神经所于翔组



AAV-hSynapsin-GFP， 10~13滴度，FC，14day

Patricia
马普计算所Khaitovich lab



Adeno-Associated viruses

Advantages

- Infects dividing and non-dividing cells
- Non-pathogenic, non-inflammatory, non-immunogenic
- Can integrate into host genome (at specific site)
- Long-term expression observed

Disadvantages

- Max insert size 5 kb
- Difficult to produce on large scale – it kills the packaging cells
- Potential problems with insertional mutagenesis
- Risk of generation of replication competent virus

Detect gene expression

Protein level

--Western blot

--Immunostaining, FACS

--Activity (Lucf, β-gal, GFP, etc)

mRNA level

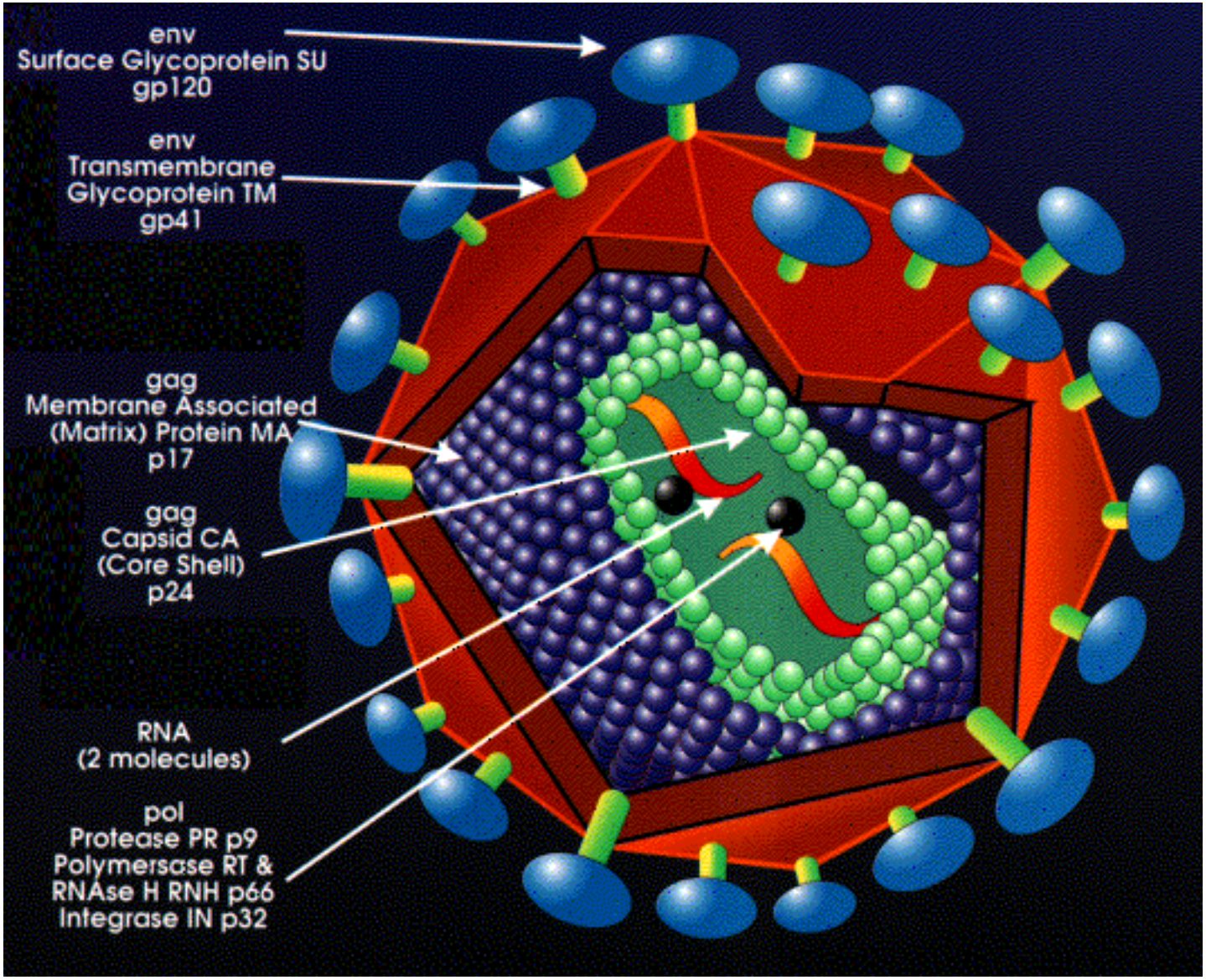
--RT-PCR, real time PCR

--Northern blot, RNase protection assay

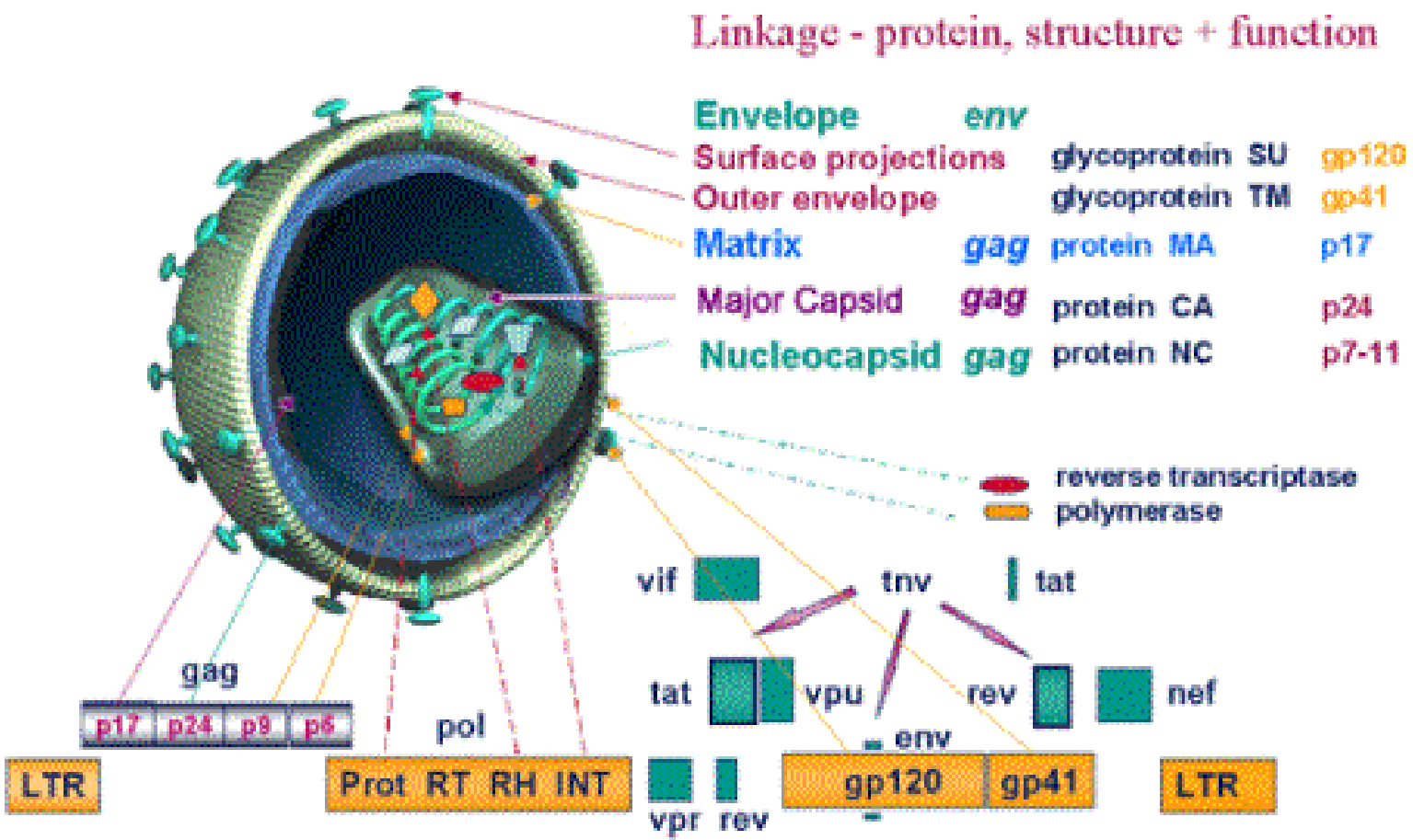
--In situ hybridization

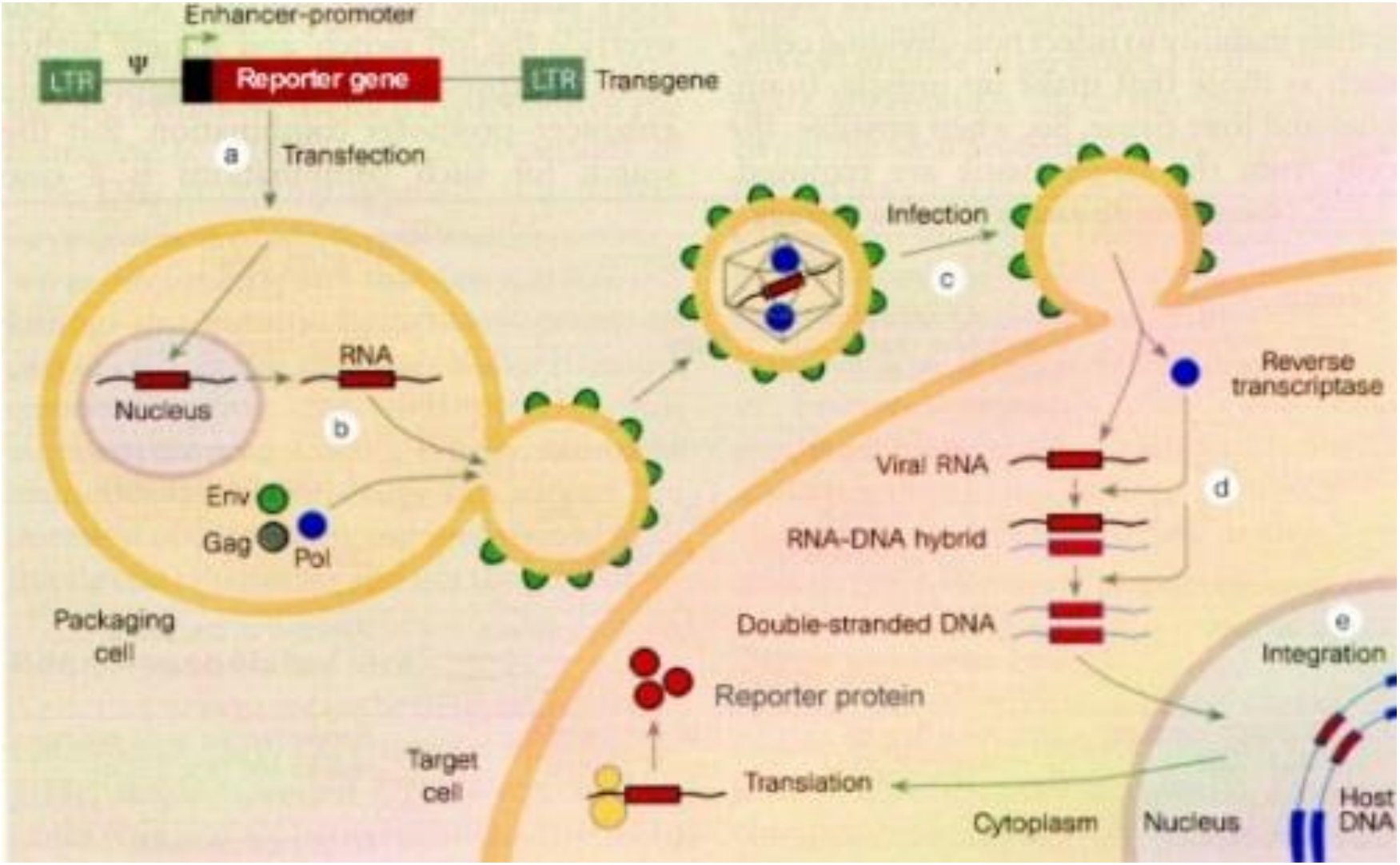
Choose the one which is:
most labor-saving, less toxic, most effective and economical

Lentivirus


env
Surface Glycoprotein SU
gp120
env
Transmembrane
Glycoprotein TM
gp41
gag
Membrane Associated
(Matrix) Protein MA
p17
gag
Capsid CA
(Core Shell)
p24
RNA
(2 molecules)
pol
Protease PR p9
Polymersase RT &
RNAse H RNH p66
Integrase IN p32

Genome map of Lentivirus




Enhancer-promoter
LTR
Ψ
Reporter gene
LTR
Transgene
a
Transfection
Infection
c
RNA
Nucleus
b
Reverse transcriptase
Viral RNA
d
Env
Gag
Pol
RNA-DNA hybrid
Packaging cell
Double-stranded DNA
e
Integration
Reporter protein
Target cell
Translation
Cytoplasm
Nucleus
Host DNA

Lentiviral vector

2-3 packaging plasmids

↓ **293T**

Lentivirus particles

A.Neurons of interest
B. Brain regions of interest

↓ **Or, C. make a mice**

Integration into host cell genome

Stable integration ↓ **Cannot replicate**

express anything you want

How to kill them?

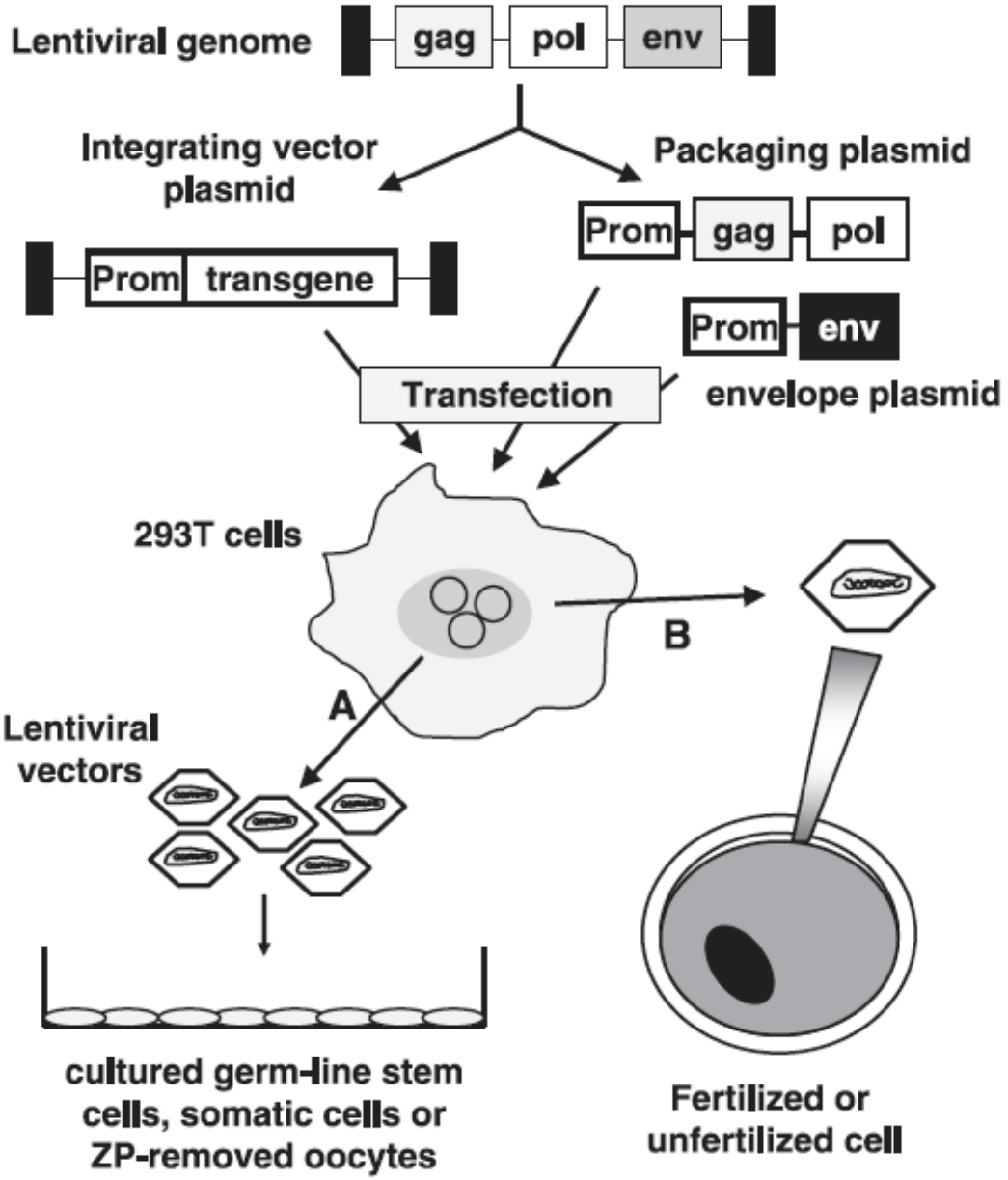
Lipids in envelope, so 75%EtOH

Lentiviral vectors: are they the future of animal transgenesis?

Frank Park

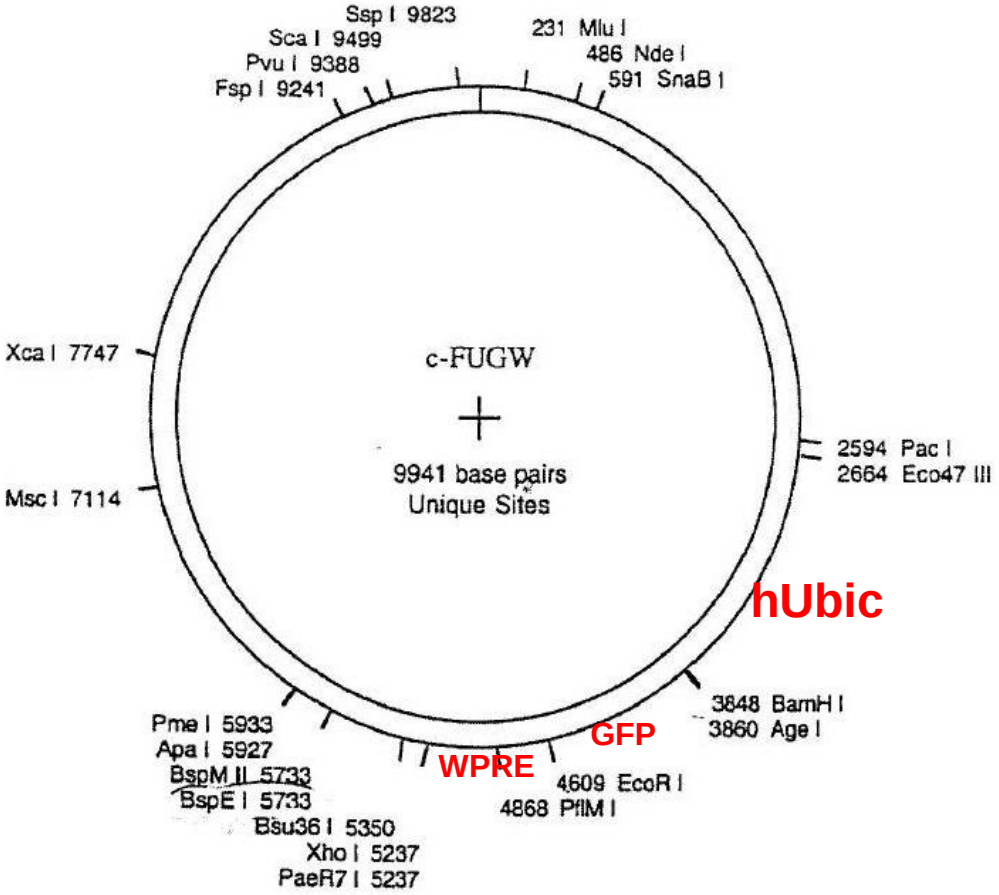
Department of Medicine, Kidney Disease Center, Medical College of Wisconsin, Wauwatosa, Wisconsin

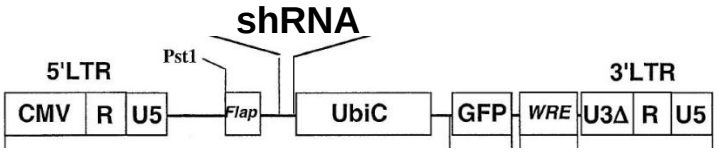
Submitted 27 March 2007; accepted in final form 5 August 2007



The actually useful lentiviral vector: FUGW

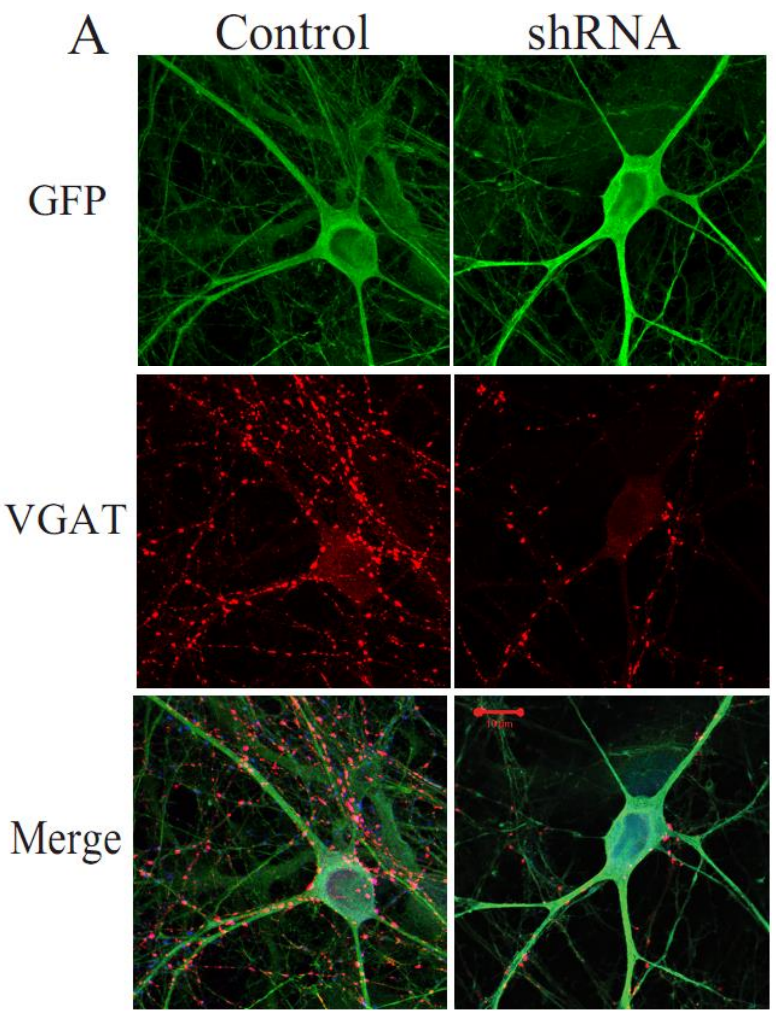
Source: Lois et al. *Science* 2002 Feb 1;295(5556):868-72 Germline transmission and tissue-specific expression of transgenes delivered by lentiviral vectors.

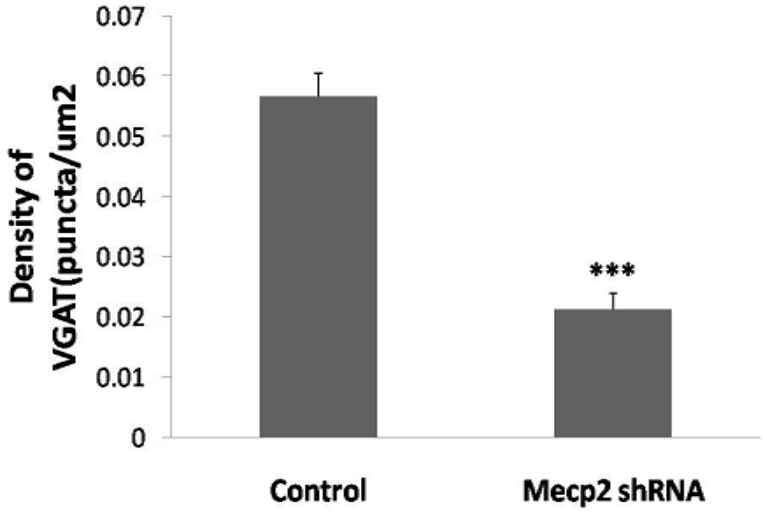


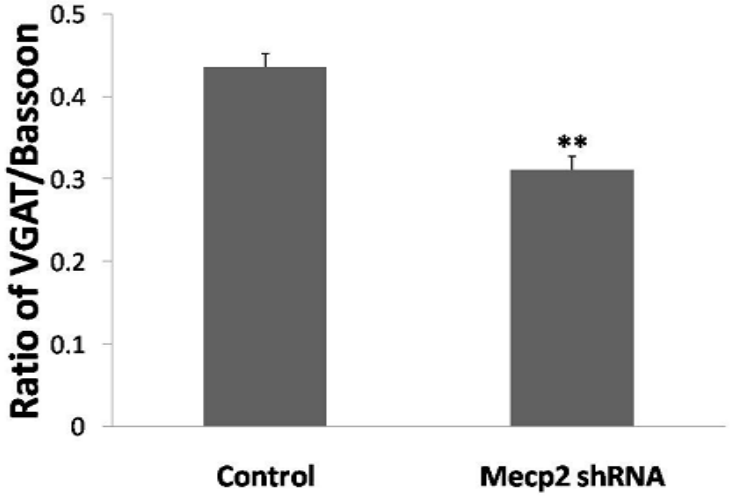


Inhibitory synaptic development defects in MeCP2 RNAi

Lenti-shMeCP2-H1—Ubi-GFP-WRE







Xinyu Liu

PROTOCOL

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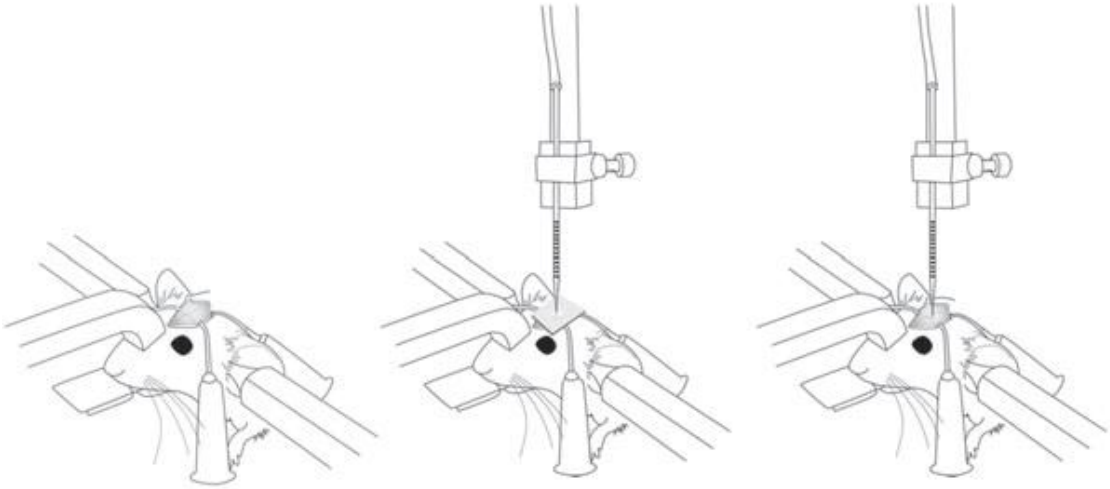
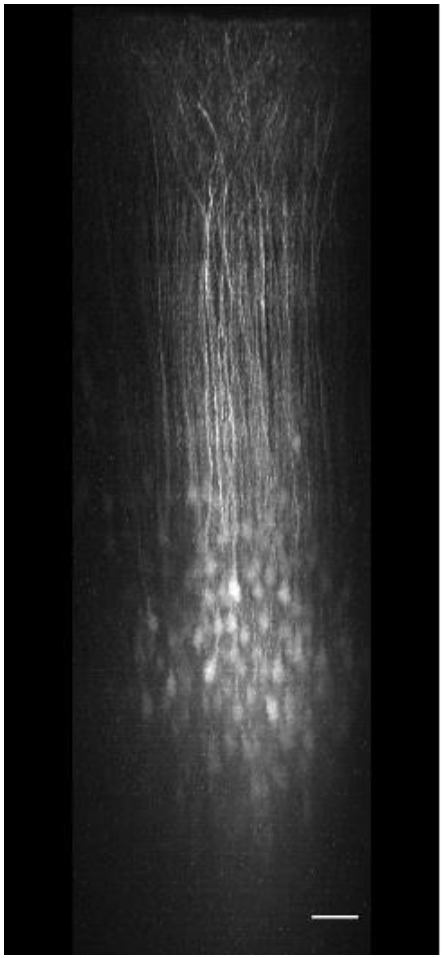
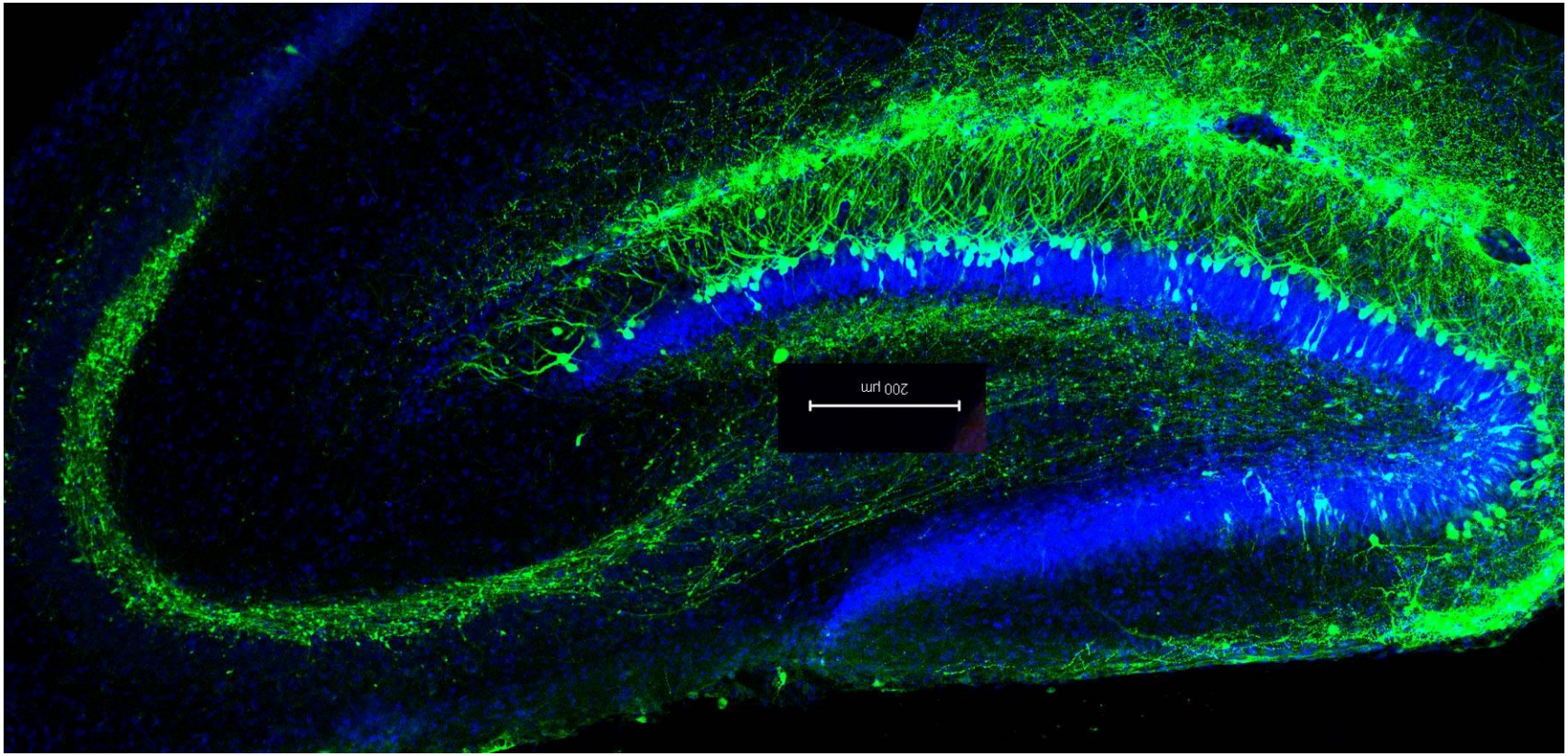


Cortical layer 2/3 neuron, *in vivo*



Three-dimensional distribution of FCK(1.3)GW-infected neurons in cortical layer 2/3 of rat neocortex (P28) in vivo.
The images are maximum-intensity side projections from a stack of fluorescence images recorded 7 days after virus infection.
Individual focal planes were recorded in 2.0-mm steps starting from 860-mm depth below the pial surface. (Scale bar, 40 mm.)

Pictures from the Ghosh lab at UCSD



Picture courtesy of Dr. Megan Williams

P5 injection, P12 image, 1 microliter not very high titer lentivirus (FSyn1.1-G

Lecture outline

- Cell culture

  dissociated from tissue

  maintenance

- Transfection

  Reporter system

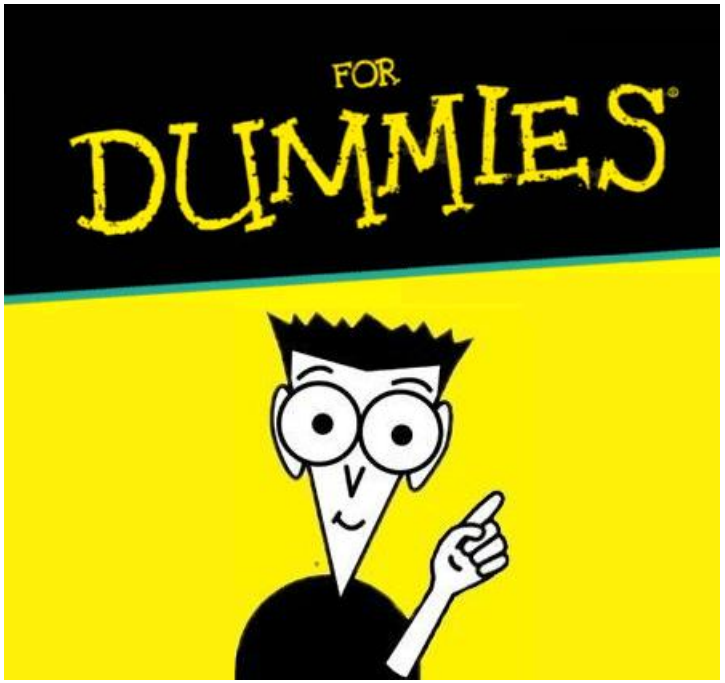
  Physical: biolistics, electroporation, injection

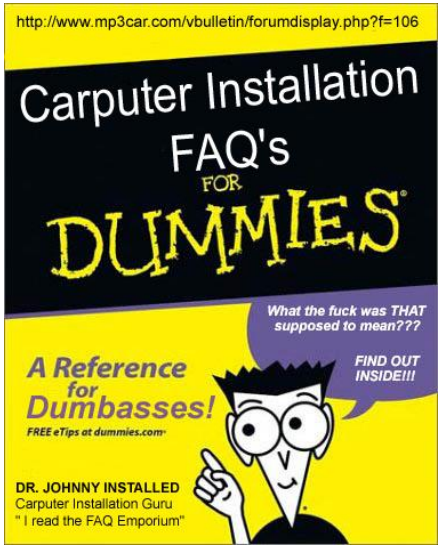
  Chemical: liposome, CaPO4

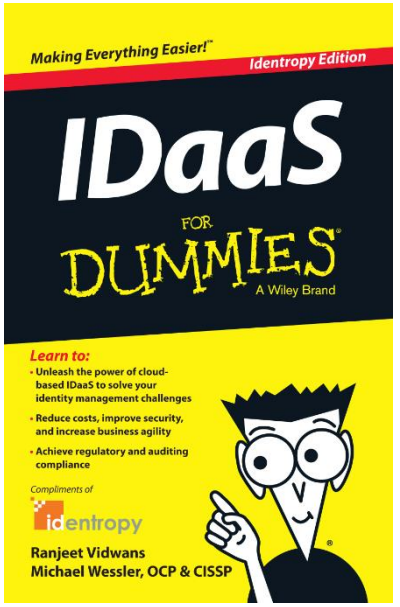
  Biological: viral-mediated

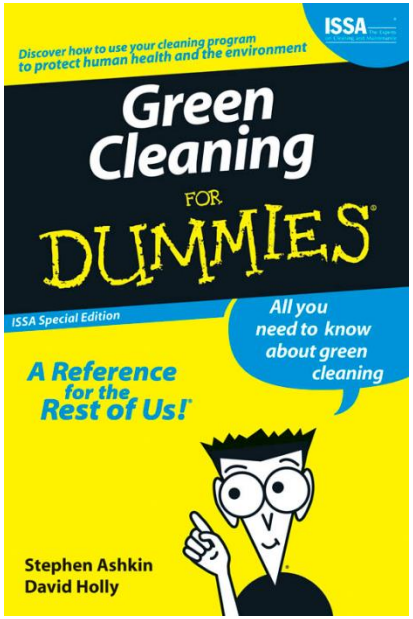
- **Crash course--Dummies book for studying gene function!**

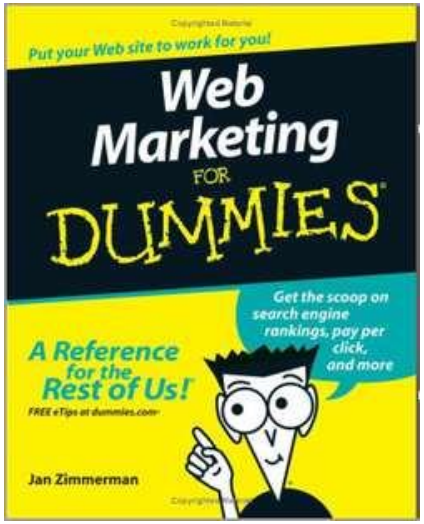
Dummies book for studying gene function











Study of the role of gene X in synapse formation

Background

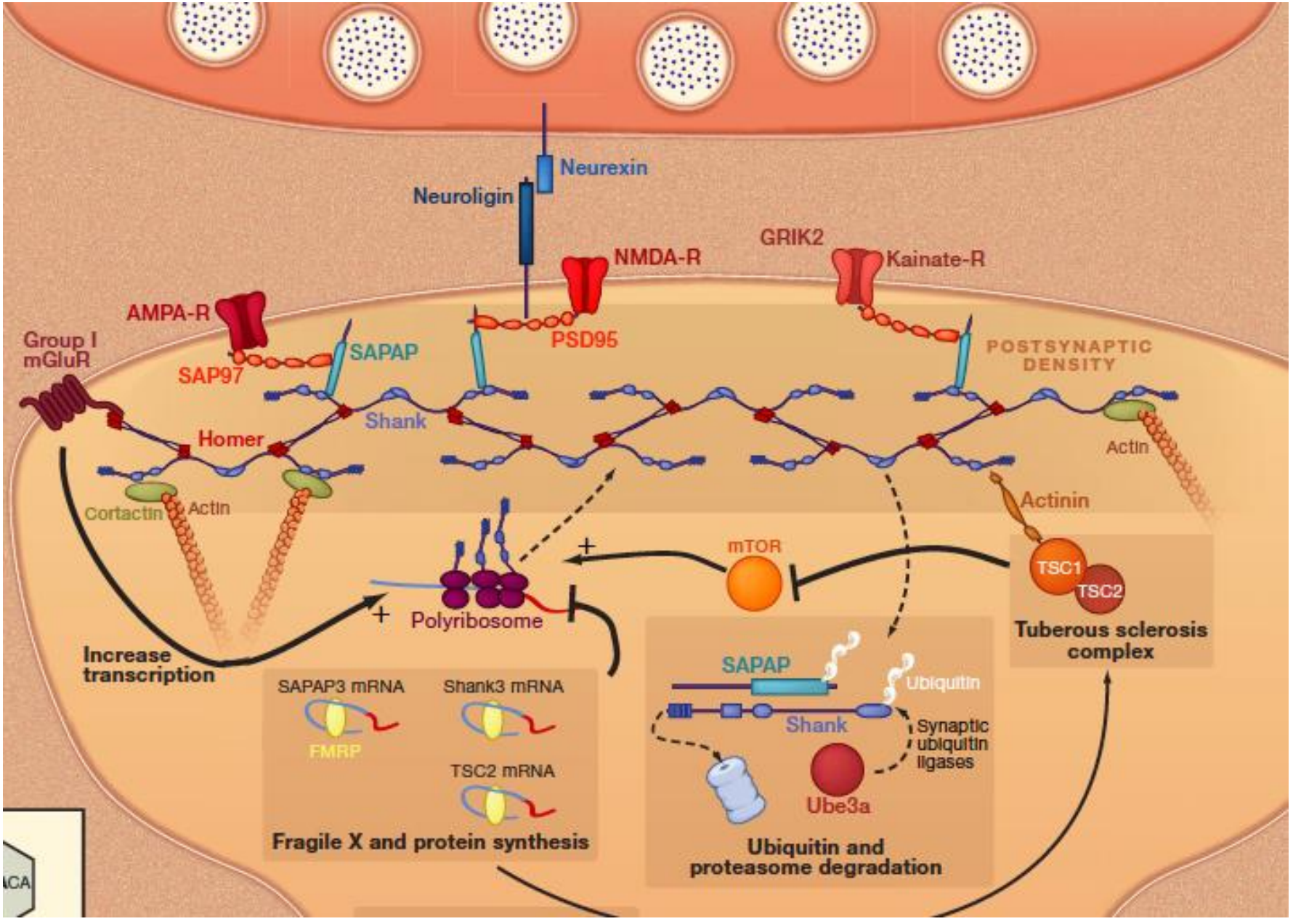
Gene X is highly expressed in mouse neuron and may encode some cytoskeleton protein. It is critical and interesting to determine whether gene X contributes to synapse formation.

Synapse :
connecting dots for neural circuitry

SnapShot: Autism and the Synapse

João Peça,[1] Jonathan Ting,[1] and Guoping Feng[1]
[1]McGovern Institute for Brain Research, Massachusetts Institute of Technology, Cambridge, MA 02139, USA

Cell



Study of the role of gene X in synapse formation

1. Design and screen short hairpin RNA again X

1. Transfection to test the role of X in neuron

2. in vitro and *in vivo* analysis for synapse

Design short hairpin RNA

- siRNA or shRNA?

  siRNA 壕的选择， shRNA价格便宜量又足

- Short hairpin RNA design

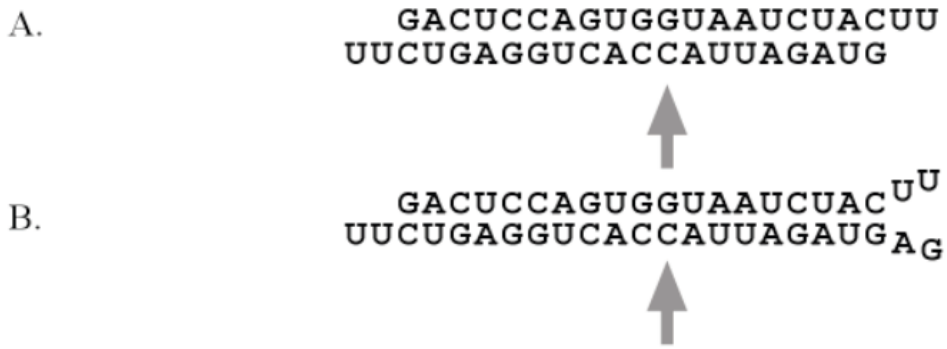
  http://www.oligoengine.com pSuper vector protocol

  http://rnaidesigner.lifetechnologies.com

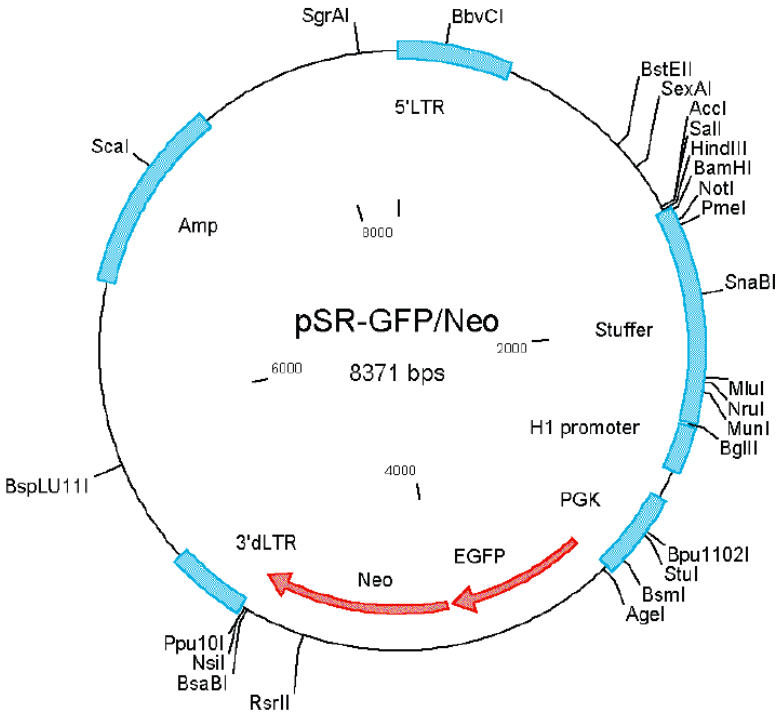
- RNAi design, 运气呢，还是运气呢？3-5对候选。

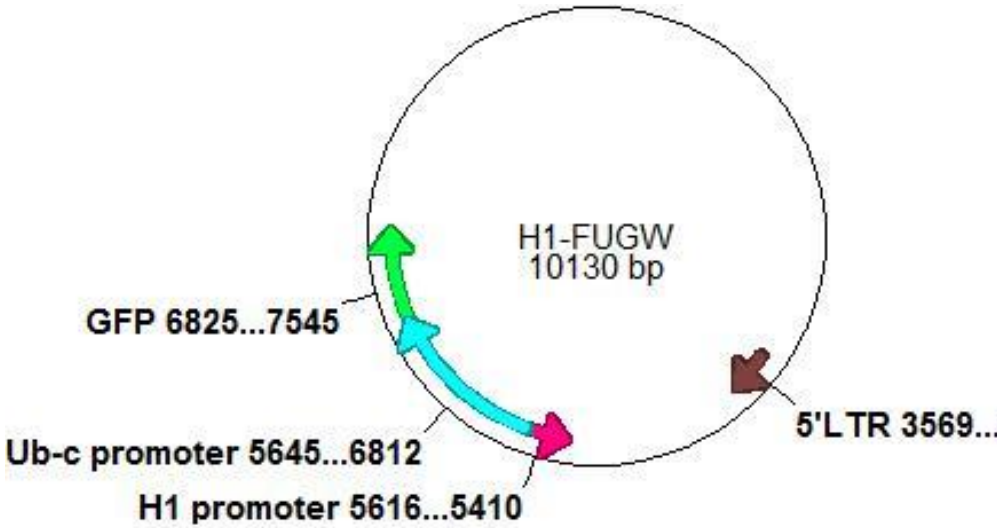
- Short hairpin RNA expression vector, U6, H1, RNApol-III

- http://www.addgene.org/25870/ FUGW-H1 vector



ssed to functional siRNA.





Validation of shRNA efficiency

- Test on endogenous proteins!

Working on transfected protein, may not working on endogenous protein

- For primary culture neuron

Electroporation pSuper-shRNA, q-pcr or western blot.

Need to over 50% transfectin efficiency.

- For human proteins, may use Hela or 293T

Difference between 293T and 293

Transfection

- On culture neuron
  lipofectAMINE 2000, Ca_2PO4, lentivirus

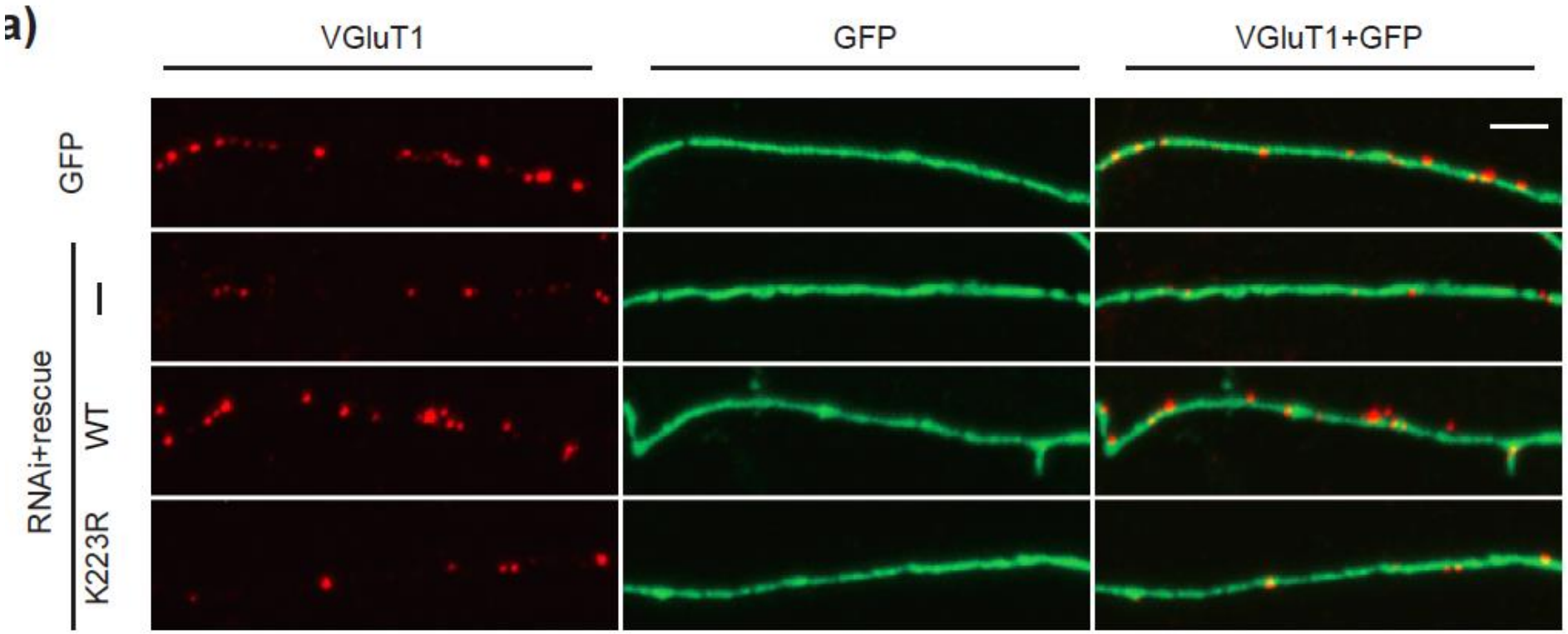
- Ex vivo
  gene gun, In Utero Electroporation

- In vivo: gene knockout mouse

Primary culture neuron

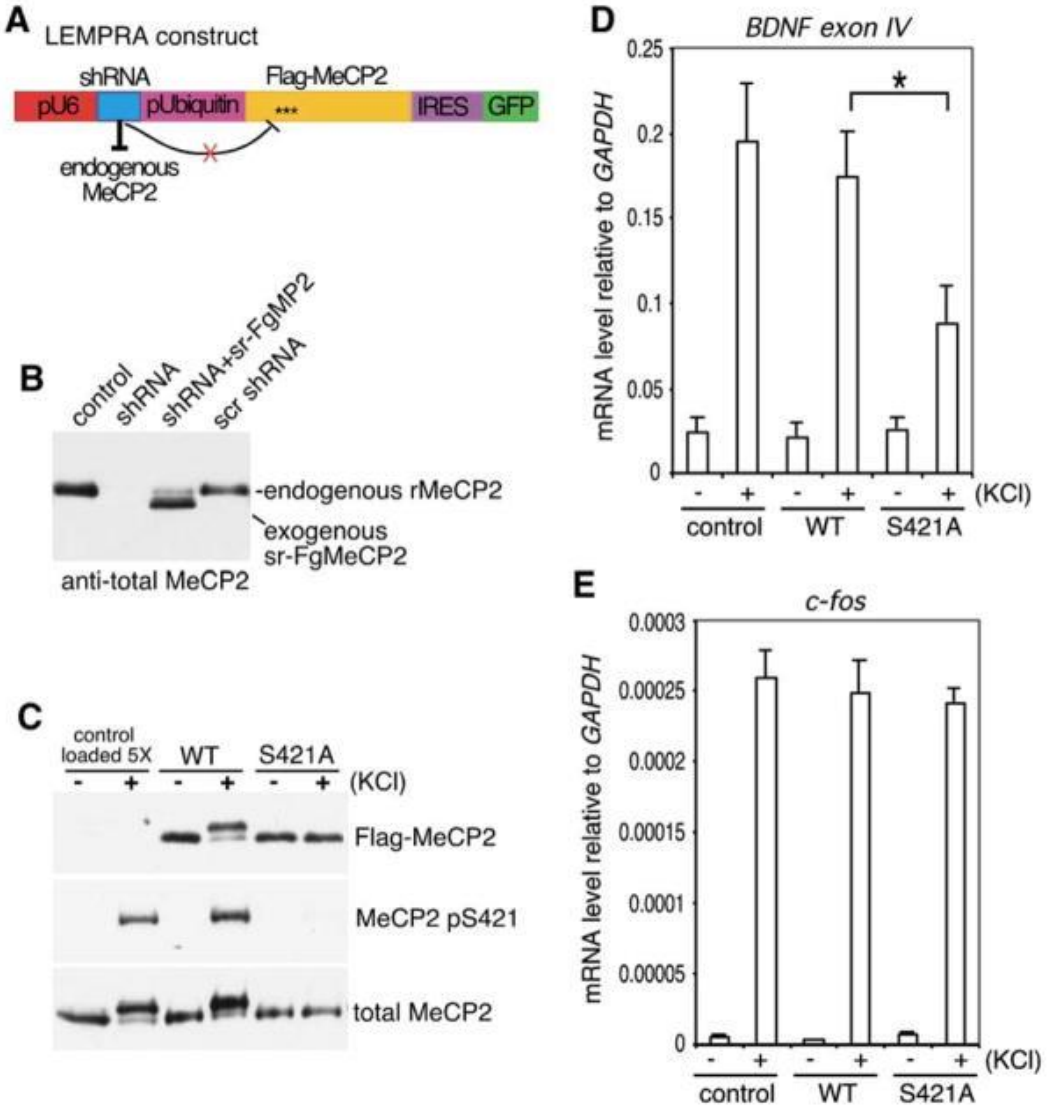
1. shRNA co-transfection with GFP

2. Rescue construct for shRNA! CRITICAL!!!
19nt, Same sense mutations for 4-6 amino acids, swing the third nt



Cheng et al. *J Neurochem*. 2014

shRNA and rescue constructs

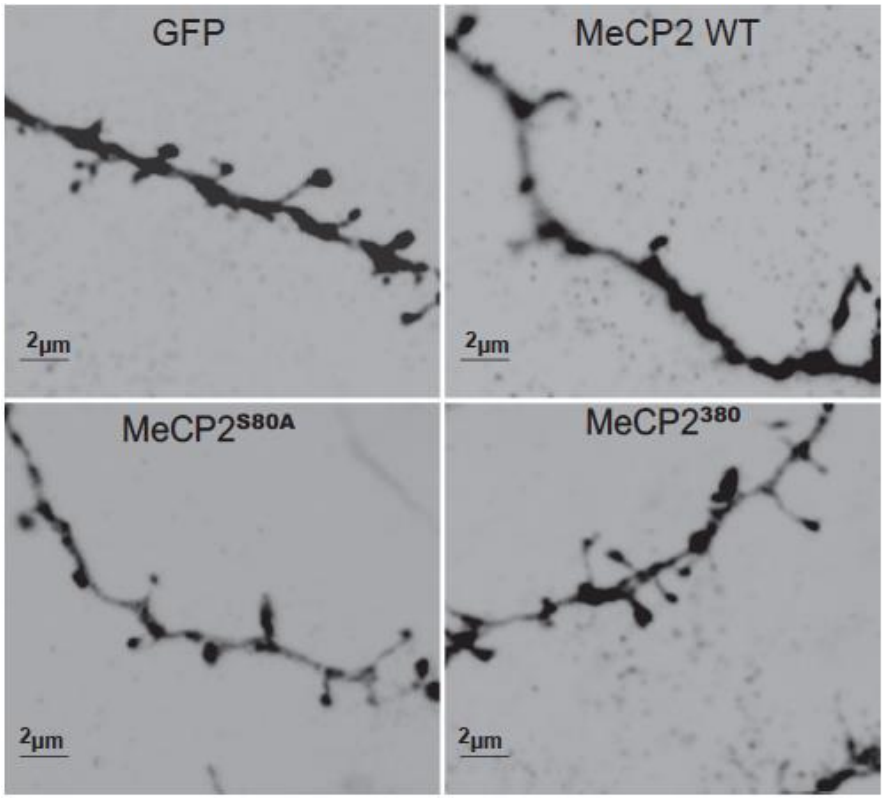


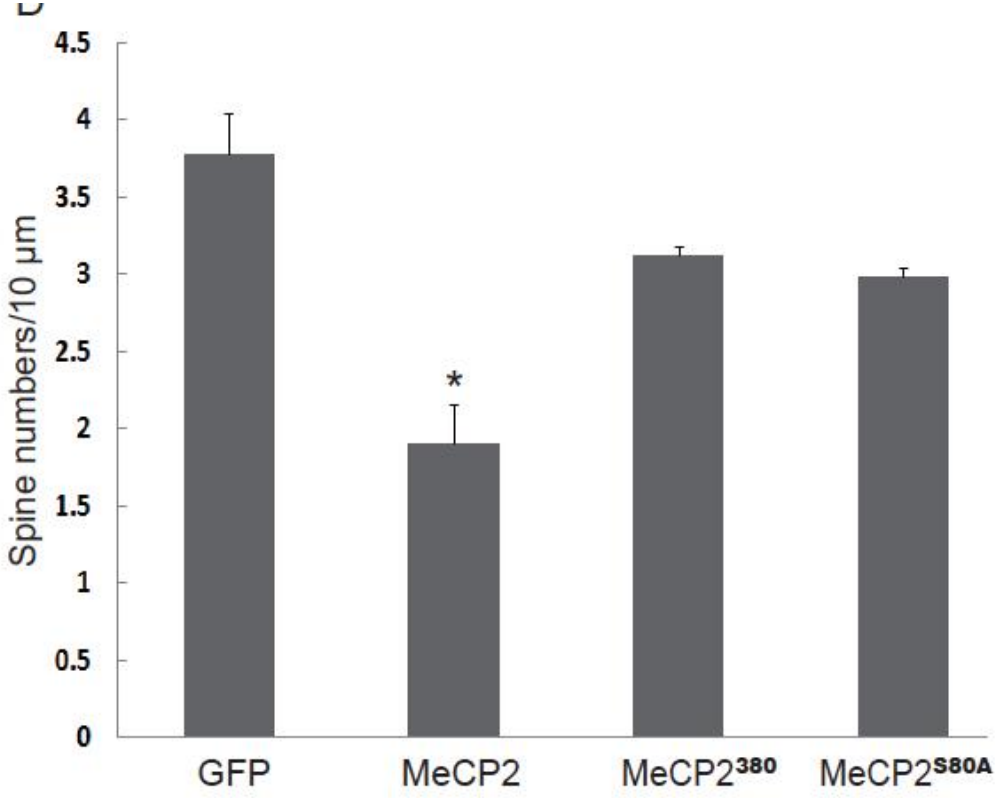
1. **shRNA knockdown endogenous MECP2**
2. **Flag-MECP2res cannot be knockdown by shRNA**
3. **shRNA + wt MECP2res rescue**
4. **shRNA + mutant MECP2 res to test whether mutant MECP2 works as wild type MECP2.**

Zhou et al. 2006 Neuron

Ex vivo 1

Gene gun-based transfection for brain slices

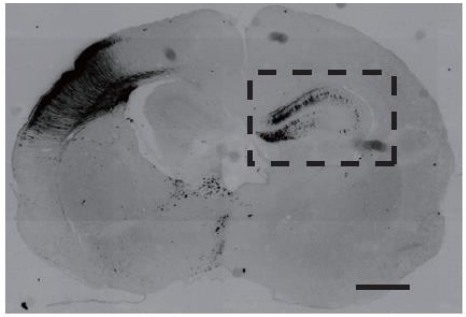


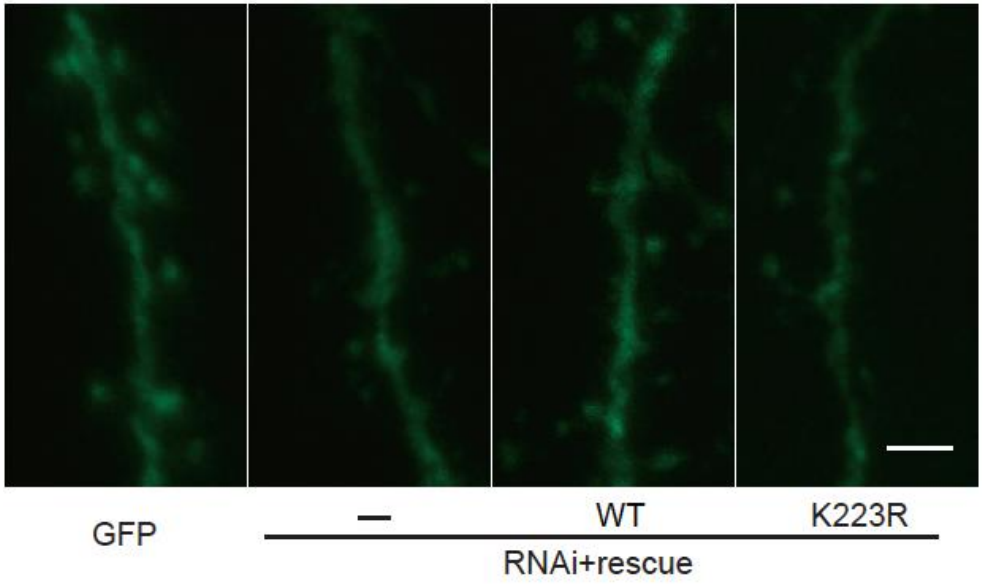


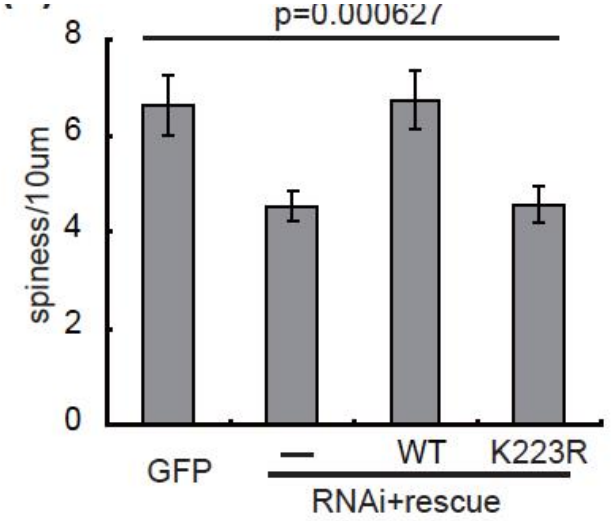
Cheng et al. *Dev Cell.* 2014

Ex vivo 2

in utero electroporation for intact animal







Cheng et al. *J Neurochem*. 2014

Lecture outline

- Cell culture

  dissociated from tissue

  maintenance
- Transfection

  Reporter system

  Physical: biolistics, electroporation, injection

  Chemical: liposome, Ca2PO4

  Biological: viral-mediated
- Crash course--Dummies book!

Thanks for your attention!

PLEASE don’t email me for final !!!