ΠΜΣ Τμήματος Ιατρικής (BIE) 2024-5

Αρχές και πειραματισμός με ζωικά μοντέλα στη βιοϊατρική έρευνα

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Lecture objectives

- Why do we need animal models?
- Types of animal models
- Humanized mice
- Allometric dose scaling
- Basic techniques applied in animal models (H&E, fluorescence, bioluminescence)
- Animal handling: processes, indications, regulations, rules

Why do we need and use animal models?

- Ethical and legal reasons against human studies without substantial evidence or justification from animal models (exception: Ebola crisis).

- Animal models help to understand the normal physiology and diseases conditions (pathology) (targets).

- Animal models are crucial for toxicology studies, dose estimation, efficacy assessments and pharmacology (PK and PD).
  (drug candidates)

Institutional Animal Care and Use Committee (IACUC) 1986

- Identification of the species and approximate number of animals to be used.
- Rationale for involving animals, and for the appropriateness of the species and numbers used.
- A complete description of the proposed use of the animals.
- A description of procedures designed to assure that discomfort and injury to animals will be limited to that which is unavoidable in the conduct of scientifically valuable research, and that analgesic, anesthetic, and tranquilizing drugs will be used where indicated and appropriate to minimize discomfort and pain to animals.
- A description of any euthanasia method to be used.

Invertebrates

- *Drosophila Melanogaster* (fruit fly)
- *Caenorhabditis elegans* (round worm)

- Cost effective
- Short life cycle
- Great for genetics, pathway dissections

Vertebrates

- Fish
- Frogs
- Chicken, birds

- Rodents (Mice, Rats)
- Rabbits
- Dogs
- Cats
- Pigs
- Monkeys

Mice

Availability

Size

Low cost

Ease of handling

Fast reproduction rate

Genetic manipulability

Non-human primates

- Closeness to human in so many ways
- Their brains share structural and functional features with human brains. They feel pain similar to humans
- Many ethical concerns (decreased NIH funding is expected in the future)

- Studies:
  - Infectious disease
  - Toxicity
  - Organ transplants
  - Etc.

Animal models

Strengths

- Investigators control environments, genetics, diets
- Independent variable(s) can be manipulated at will
- Gene/Genetic approaches can be used to establish functions and targets, cause-effect
- Shorter experimental cycle, cost-less than human studies

Animal studies

Weaknesses

- Models do not always correspond to human metabolism and disease
- Dose ranges are often extreme and unrealistic for human translation
- Routes of exposure and testing not practical for humans

Need for a new preclinical model

Real incident:

1993, NIH: Small phase 2 trial of Eli Lilly's "fialuridine" (experimental hepatitis B drug) fails:

From 15 patients, 5 died and two required emergency liver transplants

Reason??

Toxicity testing in mice, rats, dogs and non-human primates had shown no ill effects

Years later… Drug uptake via transporter expressed in human mitochondria, but not in other organisms

Need to improve translation from animal models to the clinic

To make lab mice more “human”
(genetically engineer mice to express human proteins or implant human cells or tissues)

“Humanized mice”: Mice genetically engineered to express human proteins or to implant human cells or tissues.

Types of “humanized” mice

Nude mice: Abnormal hair growth and defective development thymus. Deficiency of mature T cells and partial deficiency of B cells.

SCID mice: Mice with Severe Combined ImmunoDeficiency (SCID). No mature T and B cells. Generated in 1983 and tested for HIV in 1988 (Science, 241;1632-9).

NOD SCID mice: SCID combined with NOD (non-obese diabetic; autoimmune diabetes due to impaired immune system; defective macrophages and dendritic cells).

NOD SCID NSG or NOG: NOD SCID with non-functional IL-2 receptor gamma chains (no functional NK cells). For implantation of human immune cells.

http://www.jax.org/

Top of the line…

The NOD SCID NSG were used as recipients for implantation of human bone marrow, liver and thymus tissues (Bone Marrow Liver Thymic mice: **BLT** mice)(robust human hemato-lymphoid system).

For research on infectious agents:
HIV
dengue
herpesviruses
Ebola

Humanized for what?

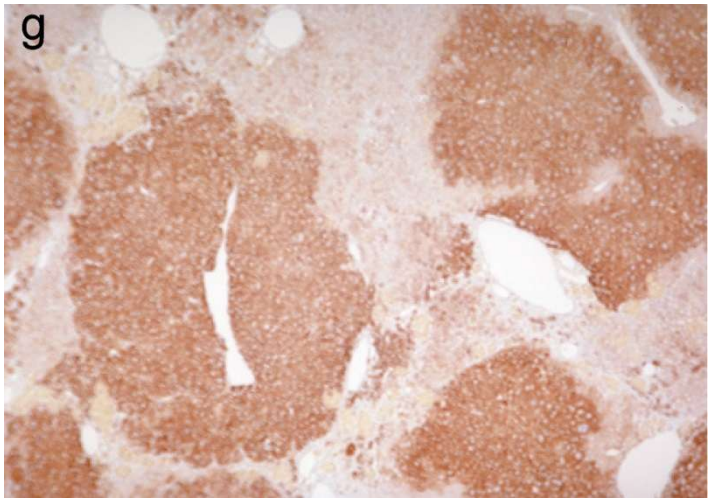
Development of mice with implanted human:
Livers
Tumors
Pancreatic cells
Immune cells
Glial cells

Which diseases can benefit from the “humanized” mice research?

- Infectious diseases
- Cancer (cancer cells, patient-derived xenografts)
- Transplantation
- Autoimmune diseases (lupus, rheumatoid arthritis, multiple sclerosis)

Humanized mice (examples)

1. Mice with chimeric livers (hepatocytes of mouse and human origin) were used as the first model to study hepatitis C.



2. Immune-deficient mice with chimeric livers (hepatocytes of mouse and human origin) were treated with fialuridine: Mouse livers failed, showing pathology similar to humans. *PLOS Med; 2014*.

3. Models for human metabolic diseases (familial hypercholesterolemia). Transplanted liver cells from 7-year-old girl with disease. Mice suffered from reduced ability to metabolize LDL, treated with LDLR gene therapy. Nat Commun 6:7339, 2015.

4. Diabetes model

5. “Humanizing” the brain: Progenitors of human glial cells into the brains of newborn mice: Human glia matured and expanded through mouse forebrain. J Neurosci, 34:16153-61, 2014.

6. Models for drug metabolism and toxicity testing.

Decrease failure rate of clinical trials (time, money and human lives).

Of mice and man: Allometric dose scaling

http://www.slideserve.com/oren/allometric-scaling-to-predict-pharmacokinetic-and-pharmacodynamic-parameters-in-man

- Allometry: a term coined by Huxley & Tessier 1936: The study of size and its consequences. (Greek “alloios”: different)

- Dosage does not extrapolate linearly from animals to human based on body weight (BW)

- Scaling correctly by “metabolic weight” and “Body Surface Area (BSA)”

Reference: Sharma and McNeill British Journal of Pharmacology (2009) 157, 907-921.

Is allometric scaling important?

Example 1. Asiatic elephant at Lincoln Park Zoo in Oklahoma city, in 1962. Was given lysergic acid diethylamine (LSD), based on $mg*kg^{-1}$ dose previously tested in cats. Status epilepticus 5 min later and death 95 min later.

Example 2. Resveratrol concentration: Based on mice (22.4mg/kg) the human dose would be 1344mg/day. Correct extrapolation is 109mg/day.

Larger animals require smaller drug doses (with exceptions).

Considerations for dose scaling

1. Differences in pharmacokinetic phase

Transport and metabolizing systems vary

i.e. Faster drug distribution and elimination (glomerular filtration rate, number of nephrons) in smaller animals.

2. Differences in pharmacodynamic phase

Target cells, systems and metabolites vary

- Antihistamines effective in humans, guinea pigs and dogs but not in cattle.
- CNS distribution of neurotransmitters: opioid analgesics induce CNS depression in primates, rats, dogs, rabbits and CNS excitation in horses, cats.

Differences in receptor affinity

- Na+/K+ ATPase transporter: sensitivity in sheep and human is 1000 greater than mice and rats

Rules for dose scaling

1. Allometric dose scaling should be applied among species, not within species.

2. Allometric dose scaling should be derived from species whose weight differs by at least three orders of magnitude.

Calder, 1981

“Dose-by-factor” approach

The approach that the No-Observed Adverse Effect Level of a drug (NOAEL) is identified by simple allometry based on the body surface area to obtain the “human-equivalent dose” (HED) (USFDA, 2005).

Formula for Dose Translation Based on BSA

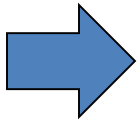
$$\text{HED (mg/kg)} = \text{Animal dose (mg/kg)} \ \textit{multiplied by} \ \frac{\text{Animal } Km}{\text{Human } Km}$$

BSA: Body surface area.
For safety reasons the HED is divided by a “safety factor”, usually it is 10.

Important: This approach assumes that the drug shows similar pharmacokinetics and pharmacodynamics in both species.

TABLE 1. *Conversion of animal doses to HED based on BSA*

Species	Weight (kg)	BSA (m^2)	K_m factor
Human			
Adult	60	1.6	37
Child	20	0.8	25
Baboon	12	0.6	20
Dog	10	0.5	20
Monkey	3	0.24	12
Rabbit	1.8	0.15	12
Guinea pig	0.4	0.05	8
Rat	0.15	0.025	6
Hamster	0.08	0.02	5
Mouse	0.02	0.007	3



Mouse/human
~ 12X

Rat/human
~6x

Many mathematical models available

“Dose-by-factor” approach: Example

Species: Rat
NOAEL (mg/kg/d): 50
HED: ?

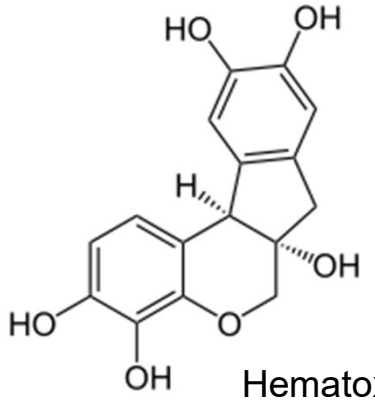
Formula for Dose Translation Based on BSA

$$\text{HED (mg/kg)} = \text{Animal dose (mg/kg)} \textit{ multiplied by } \frac{\text{Animal } Km}{\text{Human } Km}$$

HED = 50 mg/kg x 6/37 = 8.1 (mg/kg)

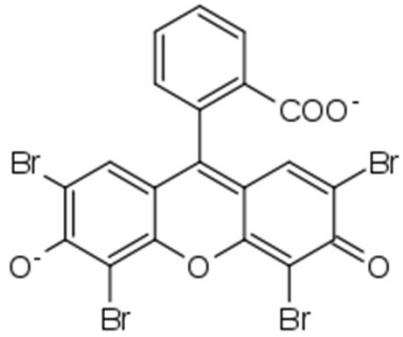
H&E staining

- Hematoxylin and eosin stain
- Primary staining method in histology
- Used for pathology reports and diagnosis



Hematoxylin

The basic dye hematoxylin stains the acidic cell compartment structures with blue-purple hue (primarily nuclei of the cells).

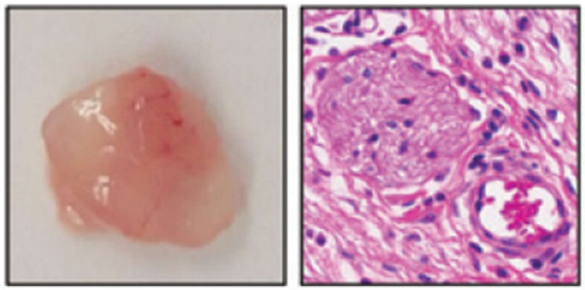


Eosin

The alcohol-based acidic eosin Y, stains the basic cell compartment structures bright pink. Eosinophillic areas are:

- Cytoplasm
- Collagen
- Muscle fibers
- RBCs

Actual image **H&E**

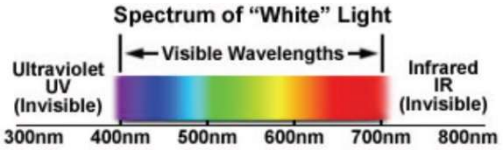


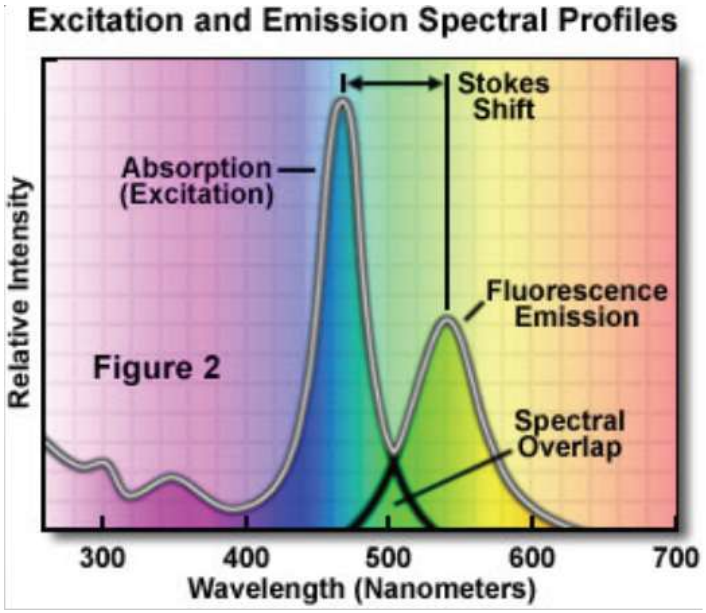
Fluorescence

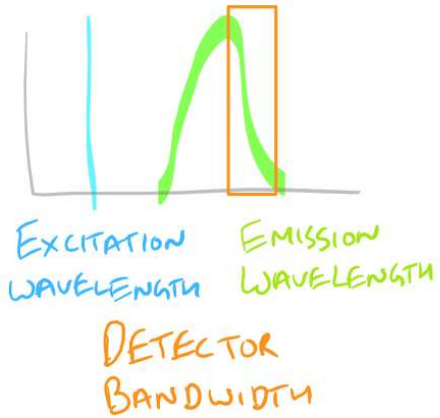
Electromagnetic spectroscopy that analyzes fluorescence from a sample. The susceptible molecules emit light from electronically excited states created either by a physical (light absorption), mechanical (friction) or chemical mechanism.

Fluorescence is governed by three important events:

1. Excitation of a susceptible molecule (in femptoseconds)
2. Vibrational relaxation of excited state electrons to the lowest energy level (in picoseconds)
3. Emission of a longer wavelength photon and return of the molecule to the ground state (in nanoseconds)

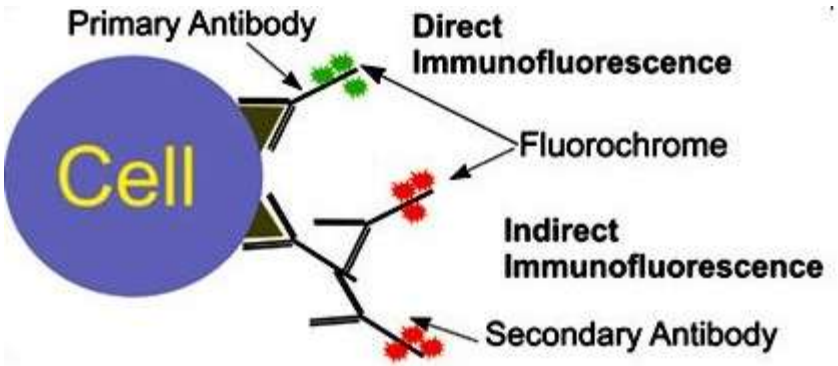


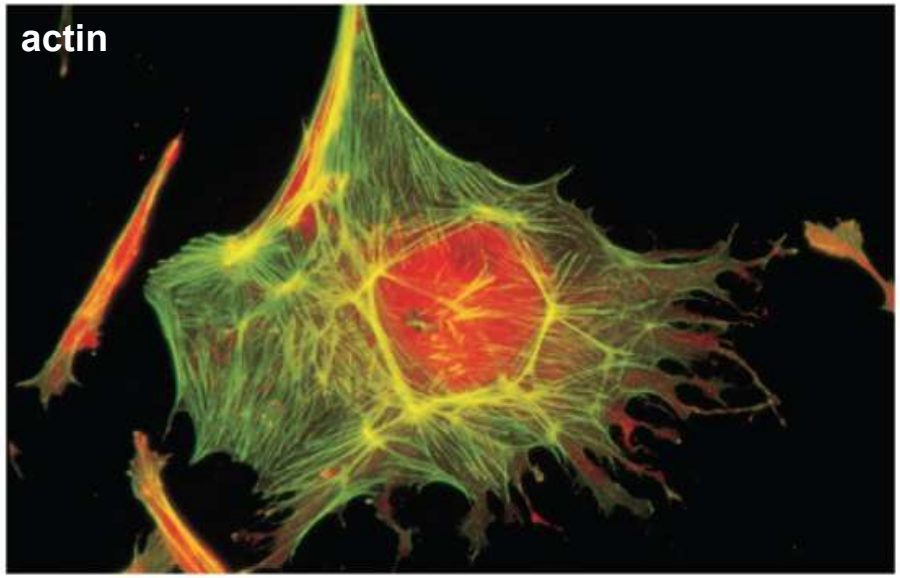




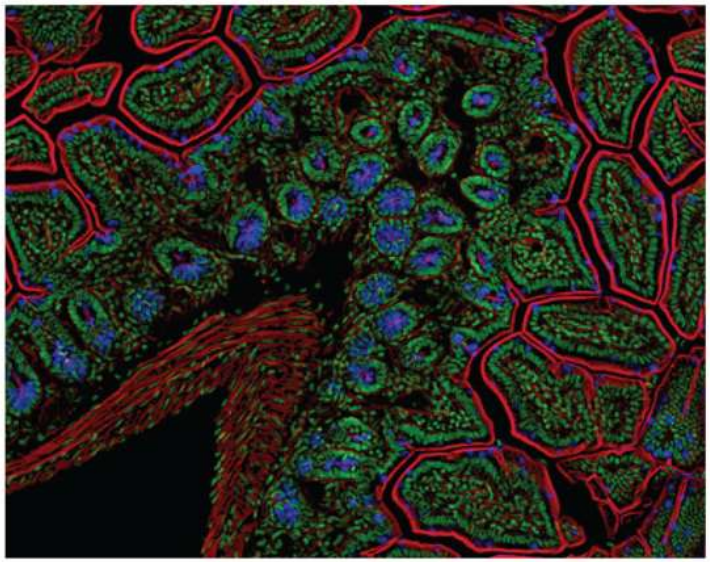
www.scigine.com

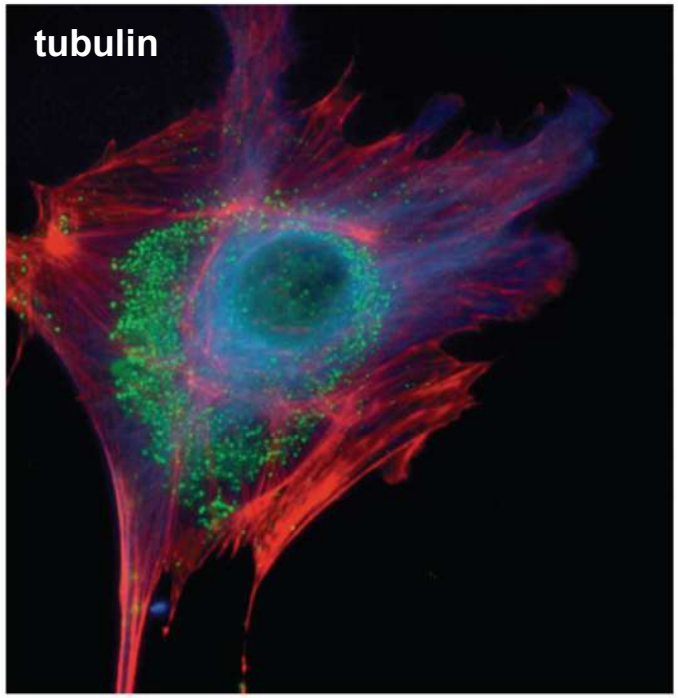
Immunofluorescence





tissues

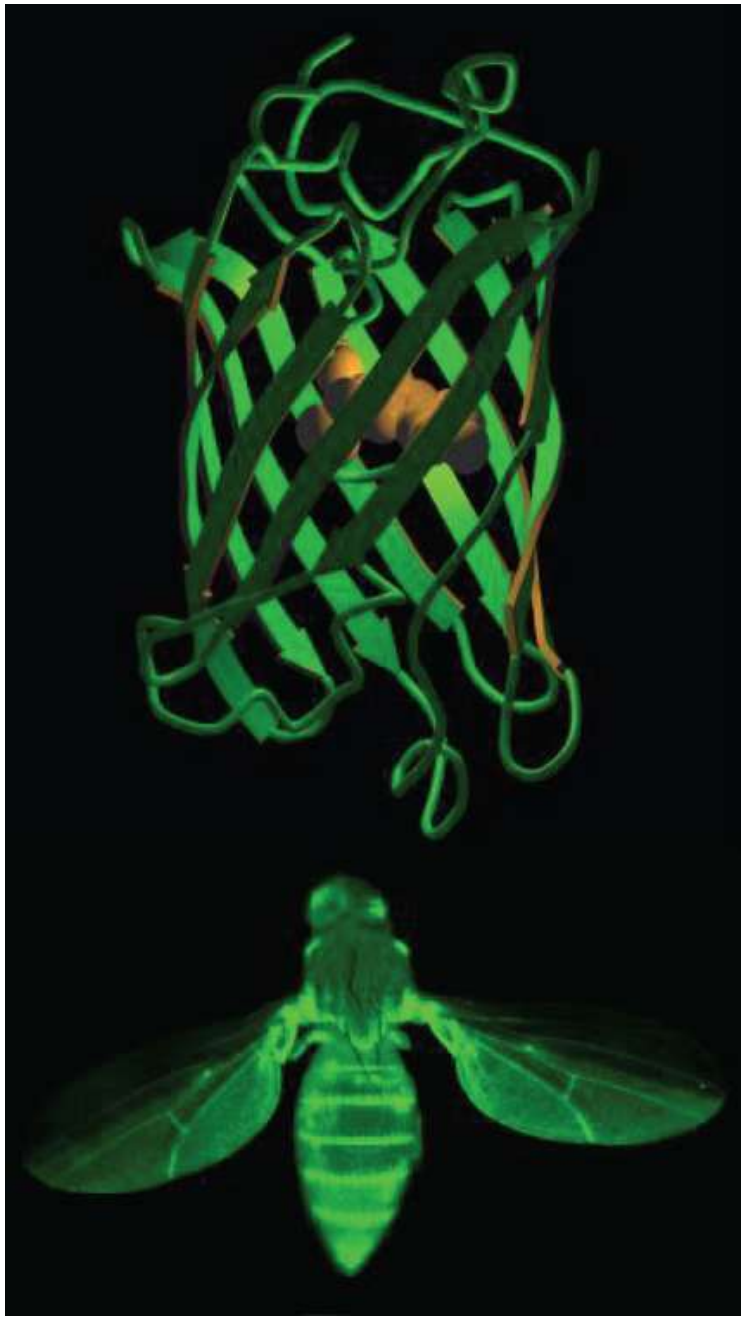




GFP

GFP: Green Fluorescence Protein

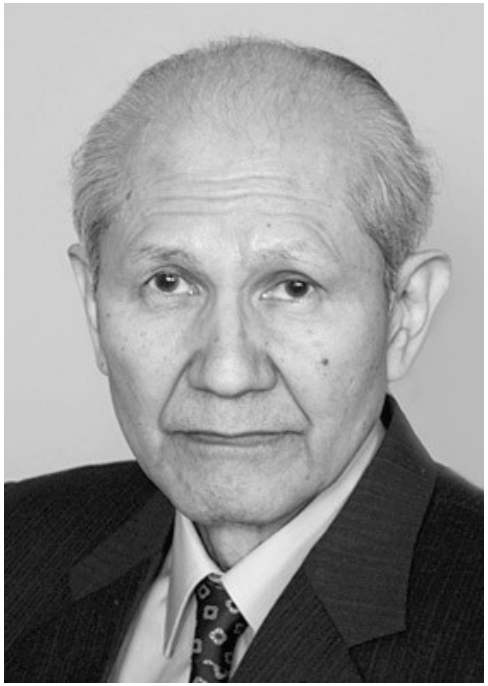
- 27kDa protein, comprised of 238 aminoacids.
- Comes from the jellyfish *Aequorea victoria* that fluoresces green when exposed to wavelengths of light of ~480nm,
- Has an 11-strand β-barrel with a single alpha helical strand.
- The barrel shape permits the chromophore to be in the center and protect it from quenching be the surrounding environment.
- Typically the GFP is used as a reporter of expression.
- Minimal to no toxicity or alterations in cell biology.



GFP

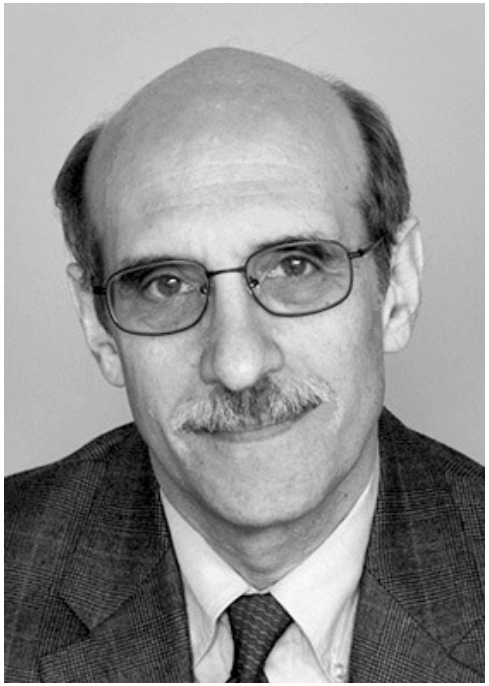
Nobel Prize in Chemistry 2008

Studied the bioluminescent jellyfish Aequorea victoria (~1960s)



Osamu Shimomura

Used jellyfish's GFP on cells (1990s)

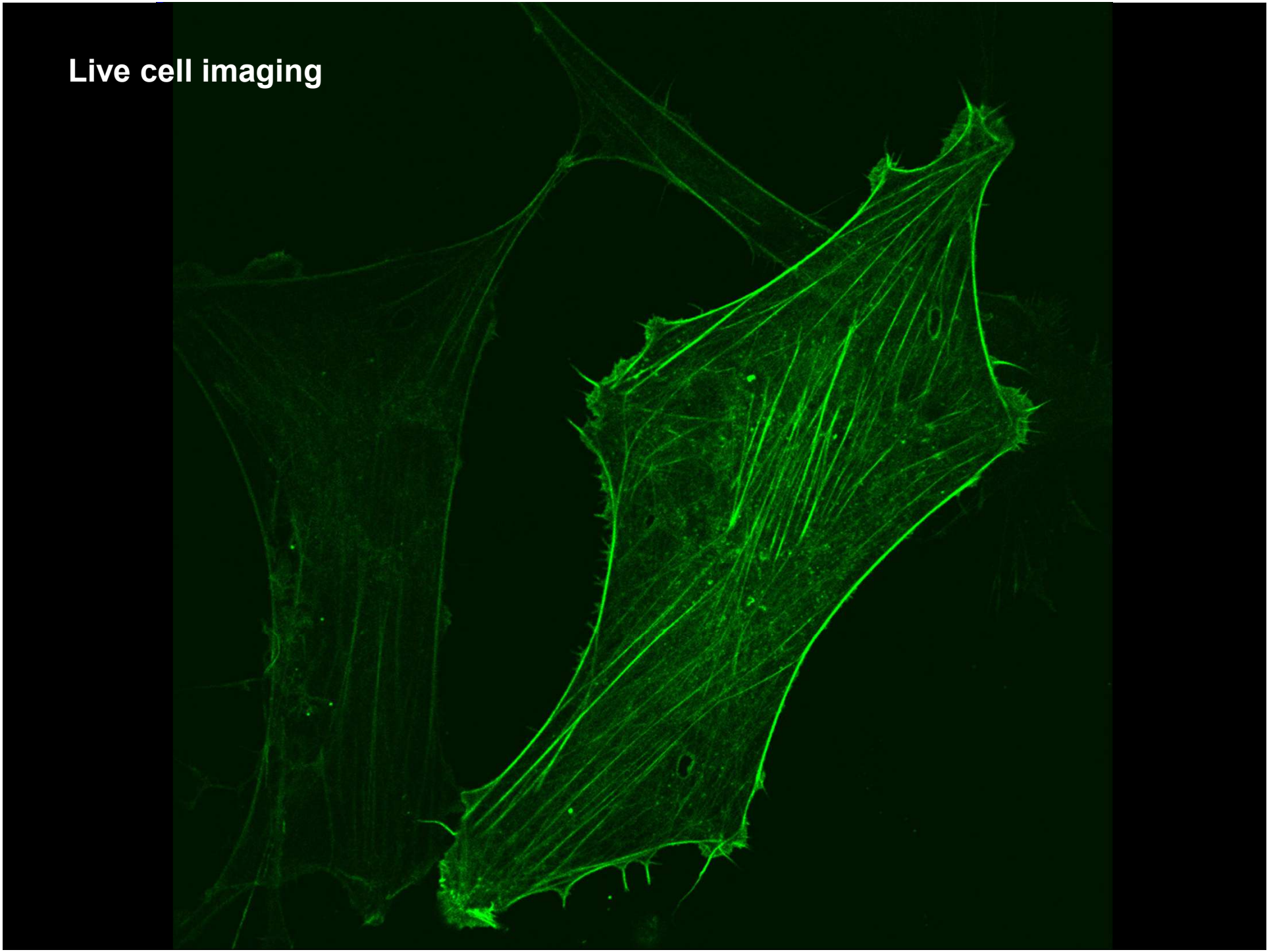


Martin Chalfie

Made new variants of GFP that shine more strongly and in quite different colors (2007)

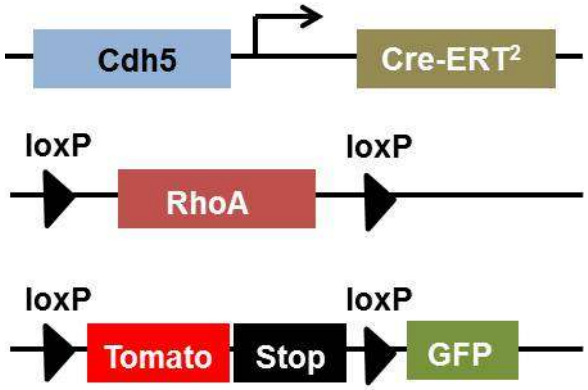
Roger Y. Tsien

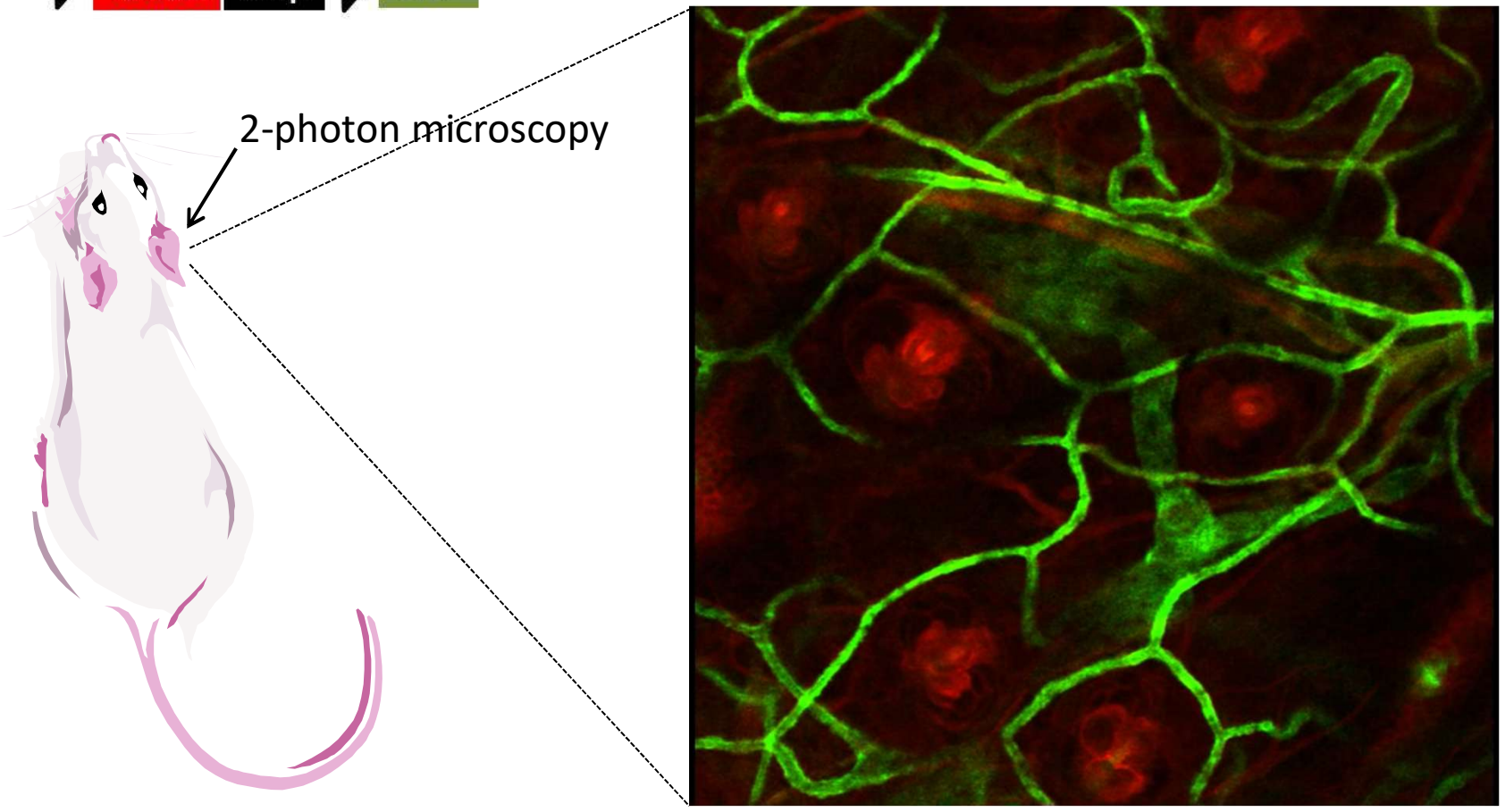
Live cell imaging



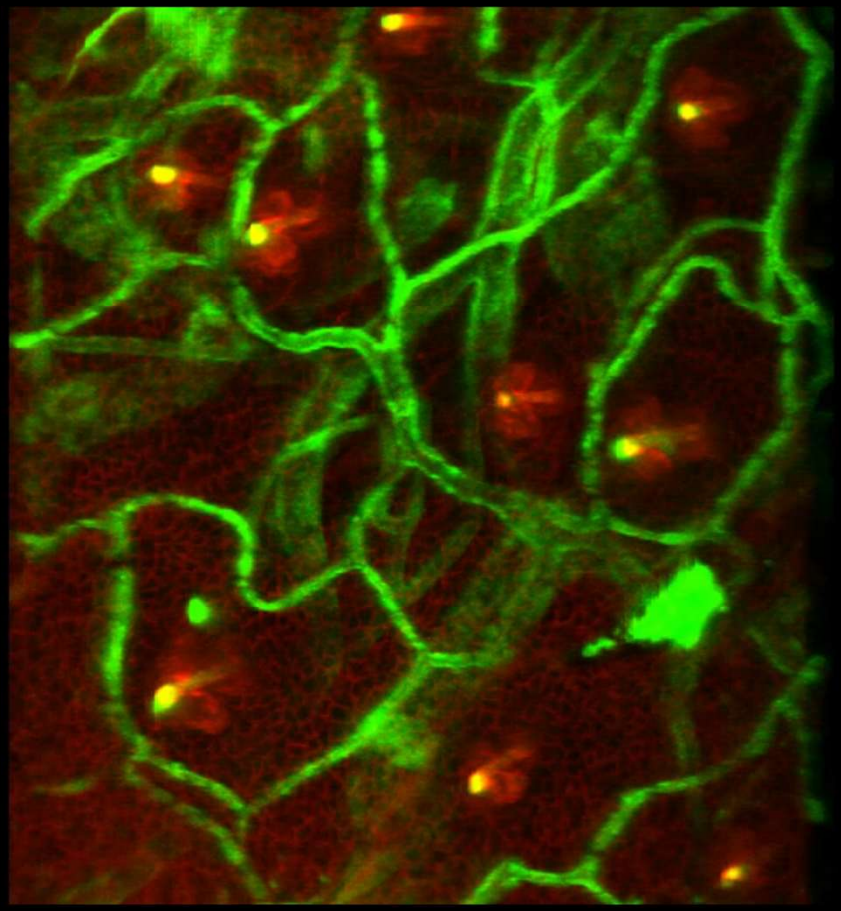
Fluorescent proteins can be expressed in entire organisms

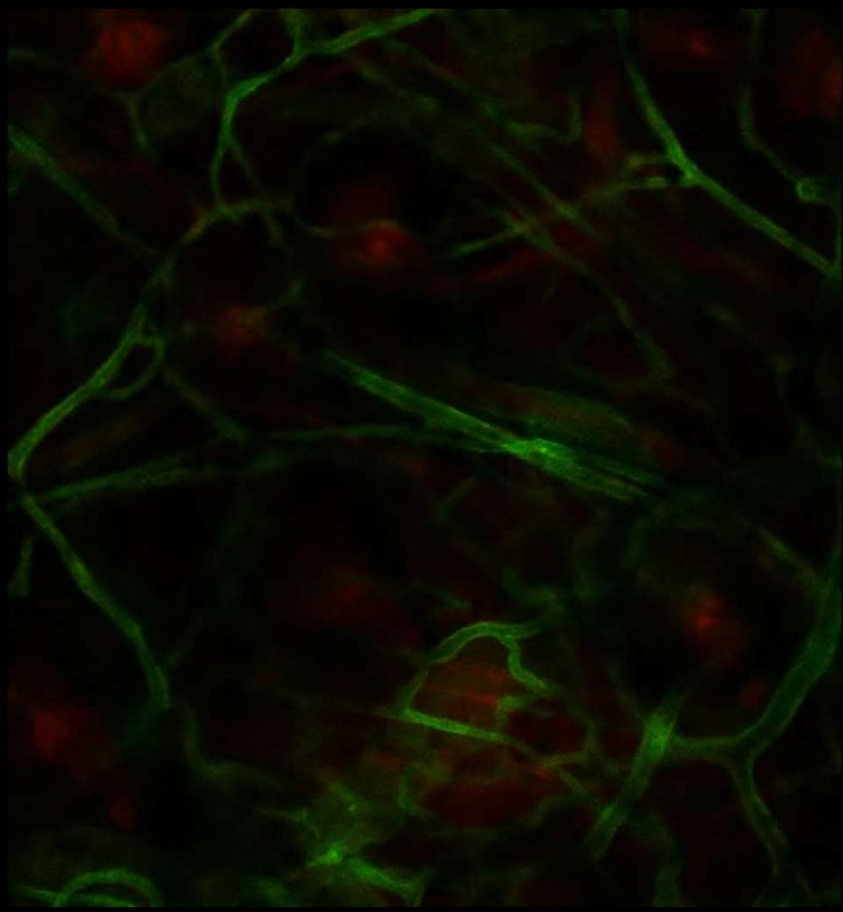
…or in specific tissues



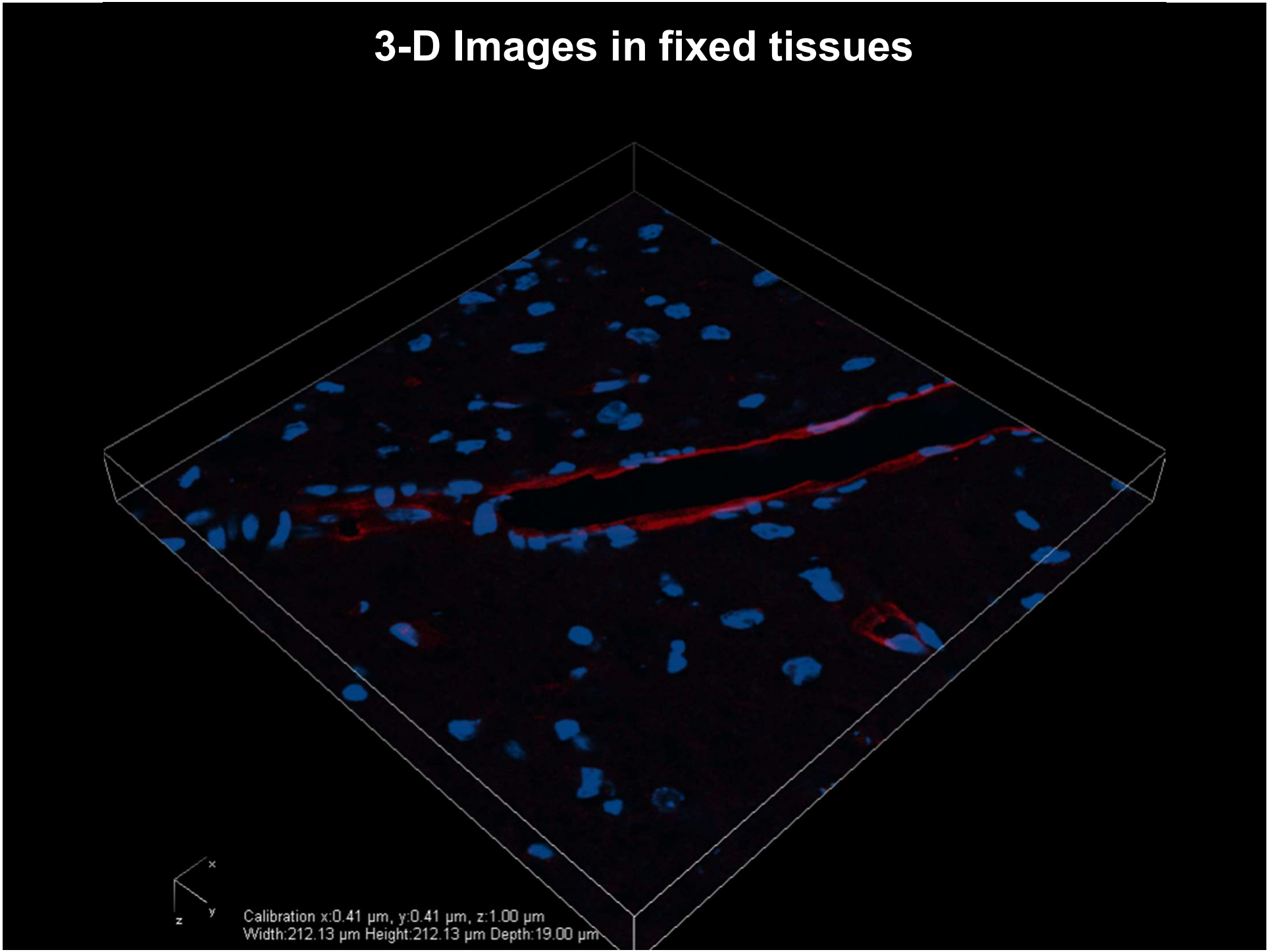


Evaluation of promoter efficiency

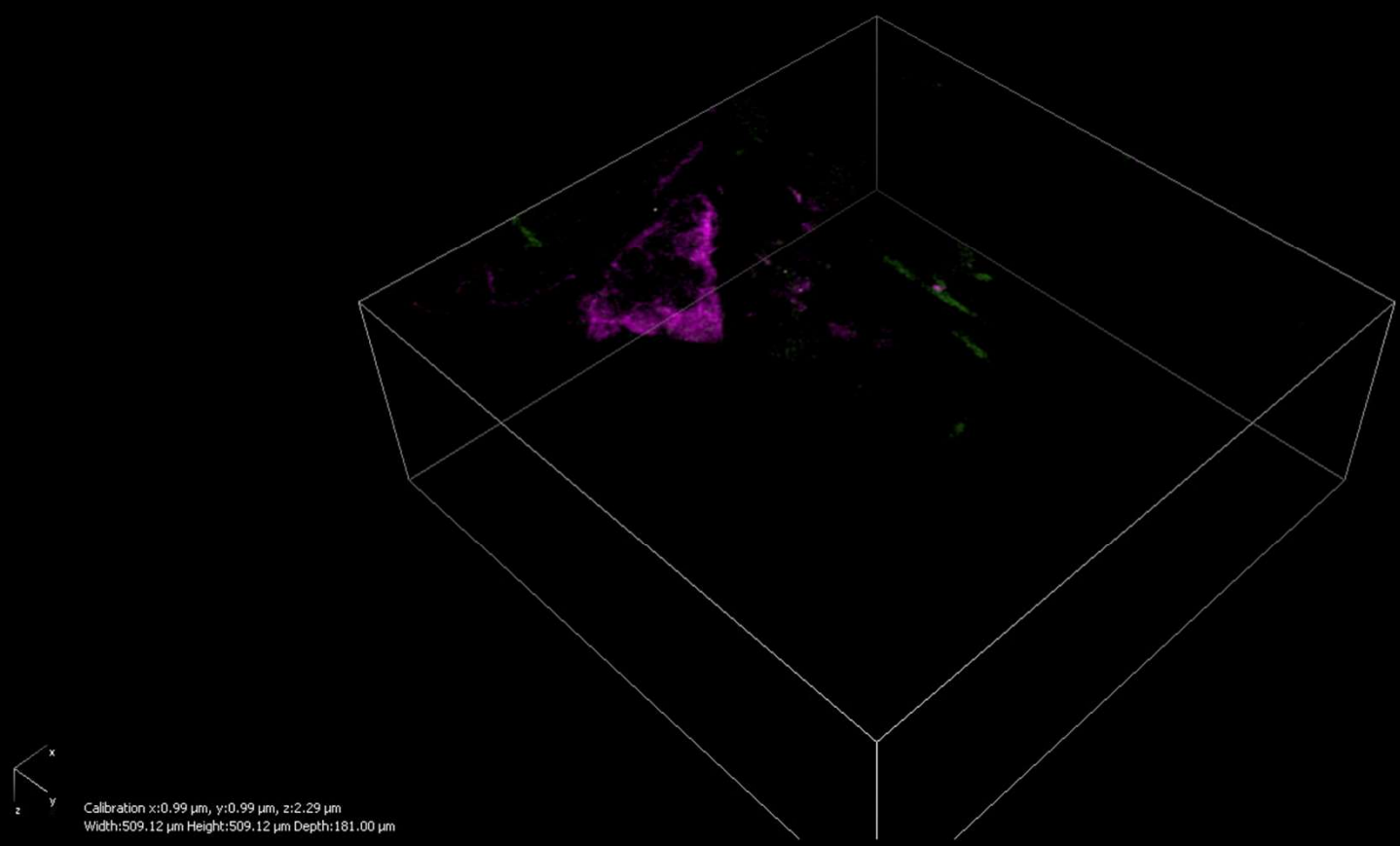




3-D Images in fixed tissues



3-D images for live cell imaging

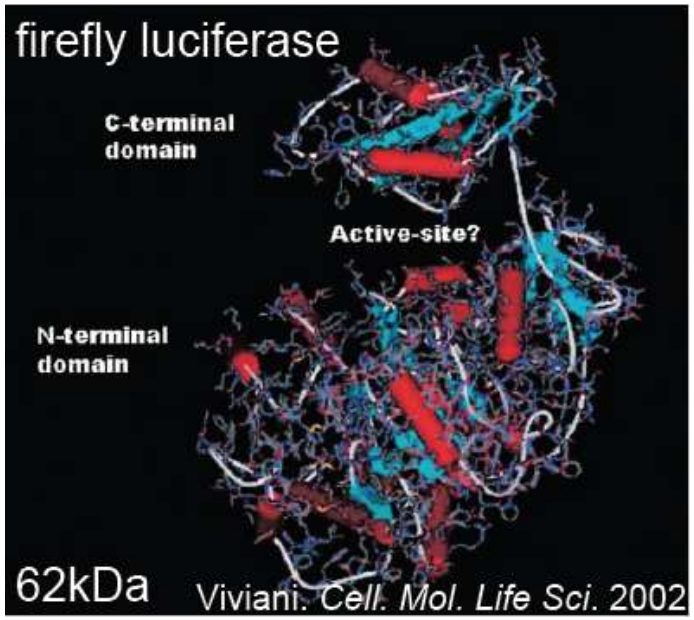


Bioluminescence

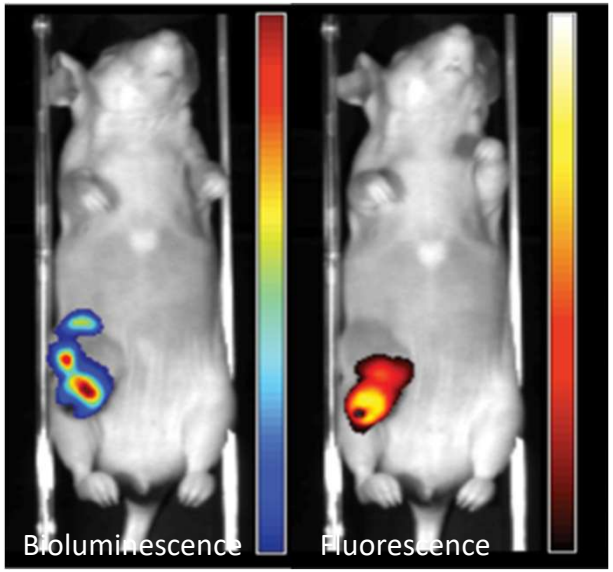
- The emission of light by a living organism.
- Typically a result of a chemical/enzymatic reaction.
- Firefly luciferase is a very common one (requires luciferin to produce light).
- Gene coding the firefly luciferase can be inserted into other cells. Stable or reporter based expression systems result in expression in cells of interest.

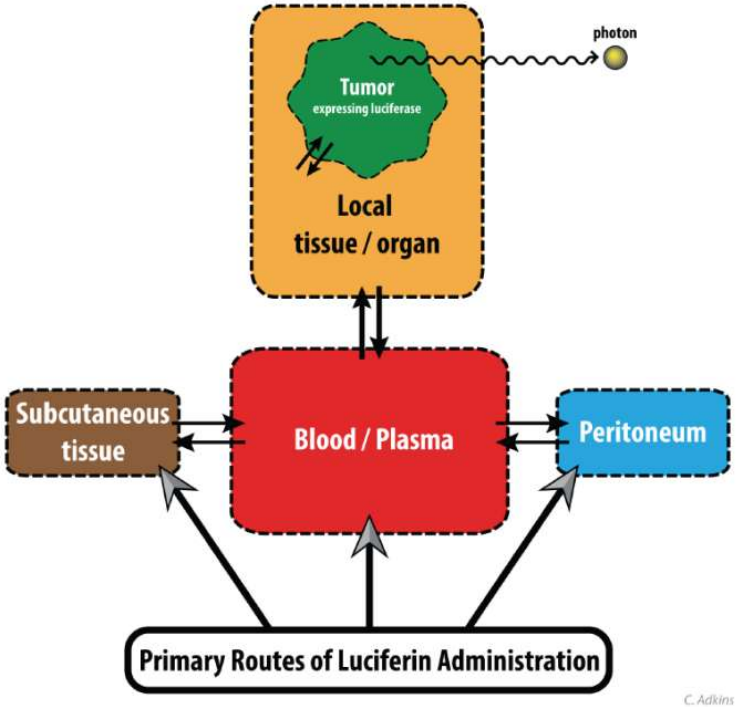
Advantages of luciferase-based luminescence:

1. No excitation light required for activation (enzymatic reaction; requires ATP, oxygen and luciferin).
2. Enhanced signal-to-noise ratio (lack of natural bioluminescence background in mammalian models).
3. Can be quantified temporally and spatially (proper imaging systems are required).

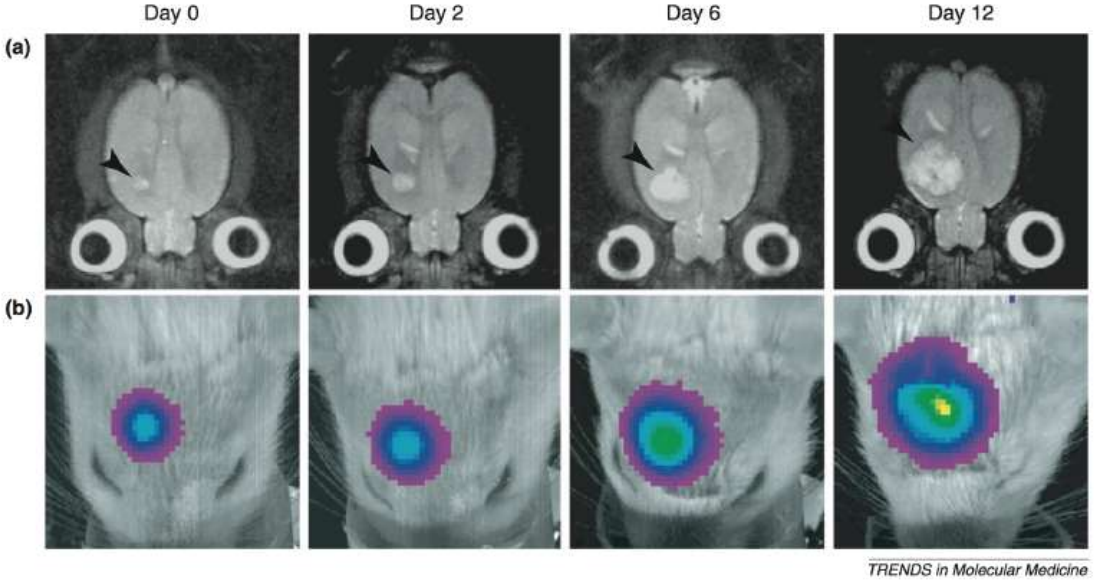


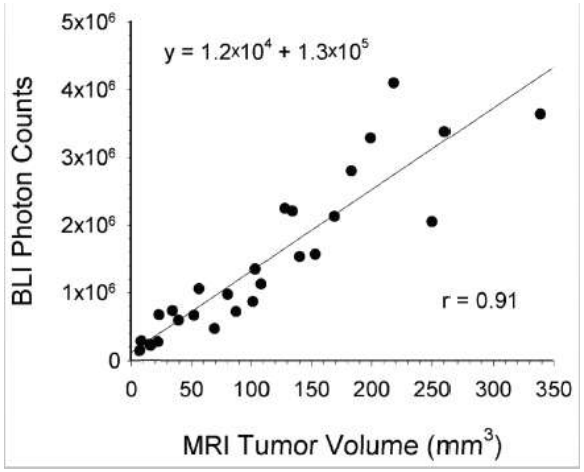
Bioluminescence





Bioluminescence can be used to approximately quantify tumor mass





Rehemtulla et al, 2000 Neoplasia 2, 491-5.

Bioluminescence

- (Caliper) Perkin-Elmer IVIS Lumina XR system available in basement of ARB

Ποιους συμβουλεύομαι όταν χρειαστεί

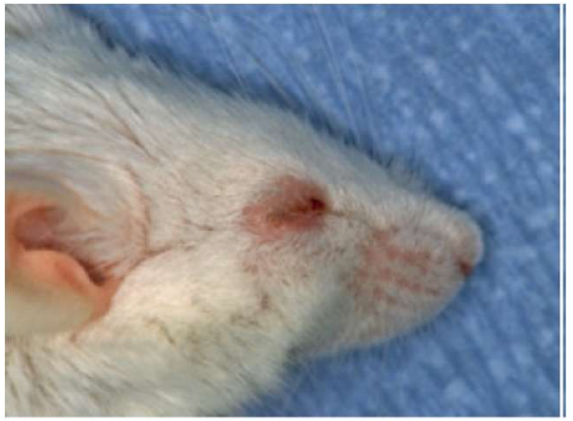
Ανάλογα με τη φύση του προβλήματος:

- Επιστημονικός υπεύθυνος (PI)
- Τεχνικός στο ζωοστάσιο
- Κτηνίατρος

Αντιμετώπιση πόνου και άγχους του ζώου

Οι μύες όταν πονάνε:

1. Χάνουν βάρος (για μακροχρόνιες διαδικασίες)
2. Δεν κουνιούνται πολύ (ή κουνιούνται υπερβολικά πολύ)
3. Δεν αντιδρούν αποτελεσματικά στα ερεθίσματα
4. Περίεργο περπάτημα
5. Καμπούρα στην πλάτη ενώ περπατάει.
6. Μαζεμένη ουρά
7. Κόκκινα (ερεθισμένα) μάτια
8. Τρόμος
9. Το τρίχωμα είναι τσαλακωμένο ή/και όχι λείο (αφυδάτωση)
10. Ανοίγουν στόμα αναπνέοντας, έκταση του θώρακα.
11. Αυτοτραυματίζονται



Αντιμετώπιση πόνου και άγχους του ζώου

Δεν είμαι σίγουρος: Ανοίγω από δίπλα ένα κλουβί με φυσιολογικά ποντίκια και συγκρίνω.

Αντιμετώπιση:

- Χορήγηση παυσίπονης ουσίας (π.χ. Καρπροφαίνη, (Carprofen: Rymadil) 5 mg/kg υποδόρια (δόση 1 φορά ημερησίως για 5 ημέρες), άλλο: κετοπροφαίνη και μπαναμίνη) – Πολύ πιο δύσκολη η χορήγηση οπιοειδών (Buprenorphine hydrochloride).
- Ευθανασία

References:

- Semenova S, Kuzmin A, Zvartau E: Strain Differences in the Analgesic and Reinforcing Action of Morphine in Mice. Parmacol. Biochem. Behav. 50: 17-21, 2005.
- Ong CK-S, Lirk P, Seymour RA, Jenkins BJ: The Efficacy of Preemptive Analgesia for Acute Postoperative Pain Management: A Meta-Analysis. Anesthesia and Analgesia. 100(3): 757-773, 2005.

Έχουμε εξαντλήσει τις εναλλακτικές για τη μελέτη μας

Γιατί πρέπει να κάνεις πειράματα με ζώα;

Έψαξες τη βιβλιογραφία για εναλλακτικές;

Ποιες databases (π.χ. pubmed);
Πότε έγινε ο έλεγχος;
Από πότε;
Ποιες λέξεις-κλειδιά; Έβαλες λέξη “alternatives”;
Screenshots

Χειρουργικές επεμβάσεις

Όλες οι χειρουργικές επεμβάσεις σε άσηπτες συνθήκες:

- Αποστειρωμένα υλικά ή αποστειρωμένα μιας χρήσης
- Μάσκα
- Χειρουργικά γάντια

Αποστείρωση με πίεση:

- 15’ στους 121°C
- 10’ στους 126°C
- 3’ στους 134°C

Χειρουργικές επεμβάσεις

Υποθερμία: Οι μύες μπορούν να χάσουν 1°C ανά 5’. Η θερμοκρασία πρέπει να διατηρείται. (Προσοχή: όχι θερμάστρες και όχι πάνω από 39°C)

Εφύγρανση ματιών: Με ειδικό gel.

Έλλειψη τροφής (starvation) πριν το χειρουργείο: τα τρωκτικά δεν το χρειάζονται γιατί δεν μπορούν να κάνουν έμετο λόγω της φυσιολογίας του οισοφάγου τους.

Χειρουργικές επεμβάσεις (references)

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• National Research Council. 2011. Guide for the Care and Use of Laboratory Animals, Eighth Edition. Washington, DC: National Academies Press.
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Μετά το χειρουργείο: Επιτήρηση

Εξετάζονται οι ακόλουθοι 5 παράμετροι:

1. Συμπεριφορά
2. Κοκκίνισμα
3. Στάση σώματος και τυχόν αλλοιώσεις της
4. Βάρος (έως 10% του σωματικού βάρους επιτρέπεται να χάσουν)
5. Κατανάλωση τροφής (όρεξη)

Σε περίπτωση αφυδάτωσης μπορούν να γίνουν ενέσεις με φυσιολογικό ορό (στους 37°C):

- 3 ml/25 g/ημέρα για μύες
- 15 ml/250 g/ημέρα για αρουραίους

Lab Animal 29:5, 40-45, May 2000

Φυσική εξέταση

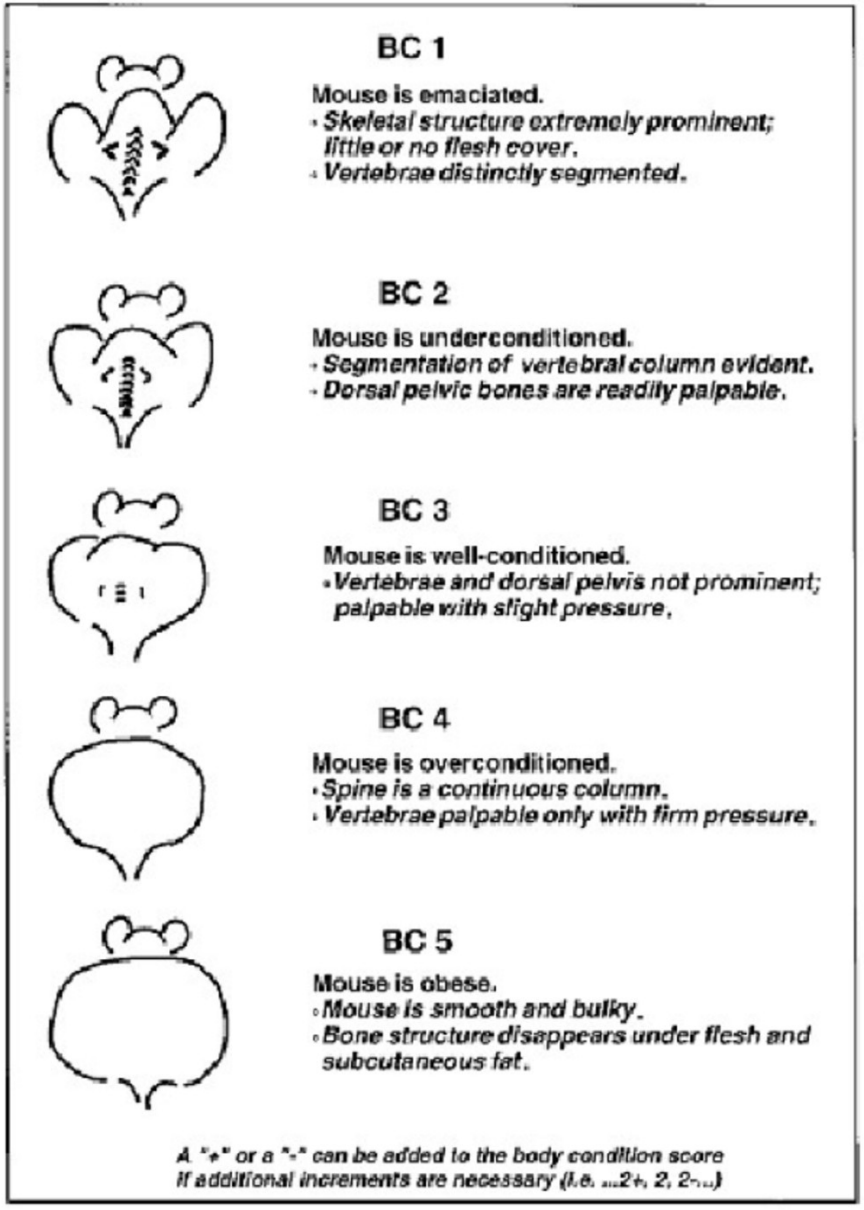
Σε κάθε πείραμα γίνεται σε τακτά χρονικά διαστήματα (**υποχρεωτικά!**).

Τι εξετάζεται:

1. Συμπεριφορά
2. Βάρος σώματος
3. Πληγές στο σώμα και τυχόν αλλοιώσεις
4. Αφυδάτωση
5. Θερμοκρασία σώματος
6. Παράμετροι του αίματος (υπό αναισθησία)

Η κατάσταση των πειραματοζώων παραπέμπει στον ερευνητή!!

Βάρος σώματος



BC 1

Mouse is emaciated.
- *Skeletal structure extremely prominent; little or no flesh cover.*
- *Vertebrae distinctly segmented.*

BC 2

Mouse is underconditioned.
- *Segmentation of vertebral column evident.*
- *Dorsal pelvic bones are readily palpable.*

BC 3

Mouse is well-conditioned.
- *Vertebrae and dorsal pelvis not prominent; palpable with slight pressure.*

BC 4

Mouse is overconditioned.
- *Spine is a continuous column.*
- *Vertebrae palpable only with firm pressure.*

BC 5

Mouse is obese.
- *Mouse is smooth and bulky.*
- *Bone structure disappears under flesh and subcutaneous fat.*

A "+" or a "-" can be added to the body condition score if additional increments are necessary (i.e. ...2+, 2, 2-...)

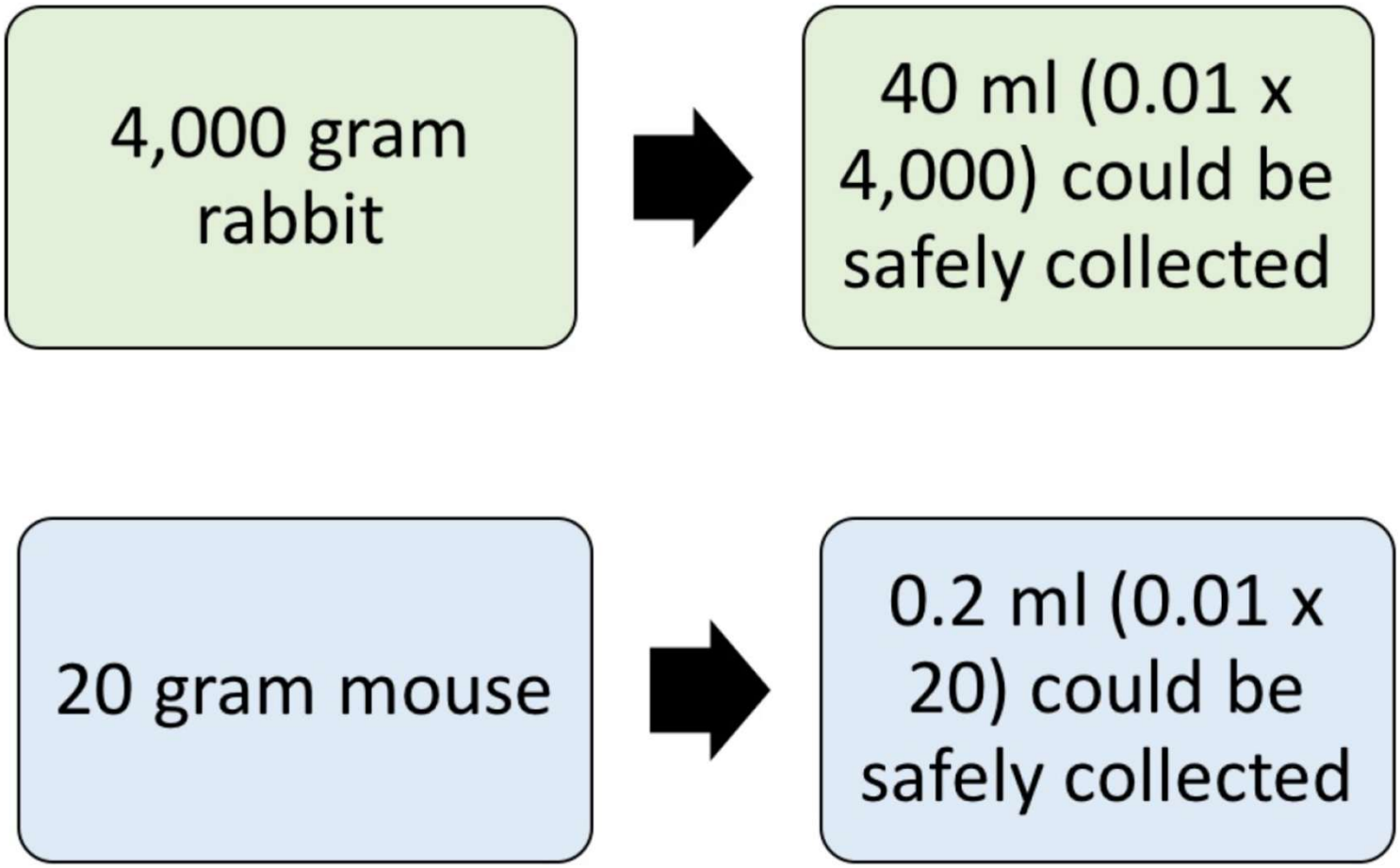
Απομόνωση αίματος

Volume recommendations (ml) for acute intravenous fluid administration and blood collection in adult mice (average 20 g):

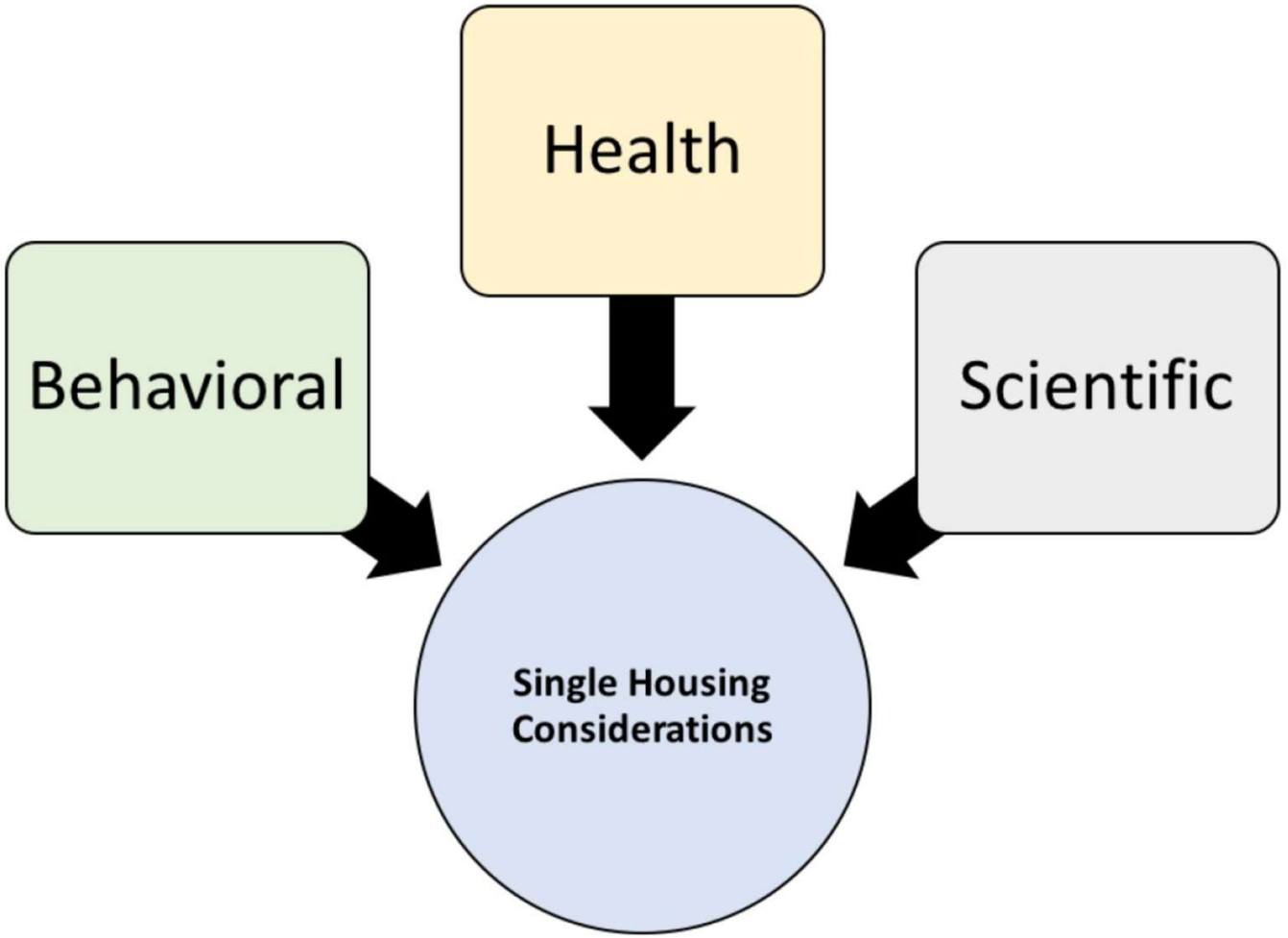
IV Fluid Volume (ml) max. acute admin.	**Total Blood Volume (ml)**	**Safe Bleeding Volume (ml)**[a]	**Tot. Bleed-out Volume (ml)**[b]
0.2	1.0 - 2.4	0.1 - 0.2	0.6 - 1.4

>0,1 ml/10g of body weight: hypovolemic shock. Το ίδιο με επαναλαμβανόμενες λήψεις. Διαδικασία υπό αναισθησία.

Απομόνωση αίματος



Single housing



Single housing

- Αρσενικοί μύες C57BL/6 και BALB/c δεν μπορούν να μπουν στο ίδιο κλουβί αν δεν έχουν μεγαλώσει μαζί (τσακώνονται).
- Αν έχουν προβλήματα υγείας δεν μπορούν να ανταγωνιστούν το φαΐ ή το νερό.
- Αν έχουν προβλήματα υγείας και θα δεχθούν εξατομικευμένες θεραπείες.
- Επιστημονικοί λόγοι: Πειράματα συμπεριφοράς, έλεγχος κατανάλωσης νερού, πειράματα μεταβολισμού κλπ.

Αποδεκτές μέθοδοι ευθανασίας (ΗΠΑ)

- Βαρβιτουρικά (IV injection)
- Εισπνεόμενα αναισθητικά (ισοφλουράνιο) (περιορισμοί για το προσωπικό)
- CO_2
- CO
- Αυχενική εξάρθρωση
- Αποκεφαλισμός
- Αντινοβολίες συντονισμού με μικροκύματα
- KCl μαζί με αναισθησία

2 μέθοδοι υποχρεωτικά για να εξασφαλισθεί ο θάνατος

Κανονισμοί στις ΗΠΑ (CITI Program and Database)

https://about.citiprogram.org/

Biomedical Investigator
Responsible Conduct of Research
Conflict of Interest
Working with IACUC
Reducing Pain and Distress in Laboratory Mice & Rats
Working with Mice in Research Settings
Aseptic Surgery
Working with Animals in Biomedical Research
Post-Approval Monitoring (PAM)
Animal Care and Use
Biosafety/Biosecurity (BSS) – NIH Recombinant DNA Guidelines

Κατά την εκπαίδευσή σας στο ζωοτροφείο υποχρεωτικά όλοι με εργαστηριακή ρόμπα!!

Questions?