Laboratory Techniques II (SoSe 2019)

This is just my self-made script. No guarantee for completeness and correctness! 😉

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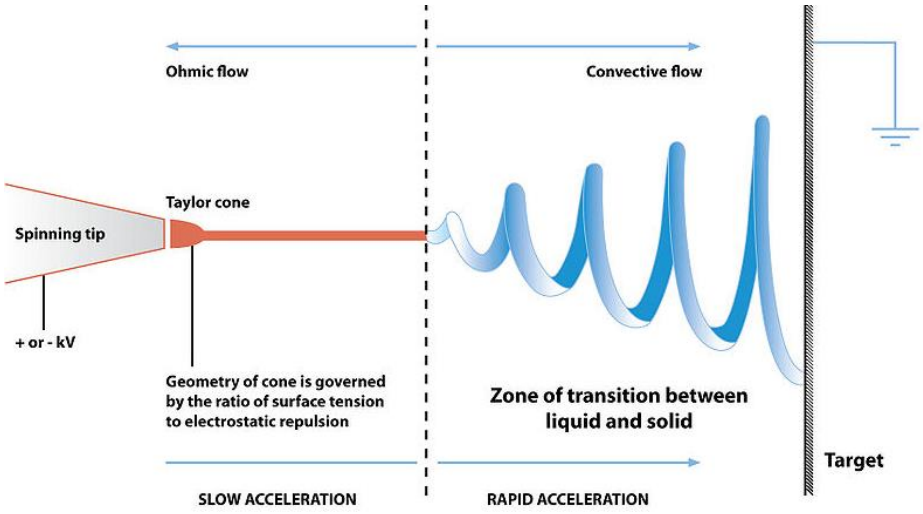
Electrospinning (15.04)

- First commercialization 1991
- From 2008 clinical application

The electrospinning proces

Wikipedia:

- When a sufficiently high voltage is applied to a liquid droplet, the body of the liquid becomes charged
- electrostatic repulsion counteracts the surface tension, droplet is stretched
- at a critical point a stream of liquid erupts from the surface (Taylor cone)
- If the molecular cohesion of the liquid is sufficiently high, stream breakup does not occur, and a charged liquid jet is formed
- As jet dries in flight, the mode of current flow changes from ohmic to convective as the charge migrates to the surface of the fiber
- jet is then elongated by a whipping process caused by electrostatic repulsion initiated at small bends in the fiber, until it is finally deposited on the grounded collector



Factors influencing the spinning process

- Polymer
  - Viscosity, concentration, molecular weight, type of polymer
- Solvent
  - Conductivity, volatility (→ wie schnell es verdampft)
- Device
  - Flow rate, voltage, electrode distance, nozzle size (Nadelöffnung)
- Humidity, temperature, air pressure

Examples of correlations:

- Acceleration impacts fiber size (faster → thinner)
  - Increase voltage
  - decrease distance of electrodes
- Thinner fibers when:
  - Higher conductivity
  - lower volatility (fiber only gets thinner until it is dried)
  - lower nozzle diameter
- Morphology
  - Humidity (high → pores on fiber surface because of little water drops on the fibers)
  - polymer concentration (low → beads, polymer clumps form and in between there is only solvent)
  - volatility and collector distance (low → flat fibers)

Types of polymers

- Synthetic:
  - Polyurethane, Poly-ε-Caprolactone, PLA, PEG
  - Advantages: Stability, controllable, reproducibility
- Natural:
  - collagen, elastin, hyaluronan, chitosan
  - Advantages: Biochemistry, Biocompatibility, Patient-specific

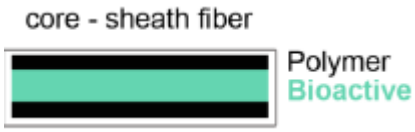
- Hybrid: Polyurethane/Cellulose, PLA/Collagen, …
    - Brings both advantages together

Interesting scaffolds for regenerative medicine

- 3D fibrous and porous structure
    - similar to the extracellular matrix (ECM)
    - improved implant integration
    - impact on cell behavior (proliferation, migration and differentiation)
- Application in regenerative medicine:
    - “advanced” cell culture
    - implant / drug delivery system
    - test system

Collectors and Electrodes

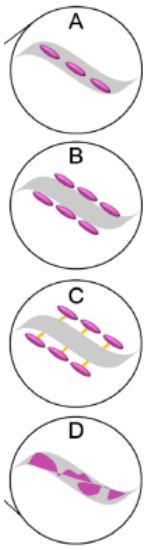
- collector design
    - flat collector
    - rotating collector
    - customized shape
    - frame collector
    - yarn producing collectors
- Need to be conductive, e.g. Al, Cu, Au, …
- Nozzle design
    - Single/multiple
    - Coaxial → gives two layers in the fiber



Fiber morphology

- Random vs. aligned
- Beads
- Core-shell
- Porous
- Tape-like
- Crosslinked

Functionalization



A Encapsulation of proteins, growth factors or drugs

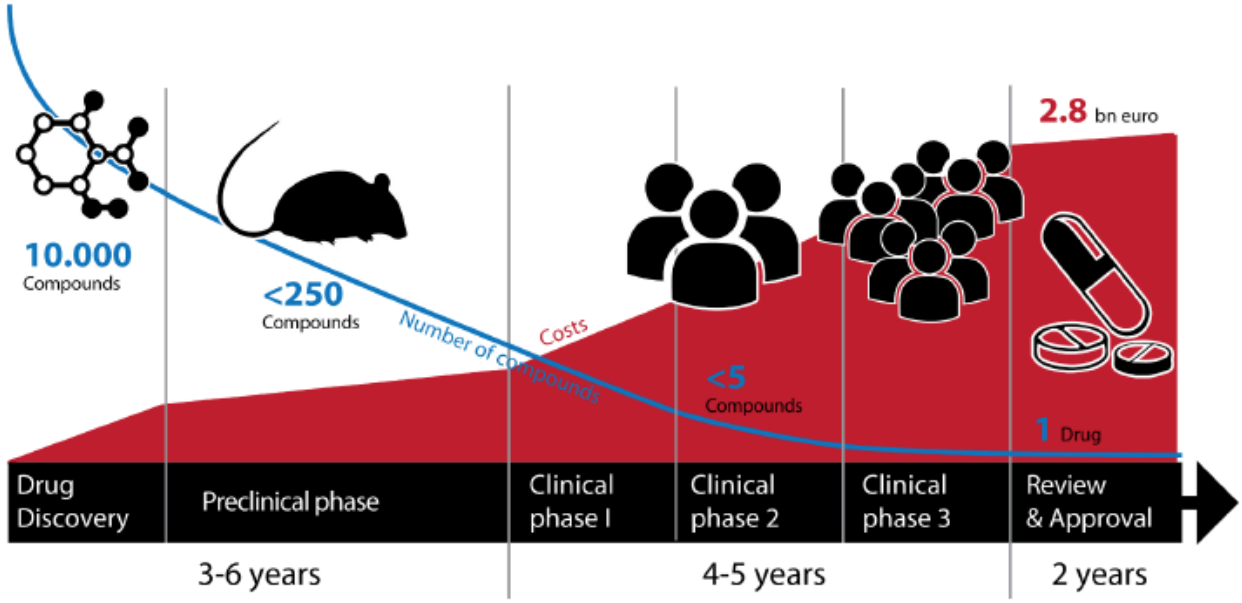
B Coating techniques to functionalize scaffolds after electrospinning

C Covalent binding after electrospinning

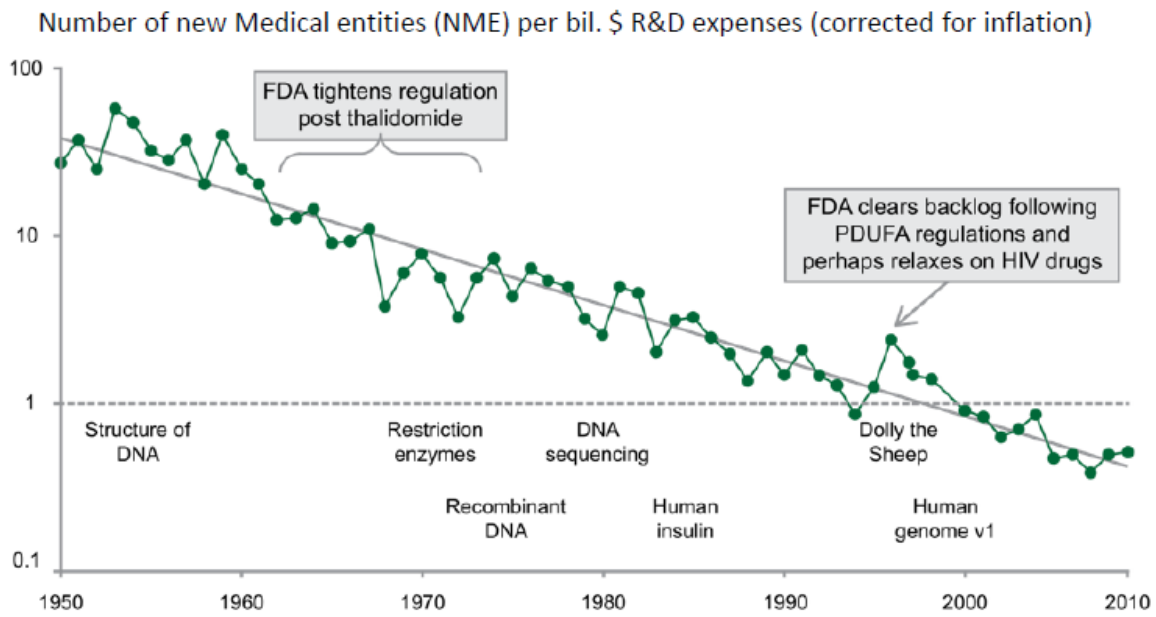
D Blend: Electrospinning of a protein-polymer mixture

Microphysiological systems as next-generation in vitro models (29.04.19)

Drug development



- **out of 10.000 compounds 12 years later 1 will get into the market**
- Extremely long process: approx. 10-12 years
- Extremely inefficient: 10 000 compounds → 1 drug
- Extremely expensive: >1 billion €



- „EROOM's law" – Continuous decline in productivity over the last decades

Preclinical research

Advantages of in vitro models	Disadvantages of in vitro models
- Cheaper - ethical less debatable - High number of samples easily possible (→small space, automatization) - Can use human cells - Standardization → reproducible	- Not always transferable results - Other biomechanical and physiological environment - No perfusion, no mechanical forces - Non-physiological mono culture - Cell / media ratio magnitudes too low - Only short-term effects can be explored

Advantages of animal models	Disadvantages of animal models
- Similar genome to human - Complex tissues + circulation → physiological and good biomechanical environment - Effects of drug on behavior can be observed	- Not always transferable results - Different physiology and immune system - Different sizes of animals vs. humans - Multiple species necessary - Each animal behaves different - Specific environment & diet - Complicated analysis - Variability - less standardization - Strain specific results due to (in)breeding

3R-principle

- Replace animal experiment by other methods
- Reduce animal experiments (make only necessary tests)
- Refine → minimize the stress and burden of the animals

Animal models - Rodent models

- Similarities
    - 95% of genes are identical to humans
    - Similar organ structure
- Advantages
    - Fast breeding
    - Small: Advantageous in terms of housing and facility size
- Disadvantages
    - Specific environment and diet
    - Different immune system / strategies to fight infections and injuries
    - Different physiology heart rate 500-700 bpm
    - Strain specific results due to breeding

New opportunities provided by organ-on-a-chip

- Combines advantages of both models
    - Human engineered tissues
    - In vivo structure and function
    - Microphysiological environment
        - Microfluidic perfusion
- Homunculus = miniature representation of a human
- Organ on a Chip as enabling technology for the transition of compounds from the preclinical phase into the clinical phases.

➔ Increase in predictivity → Safer, faster and more economical drug development

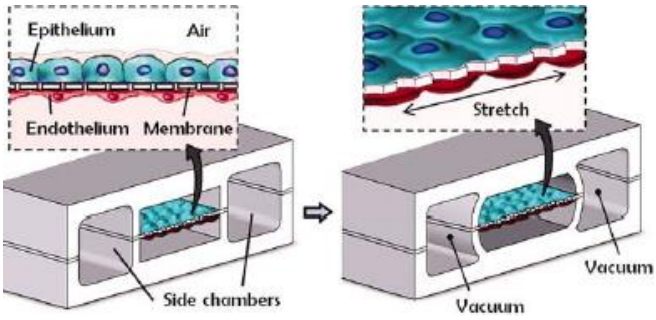
Induced pluripotent stem cells (IPS)

- Nobel prize 2012: Shinya Yamanaka, Gladstone Institutes
- Adult human cells (e.g. fibroblasts, keratinocytes) are isolated
- Reprogramming through delivery of OCT4, SOX2, NANOG and LIN28 transgenes (via retroviruses for instance)
- Induced pluripotent stem cells can then differentiate into all three germ layers / all cell types

➔ Enables patient and disease specific engineered tissues

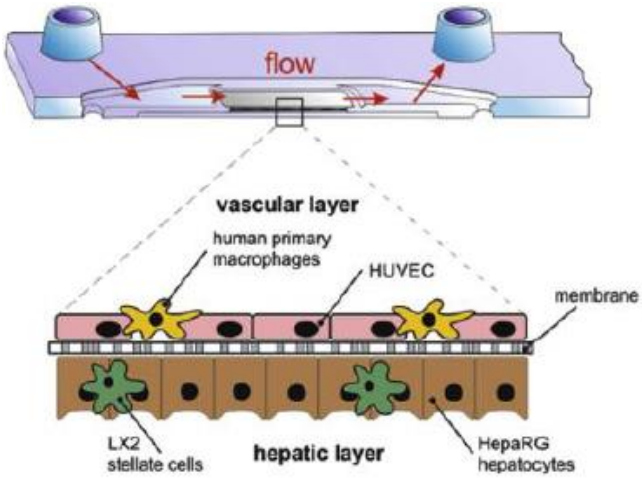
Lung-on-a-chip

- Breathing motion: Mechanical stimulation
- Air Blood interface
- Immune competence
- Flexible membranes with epithelial cells on one and endothelial cells on the other side



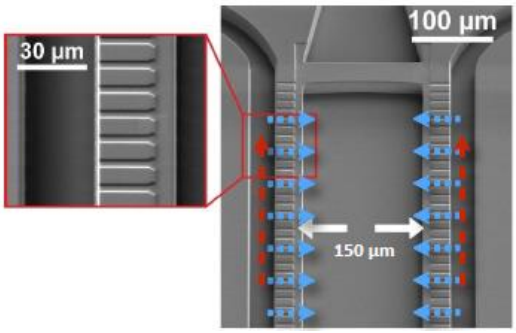
Liver-on-a-Chip

- Multilayer structure
- Integration of non-parenchymal cell types
- Long term maintenance of metabolic and clearance capacity
- Two channel platform with endothelial side (upper) and hepatic side (lower)
- Immune response
- Inflammation
    - loss of central metabolic functions
    - sustained inflammation
    - cellular dysfunction / cell death
- Regeneration
    - Reconstitution of organotypic functionality
    - immune tolerance
    - prevention of cell death



Heart-on-a-Chip

- 3D fiber structure
- Spontaneous contraction
- coordinated stimulus transmission
- Central tissue chamber and adjacent vasculature-like media channels connected via micropores
- Separation of transport processes (diffusive vs convective)
- Aligned human iPS-Cardiomyocytes
- Physiological effect of exposure to drug compounds: Increase in contraction force and beating rate



Future applications

- Drug development
- Disease modelling
- Personalized medicine
- Toxicity screening

- Mechanistic studies
- Microbiome research

Genetic Manipulation (13.05.)

Applications:

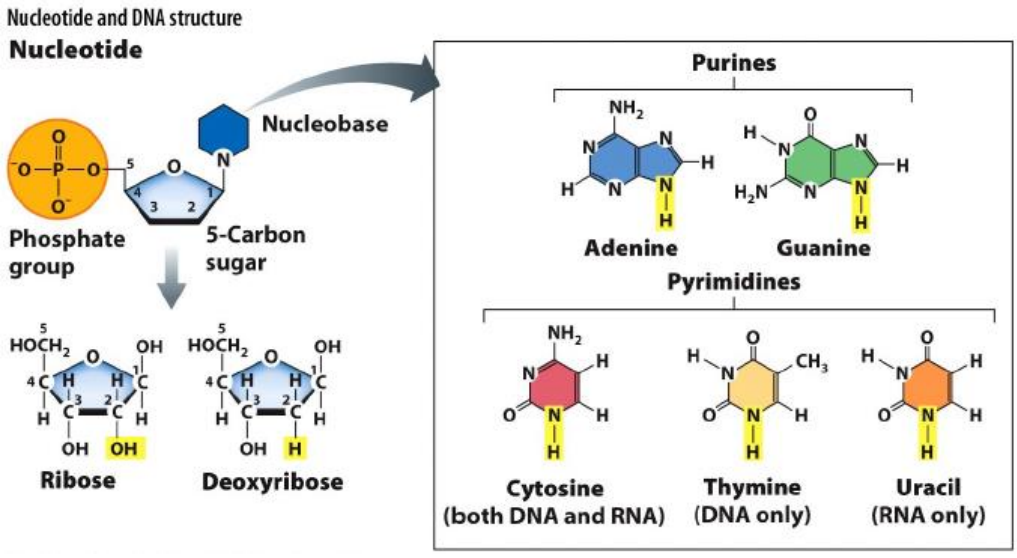
- cloning of genes for protein production (e.g. insulin)
- Mutagenesis of organisms
    - Deletions
    - Point mutations
    - Insertions
    - Chimera (more than one genome in one animal)
- Reprogramming of gene expression (inhibit oncogenes, activate tumor suppressors)
- Gene-therapy

Molecular Basics

Avery-MacLeod-McCartey experiment

- Found out that information is in DNA
- Bacteria can naturally take up other DNA → "auto-manipulation"
- In bacteria (and also mitochondria) no chromosomes, but circular DNA and plasmids, also regions of genes and something in-between

DNA structure



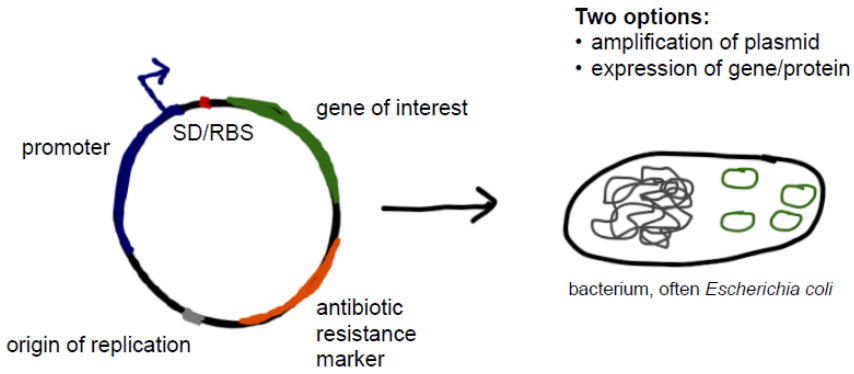
- DNA has melting temperature where the double strand is divided into two single ones.
    - For G/C: 4°C, T/A: 2°C for each nucleotide → have to add up
- 5'-3' direction can be doubled continuously, but 3'-5' only in little parts

Production of proteins

Cloning of gene on plasmid

- Plasmid contains:

- Origin of replication: how often the plasmid should be copied → more of the gene
- Antibiotic resistance marker: force the bacterium to keep the plasmid inside

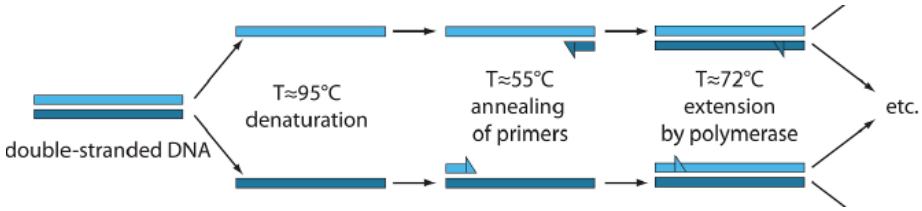


Cloning by using restriction endonuclease (old school)

- Plasmid is opened by endonuclease
- Foreign DNA is cleavage at the ends to make "sticky ends" (one stranded part with AATT/TTAA sequence)
- Foreign DNA is fused with plasmid

Excursus: polymerase chain reaction (PCR)

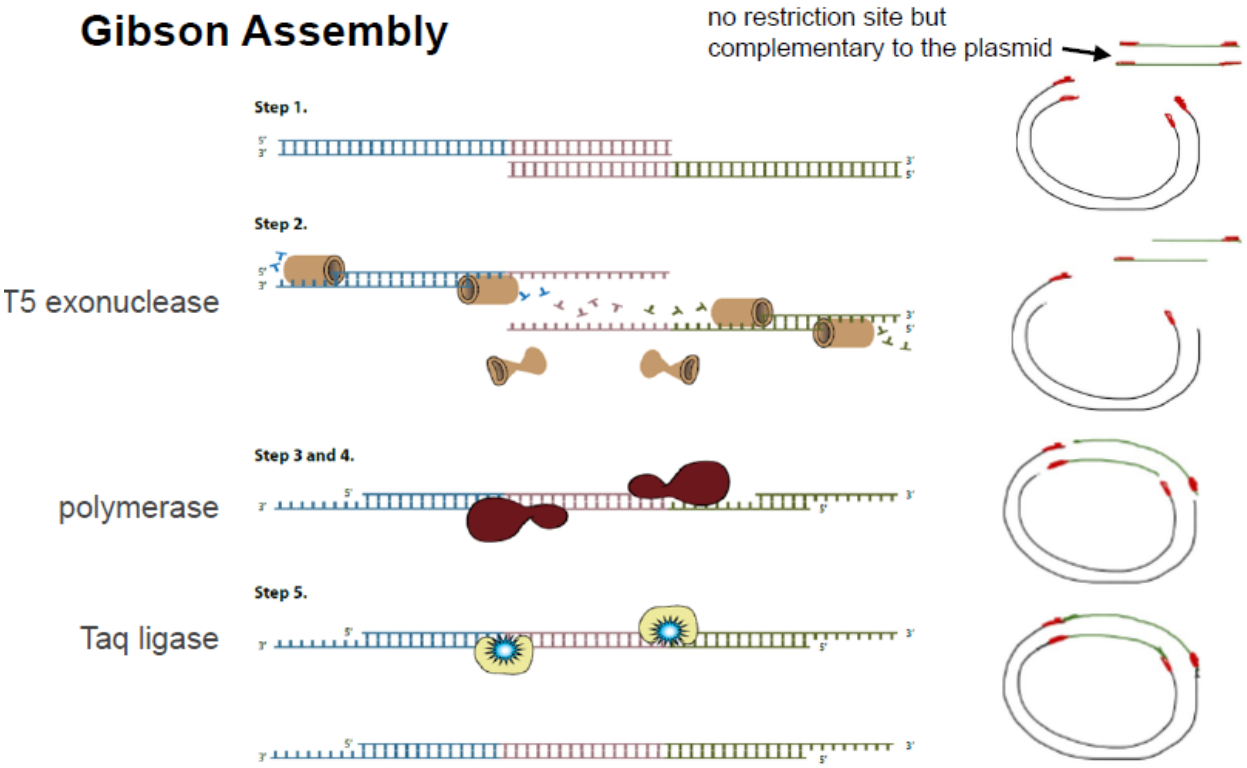
- DNA template
- DNA primer
- DNA polymerase
- dNTPs (dinucleotide-triphosphate)
- Buffer (MgCl2, ...)



- Double strand is denaturized at 95°
- Annealing of primer at 55°C
- Extension/building new double strand by polymerase at 72°
- Repeat

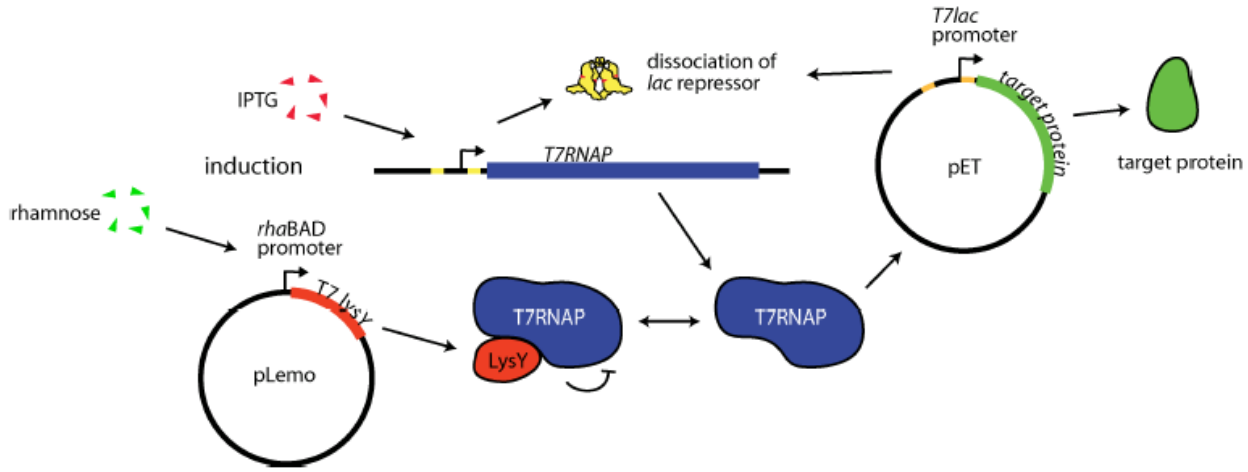
Gibson Assembly

- End of the plasmid region and end of the gene insert overlap
- Exonuclease "eats up" one strand of the ends → creates long sticky ends
- Polymerase wants to close the holes again, but before the different pieces stick together, and only the holes in between are filled
- At last the taq nuclease seals it completely
- → gene of interest in plasmid



Example: Lemo 21

- Repressed system without inducer: no expression due to the lac operator and lac oppressor
- By introducing IPTG the oppressor is detached, and the polymerase can attach to T7RNAP gene (expression system)
- The whole thing is in front of the gene for the target protein
- As soon as you add IPTG the protein is produced very much
- Control mechanism: second plasmid with rhaBAD promotor
    - Activated by rhamnose
    - inhibits T7RNAP via LysY → make sure no transcription of T7RNAP is happening and no protein is produced



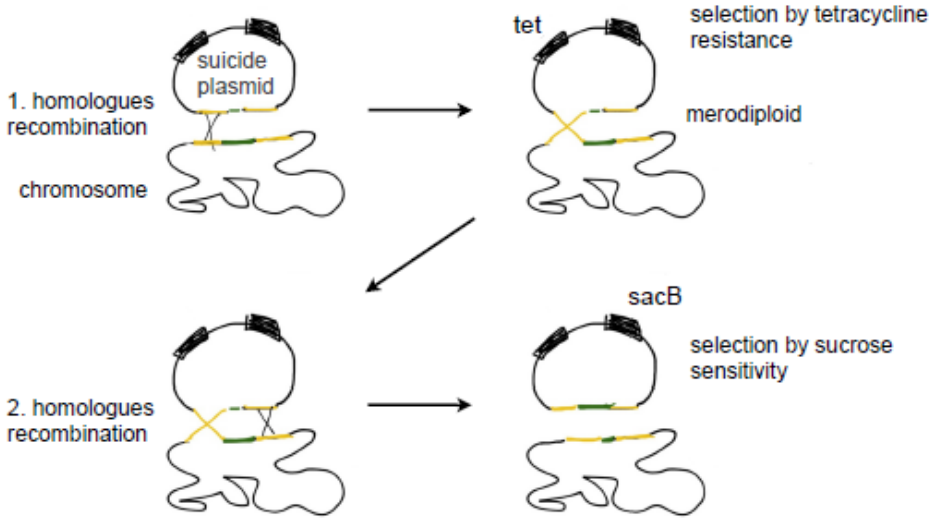
titratable system with two inducers

Mutagenesis

- Random vs. site-directed
- Random:
  - PCR products – error prone PCR, e.g. addition of Mn^{2+}, disbalance of dNTPs
  - Plasmids – amplification in strains with impaired DNA repair mechanisms
  - Organisms – addition of mutagens, infection with virus
- Site-directed:
  - PCR amplification with primers containing specific mutation

Modifying bacterial chromosomes – Allelic exchange

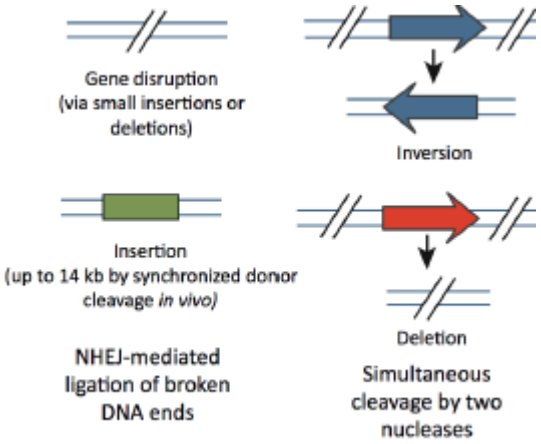
- Suicide plasmid: cannot be replicated, “single-use”
- Information on suicide plasmid has to be transferred to chromosome
- By doing a homologues recombination the gene of interest is transferred to the chromosome
- Suicide plasmid is transferred naturally from donor cell to a recipient cell via sex pilus



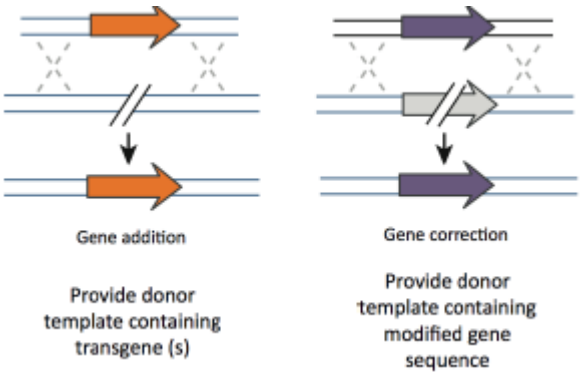
Editing eukaryotic genomes

DNA repair

- Non-homologous end joining
    - Gene disruption (via small insertions or deletions) is repaired insertion → just any nucleotide is added in between
    - Two gene disruptions are repaired by
        - Inversion → the middle piece of DNA is inverted and then everything is glued together again
        - Deletion → the middle piece is left out and the two other ends are glued together



- Homologues recombination



How to target a specific gene/DNA sequence? ZFN: Zinc finger nucleases

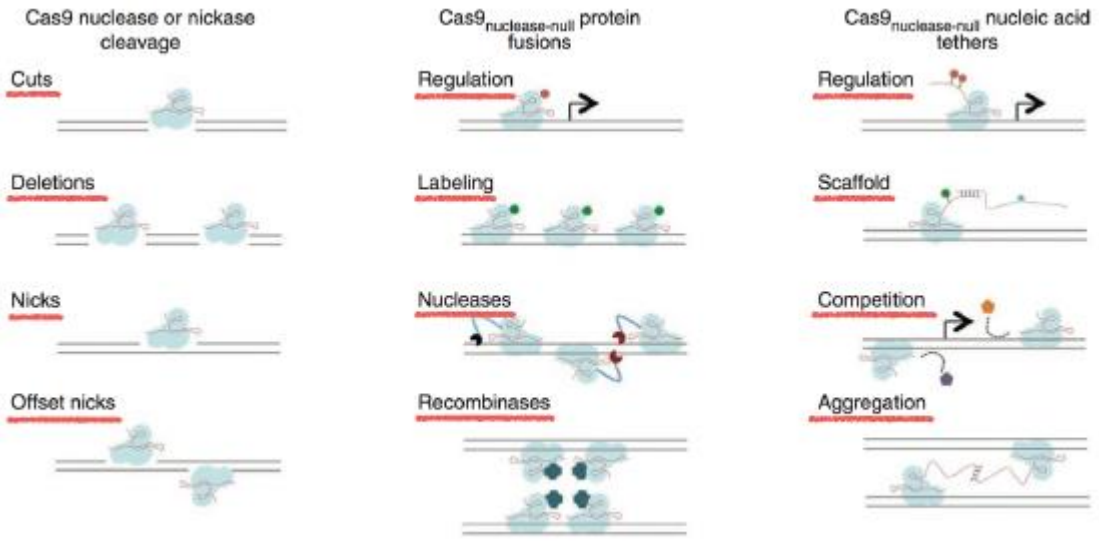
- 6 “fingers” that each recognize 3 base-pairs → very specific in the end
- Can control where this ZNF will bind to DNA
- Can define where DNA should be cut (cleaved)
- specific ZFs for nearly all 64 possible bp triplets exist to be combined

CRISPR-Cas

- RNA-mediated DNA-targeting system that confers immunity
- Immunization: bacteria uses CRISPR locus for immune defense

    - Viral DNA is cleaved in spacers, then chain of spacers with repeaters in between as a "memory" of infections
    - Out of that a RNA is made, then cleaved via RNAse III, cas9 and Tracer RNA → result: little pieces specific for each viral DNA
    - These pieces can bind to new viral DNA and again cleave it
- This principle is now used to target any DNA sequence
    - Here now the spacer and the tracer RNA are bound in the same chain
- You have DNA, RNA and protein → very powerful

Application



Concepts

- Restriction:
    - site-specific cleavage of palindromic DNA by restriction enzymes
- Assembly:
    - isothermal fusion of dsDNA fragments using exonucleases, polymerases, and ligases
- Plasmids:
    - mobile genetic elements used for cloning and expression of genes
- Homologous Recombination:
    - cross-over (exchange) of homologous DNA fragments
- Site-specific DNA editing:
    - requires double strand breaks
    - protein-guided nucleases: ZFN, TALEN
    - RNA-guided nucleases: CRISPR-Cas

- Need to know if you e.g. need a polymerase, but not if it is I or III, …

Computer simulation of human movement (20.05.19 Dr. Daniel Häufle)

Forces in the human body

- Bones:
  - gravity acting on mass, friction forces
  - shear, loading, rotational forces
- Tissue
  - Contact forces → deformation of soft tissue
  - Internal pressure
- Muscle
  - Tensile forces (passive)
  - Active forces by muscles

Newton's laws

① if $F = 0$ $\frac{dv}{dt} = 0$ (Trägheitsgesetz) inertia

② $F = \frac{dp}{dt} = \frac{d\,mv}{d\,t}$ $m = \text{const.}$

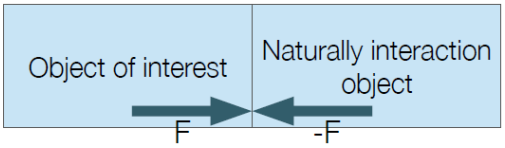
$F = ma = m\ddot{x}$

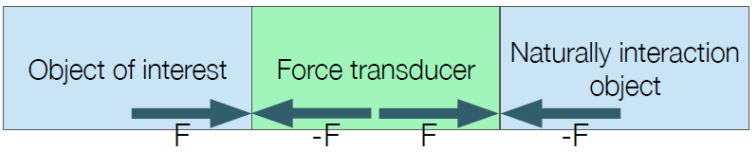
③ $F_{actio} = -F_{reactio}$

Force measurements

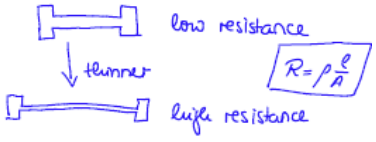
Force transducers

- Deformation caused by force
  - Hooke's law: $F = -k \cdot \Delta l$





- Use force transducer to measure the force between two objects
- Buckle force transducer
  - Strain gage on top of a rod
  - strain gage will get longer and therefore thinner during bending → resistance will increase for stronger bending
  - (calibrate the system and then measure forces with it)

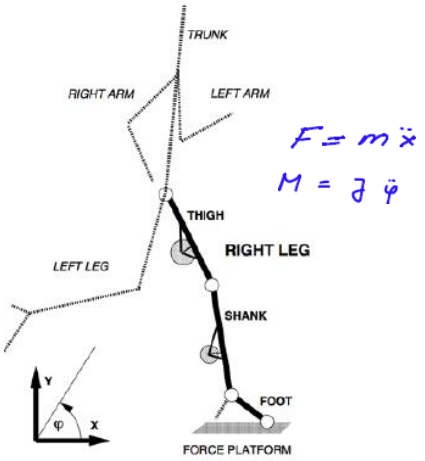


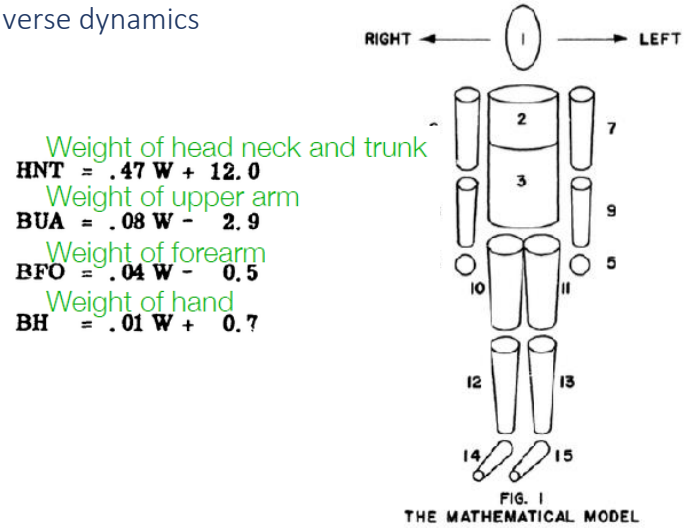
- Optical fiber in the tendon (of dead person)
    - Deformation can be detected via wavelength of reflected and transmitted light
- Pressure transducer implanted in belly

Force transducers: advantages and disadvantages

- Advantages:
    - direct measurement of the force (via deformation)
    - many options (need deformable object, measure deformation)
- Disadvantages:
    - additional device needs to be inserted (invasive)
    - analysis tool (cannot predict/simulate any outcome)

Second approach for force measurement: inverse dynamics





- Anthropometric models give mass and inertia of each segment
- Acceleration:
    - Capture motion with 12 cameras
    - Motions captured with force plates (ground force detectors) and inertia measurement units (IMUs) placed directly on the person

Inverse dynamics: advantages and disadvantages

- Advantages:
    - not invasive
    - robust algorithms exist
    - commercially available → popular in (sports-) biomechanics
- Disadvantages:
    - Analysis method
    - only net joint torque/contact forces → co-contraction is not resolved

Prediction of internal forces and loads

- forward dynamics
    - make use of Newton's law → $\ddot{x} = \frac{F}{m}$

- predict internal forces and movement by mathematical models of structures in the human body → computer simulation

Our viewpoint on natural systems



Bones Structure	Rigid bodies
Ligaments, cartilage, fat Springs	Passive force
Muscles Motors	Active force
Neurons Wires, CPU	Reflexes, commands

$$\frac{d}{dt}\frac{\partial L}{\partial \dot{q}_i} - \frac{\partial L}{\partial q_i} = Q_i$$

depend on mechanical system state

$$F_p = f(z, \dot{z})$$

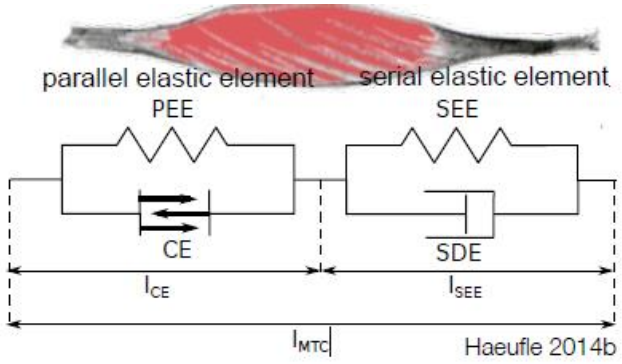
with $\mathbf{z} = [q_1, \ldots, q_n, o_1, \ldots, o_m]^T$

$$F_a = f(z, \dot{z}, u)$$

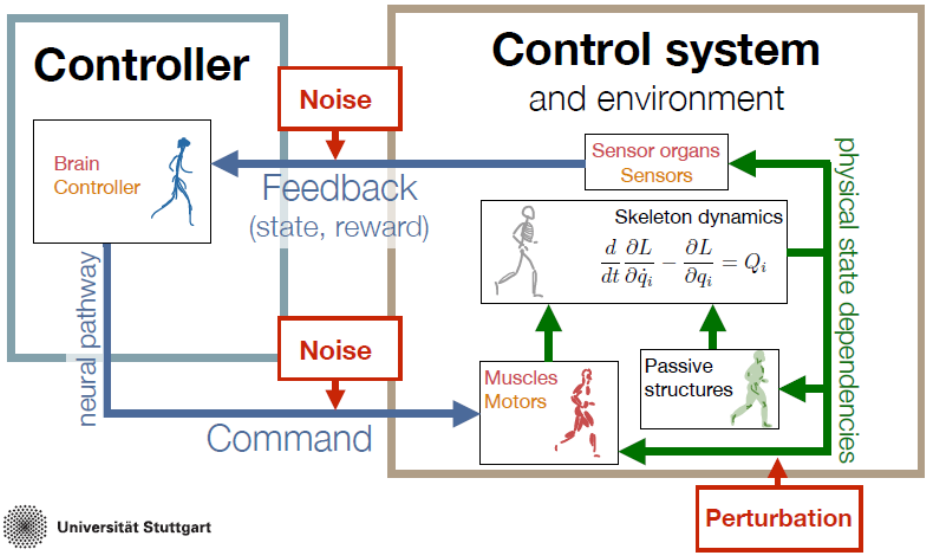
with $\mathbf{u} = [u_1, \ldots, u_k]^T$

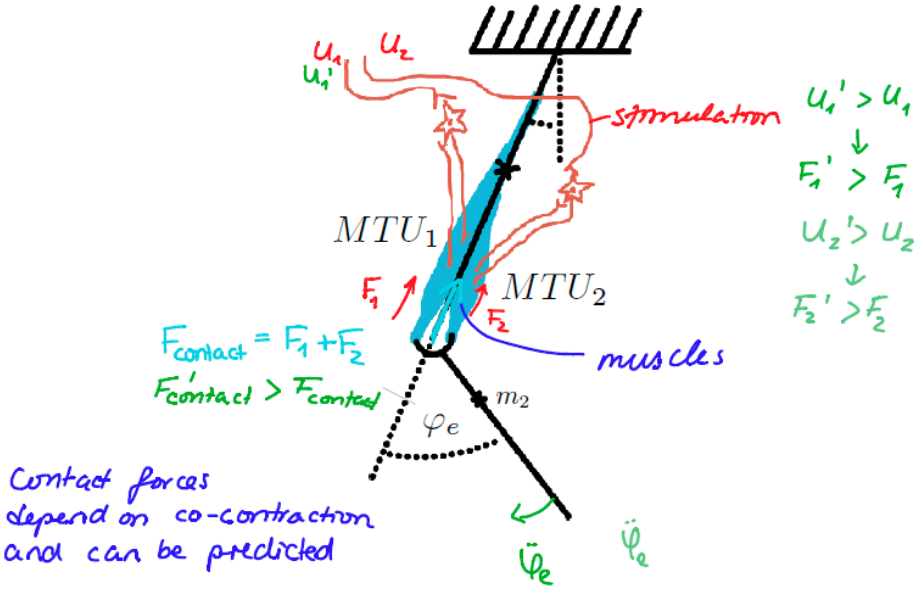
$$u = f(z, \dot{z}, \text{"brain"})$$

- Muscle:



- Soft tissue:
  - Function:
    - interaction with the environment
    - relative movement and absorbing energy
- bones and joints
  - Function: dynamics of the movement
- neurons
  - Function: perception and action





Forward dynamics computer simulation: advantages and disadvantages

- Advantages
  - consider detailed forces and loads
  - predict an outcome (no analysis tool)
  - may be used to plan a surgery
- Disadvantages
  - computationally expensive
  - difficult to model forces of all structures

➔ Synthesis (prediction)

Advanced human model - lower extremity and spine

- 13 body segments
- 452 degrees of freedom

- 48 mechanical DOFs
- 202 muscle-tendon-complexes
- 58 non-linear ligaments
- 5 IVDs (nonlinear, coupled)

A numerical application in spinal fusion surgery

- Number of spinal-fusion surgery is rapidly increasing
- Is adjacent segment degeneration and disease a consequence of spinal fusion?
    - Change of displacement, internal forces and moments

Take home:



- Knowing about internal dynamics, loads and forces is important



- Measure forces:
  + direct results
  - invasive
  - analysis tool



- Inverse dynamics
  + robust
  + clinical acceptance
  - only net joint torques
  - analysis tool

- Forward dynamics computer simulation
  + synthesis tool
  + predicts outcome
  + may be used to plan surgery
  - computationally expensive
  - difficult to model



Application for Animal Experiments (03.06.19, Schenk)

- Only 0.26% of animals are sacrificed for research (ca. 99% are killed for nutrition)
- Directive EU law on protection of animals used for scientific research (also transferred to German law: Tierschutzgesetz, Tierschutz-Versuchstierordnung)
- Nobody is allowed to inflict harm, suffering or injuries to an animal without a reasonable motive
    - "Reasonable" motives: meat production, killing male chicken in egg production

DIRECTIVE 2010/63/EU

- Article 1
    - Is only for scientific and educational purposes
    - Only applies until animals are killed → you can do anything with the dead animal
    - Animals: vertebrates & cephalopods (Oktopus), for others no law
    - If you do more than a needle injection, it is an animal experiment
    - Directive does not apply to non-experimental agricultural practice, …
        - Things like horse walkers etc. used on farms are allowed/not stopped by this law
- Article 4: Principle of replacement, reduction and refinement
    - **Replacement**: preferred use of non-animal methods over animal methods
    - **Reduction**: methods to obtain comparable data from fewer animals
    - **Refinement**: methods to alleviate or minimize potential pain, enhance animal welfare, …
- Article 5: Purposes of procedures
    - Procedures may be carried out for the following purposes:
        - basic research
        - translational or applied research
        - developments, testing etc. of drugs, food- and feed-stuffs, …
        - protection of natural environment in the interest of welfare of animals
        - research to preserve the species
        - higher education, training
        - forensic inquiries
- Article 6: Methods of killing
    - Kill with minimal pain
    - Precisely regulated which killing method can be used for which animal
- Article 11
    - Stray and feral animals of domestic species shall not be used
- Article 12: Procedures
    - Procedures may be carried out only within the framework of a project (not for a complete institution)
- Article 13: Choice of methods
    - Use minimum number of animals
    - cause the least pain, suffering, distress or lasting harm
        - Professionality (Infrastructural, Experimentators, Animal Keeping)
        - Continuous Monitoring (Health status, Expected and unexpected suffering)
        - Appreciation (most valuable team members)

- Death as end-point of a procedure shall be avoided

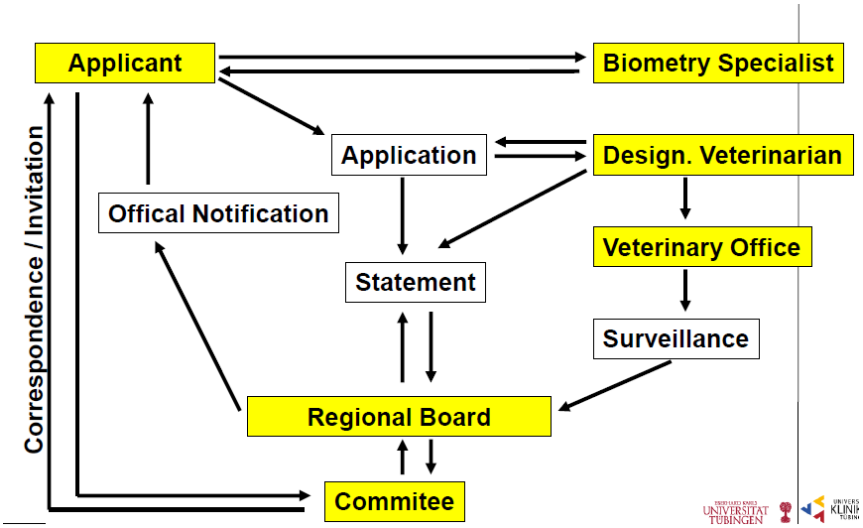
Side note: Sample number estimation

- Hypothesis
- Primary Target Parameter
- Standard Deviation of Measurement
- Effect Size
- Acceptable Error Type I
- Acceptable Error Type II

Table of error types		Null hypothesis (H_0) is	
		True	False
Decision About Null Hypothesis (H_0)	Reject	Type I error (False Positive)	Correct inference (True Positive)
	Fail to reject	Correct inference (True Negative)	Type II error (False Negative)

- Article 15: classification of severity of procedures
  - Classify procedures as “non-recovery”, “mild”, “moderate” or “severe”
  - Mild: single dose or limited number of blood samples, non-invasive imaging, superficial procedures
  - Moderate: surgery under general anesthesia with post-surgical pain; induction of tumors causing moderate pain; irradiation or chemo with a sublethal dose
  - Severe: toxicity testing with death as endpoint,
- Article 18: sharing organs and tissues
  - Member States shall facilitate the establishment of programs for the sharing of organs and tissues of animals killed
- Article 23: Competence of personnel
  - staff shall be adequately educated and trained before
  - Member States shall publish minimum requirements with regard to education and training and the requirements for obtaining, maintaining and demonstrating requisite competence
- TierSchVersV:
  - Who can additionally to Article 23 do animal experiments?
  - Everyone with finished degree who can meets the requirements (→ do a training)
  - Operations only from specially trained people
- Article 30 Animal records
  - Need to keep records about species, origin, for which project they were used, number of killed animals, …
  - Records shall be kept for a minimum of 5 years and made available to the competent authority upon request
- Article 36 Project authorization
  - Projects must not be carried out without authorization from a competent authority

- Article 37 Application for project authorization
    - Application must include project proposal, non-technical summary, …
- Article 38 Project evaluation
    - evaluation shall be performed with a degree of detail appropriate for the type of project and shall verify that the project meets the following criteria:
        - project is justified from a scientific or educational point of view or required by law
        - purposes of the project justify the use of animals
        - project is designed so as to enable procedures to be carried out in the most humane and environmentally sensitive manner possible.
- Every animal experiment has to be entered on a webpage (AnimalTestInfo)



Animal models as tools to investigate diseases (17.06.19, Erwin Bohn)

The ideal model: humans

- Not ethical, risk for life to high
- Exceptions:
    - 1. Highly controlled clinical trials and studies
    - 2. Study of inborn errors rare genetic disorders of humans, best evidence to understand gene functions
    - 3. Association studies Determination of SNPs single nucleotide polymorphisms which are leading to increased disease risk. Animal models helpful to further investigate candidate genes
    - E.g. patients with a genetic defect give the best information about this pathway, if you don't have patients you need animals to investigate pathways

Why use mammal models to study human disease?

- Models selected because of similarity to genetics, anatomy, and physiology
- Unlimited supply and ease of manipulation
- Defined nutrition, defined environment, defined genetic background

- Important manipulate only one variable and observe in consequence change
- Perform statistics

Most important species of laboratory animals

- Mouse
  - Most frequently
  - Similar genome to human
  - Easy to handle
  - Unique technologies to manipulate mouse genome
  - High reproductive rate
  - Low costs
- Rat
  - physiology of cognitive processes, behavior, models of diabetes
- Rabbit
  - serology, insulin quantification
  - pyrogens quantification
  - tests of irritable effect of chemical substances on the cornea
- Cat
  - study of CNS and respiratory system
- Dog
  - e.g. beagle
  - use in electrophysiology, neurophysiology
- Guinea-pig
  - in microbiology and serology
  - physiology of the auditory system
- Hamster
  - genetics
- Pig
  - training of surgical techniques
  - temporary covering of burns with porcine skin
- Primates
  - rhesus monkey, baboon, chimpanzee
  - use in neurology, virology, behavior
- Frog
  - physiology of blood circulation, electrophysiology
- Fish, molluscs, insects
  - development, infections

GENETICS OF LABORATORY ANIMALS

1. Isogenic = genetically defined strains (isogenicity = genetic uniformity of all individuals)
2. Non-isogenic = genetically undefined strains
3. Genetically semi-defined strains

Isogenic strains

- Inbred strains
    - close breeding for more than 20 generations (brother + sister or offspring + one of the parents)
    - homozygosity higher than 98 % (possessing two identical forms of a particular gene, one inherited from each parent)
    - isogenicity, phenotype uniformity (low variability of reactivity), usually low fertility, disposition to diseases
    - advantages: homogenous statistical set, lower number of individuals is sufficient
    - disadvantages: risk, that the findings are strain-specific and are not valid for other strains, problematic generalization of the results
- Coisogenic strains (mutant strains)
    - differ from the original strain only in one gene, in which a mutation occurred
- Congenic strains
    - strains originated by cross-breeding of two strains and following back-cross- breeding with one of the original strains (at least 10 times, selection of some specific feature)
    - presence of specific genes of one strain on genetical background of the second strain
- Recombinant-inbred strains
    - crossing of 2 strains, the hybrids give origin of new lines which are then crossed brother x sister, what leads to establishing of a new strain
- Consomic strains
    - a complete chromosome of one strain is transferred on the background of the second strain with back-crosses (similarly as for individual genes in congenic strains, but the process is more complicated)

Non-isogenic strains

- Outbred lines
    - genetically heterogeneous
    - no crossing with individuals coming from different …
    - close crossbreeding is avoided so that the coefficient of inbreeding remains as low as possible
    - features: some level of phenotype variability (higher variability of reactivity), higher fertility and resistance to diseases
    - advantages: cheaper and easier production, the findings have more general validity
    - disadvantages: less homogeneous set, higher number of animals is necessary

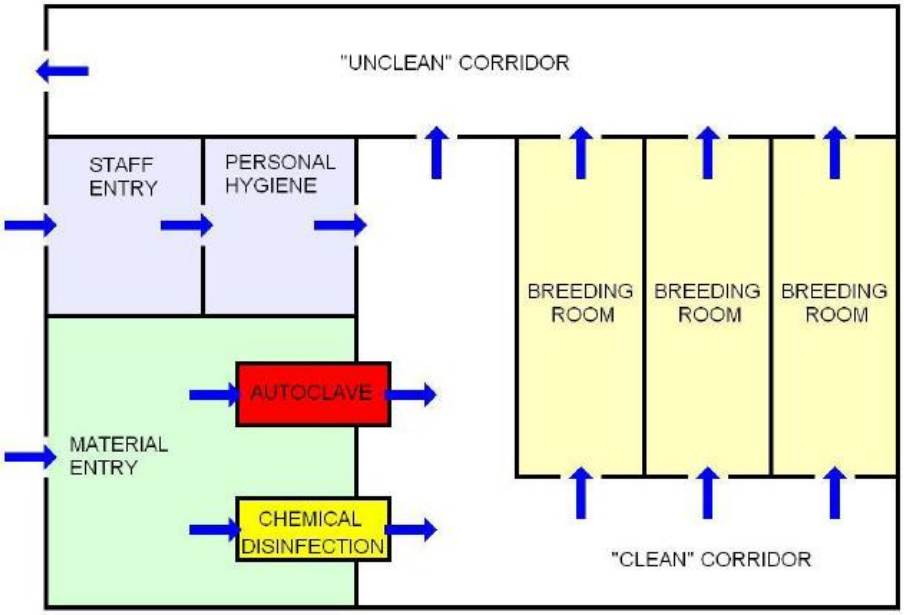
SYSTEMS OF BREEDING OF LABORATORY ANINMALS

- Open
    - communication without the barrier
- Barrier
    - animals separated from external environment
    - movements of animals, people and material are controlled to eliminate possible introduction of alien factors from the external environment (infection)

- sterilization of coming water, food, sawdust used for bedding, perfect personal hygiene of the personal

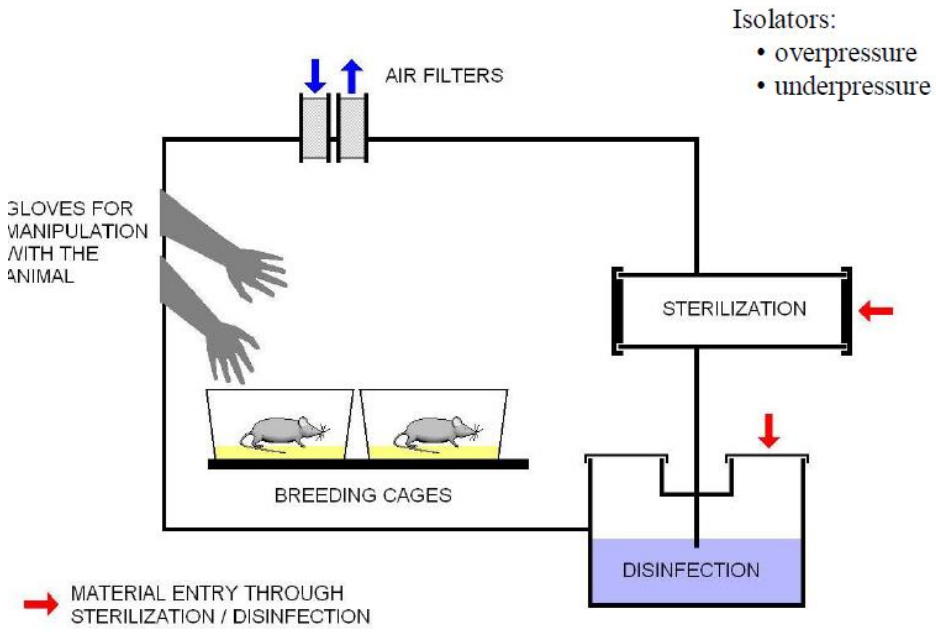
- Isolator
  - space for the animals is permanently separated by a barrier from external environment as well as from people who manipulate with the animals.

➔ Question of costs



GNOTOBIOLOGY (→ study in pathogen free environment) OF LABORATORY ANIMALS

- Conventional animals
  - undefined microbiota
  - open breeding facility complying basic hygienic conditions
- **SPF animals = specified pathogen free**
  - microbiota of the animals certainly does not contain specified pathogens.
  - barrier breeding facility
  - everybody defines it different
  - variations of microbiota can be different from one room to the next
- **Gnotobiotic animals - breeding isolators**
  1. **Axenic animals = germ free**
     i. without any microbes
     ii. babies obtained with sterile hysterectomy or hysterotomy into sterile atmosphere of the isolator
  2. Associated animals
     i. derived from axenic animals
     ii. colonizing them artificially with one or more species of microorganisms
     iii. monoxenic, dixenic, polyxenic

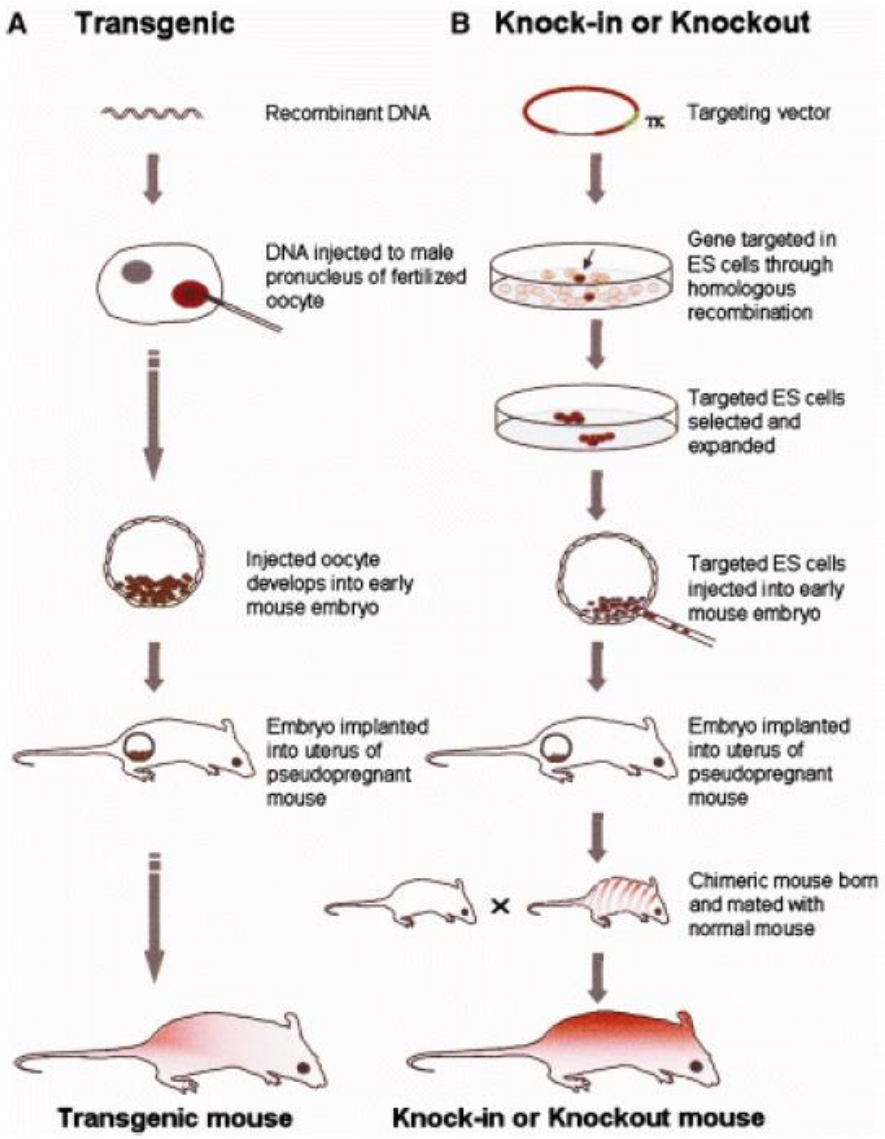


Germ-free mice

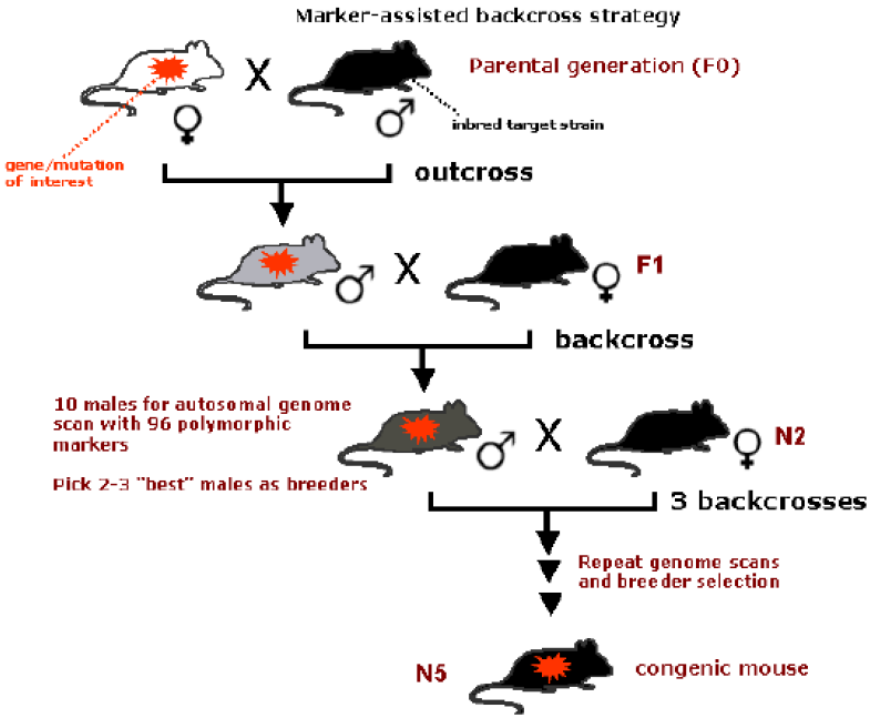
- Microbiota strongly affects immune system
- altered splenic architecture (less germinal centers)
- Less intestinal CD4 cells
- less IgA producing cells
- Addition of microbiota leads to restoration of immune system
- Used to understand the impact of the microbiota on immune system, disease development (microbiota 100-fold more genes)
- GF mice develop less inflammatory bowel disease, thus microbiota associated with IBD
- GF mice more susceptible to gastrointestinal infections (colonization resistance, altered Immune system)

Animal models of diseases – Genetics

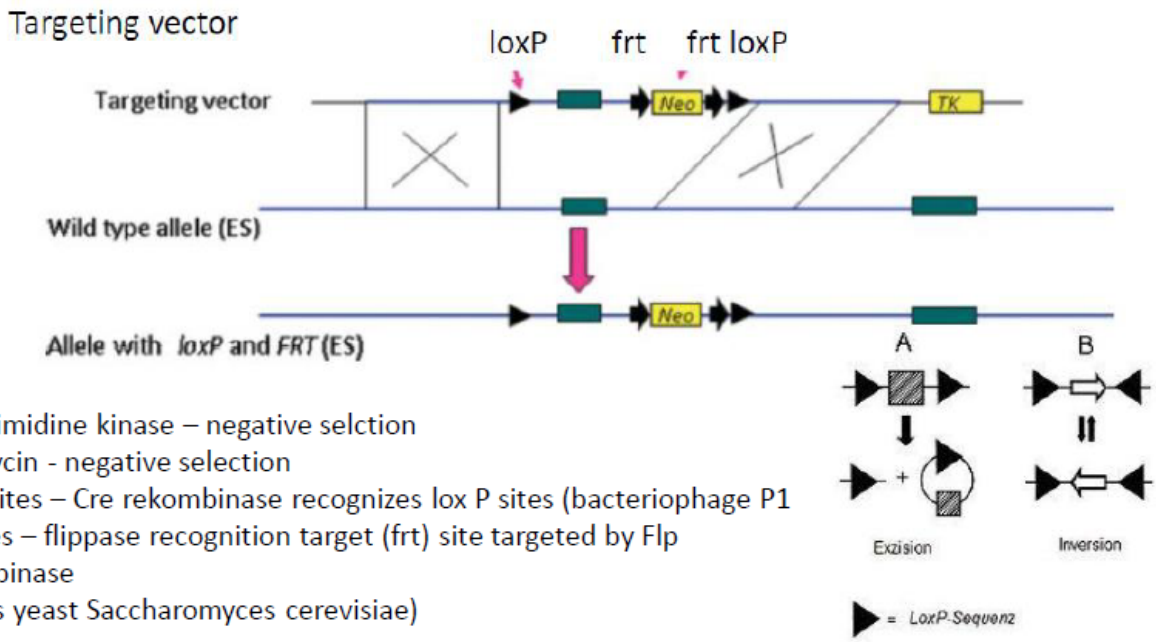
1. **Reverse genetic or gene-driven approach**
    - Targeted mutation of genes to determine the function of a gene in the context of a whole organism
    - Initial hypothesis required
    - approximately 50% of about 34000 known mice have some functional annotations
    - Sometimes inconclusive (embryonic lethality, undistinguishable because of gene redundancy (= Überflüssigkeit)
    - Single gene knock-outs
    - Knock-Ins
    - Constitutive or conditional gene modifications
    - Chromosomal rearrangements



- Injection of ES cells into early embryo
- Implantation of embryo into forster mother
- Offspring: chimera
- Mating (= Paaren) with the target strain and screening for mice with germline transmission
    - Some of their children (50%?) have then the desired gene defect
    - Back crossing takes 10-20 generations to receive inbred strain
- Other option: speed congenics



Generation of Knock-out mice:



Tk Thyimidine kinase – negative selction
Neomycin - negative selection
Lpx P sites – Cre rekombinase recognizes lox P sites (bacteriophage P1
Ftr sites – flippase recognition target (frt) site targeted by Flp recombinase
(bakers yeast Saccharomyces cerevisiae)

Transfection of embryonal stem cells (129Sv agouti coloured) with plasmid
Selection with Neomycin and gancyclovir
Neomcin-positive and Tk-negative cells survive

Finally deletion of selection marker, e.g with Flp rekombinase

2. Forward genetic or phenotypic-driven approach
    - Definition of susceptibility loci by backcrossing approaches
    - Generation of congenic/consomic strains and testing phenotypes
    - Mutation-driven approach uses radiation and chemicals to cause mutations. Large scale mutation screens to test phenotypes

Galleria melonella (Wax moth) as an infection model and for drug screening

- Cheap
- Larvae can be easily purchased (used for feeding of reptiles or fishing)
- Larvae: 150-200 mg and 2-3 cm long
- High temperature tolerance, tolerate 37°C (temperature of infection)
- Easy and rapid injection of bacteria or substances possible
- Innate immunity can be studied
- Hemocytes function similar to mammalian macrophages and neutralization

	Mouse	Galleria
Application	Yes	No
Costs	expensive	cheap
Immune system	innate and adaptive	innate
Intestinal tract	similarities	
Cell adhesion	similarities	
Antimicrobial peptides	similarities	
Adaption to oxidative stress	similarities	
High throughput screening e.g. new antibiotics	difficult	easier

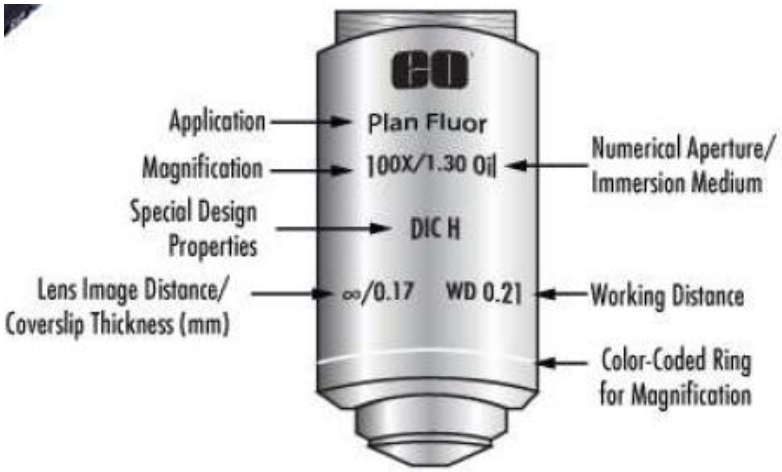
Advanced Microscopy (Eva Brauchle, 24.06.19)

Magnification:

$M_{microscope} = M_{objective} \times M_{ocular}$

Resolution: $d_0 = \frac{1.22\lambda}{NA_{obj.} + NA_{cond}}$, $NA = n \sin \alpha$

Objectives



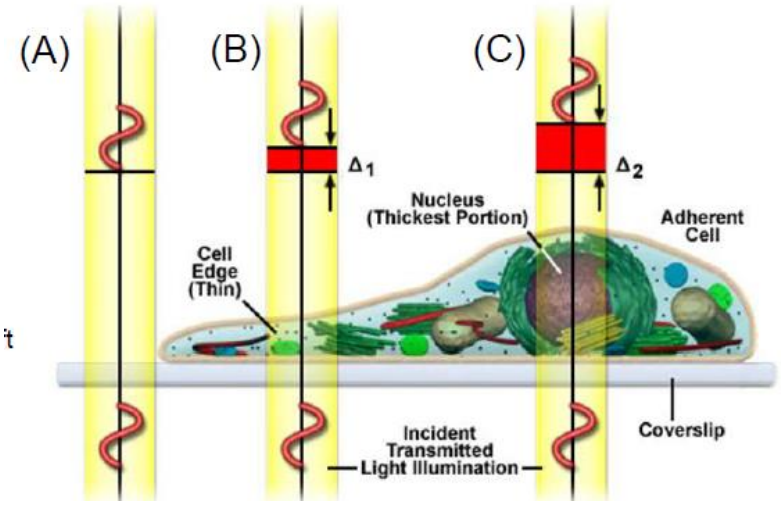
Chromatic aberration

- Failure of the lens to focus all colors on the same point, due to a different refractive index for different light wavelengths
- **Achromat** is corrected for blue and red light
- **Apochromat** is additionally corrected for green light

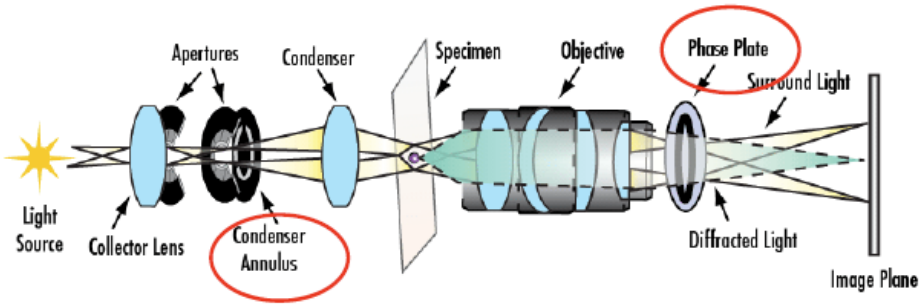
Image Contrast

= difference between the signal in one pixel and that in another that conveys information about the shape of the specimen or the difference in signal intensity between various parts of an image or between details of interest and “background”

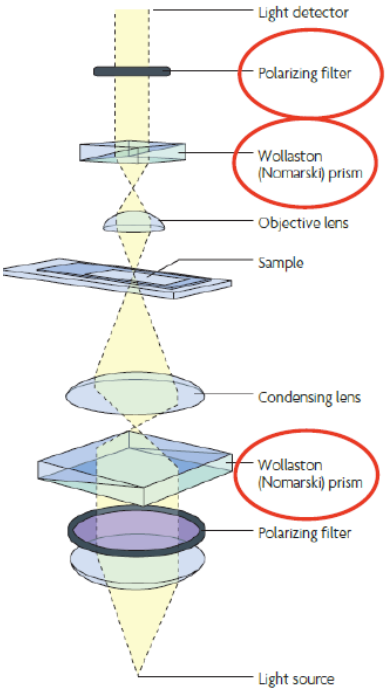
- Various physical and digital “filters” can be used to select specific signals
- Contrast enhancing techniques in optical light microscopy
    - **Köhler illumination**
        - Close blend, focus, open blend only as far so that the sample is illuminated
    - **Phase contrast**
        - Small differences between the refractive indices of the aqueous solution (A) the cell periphery (B) and the nucleus (C)
        - Shift of light rays are translated into intensity shifts



- Condenser annulus: Opaque plate that illuminates the specimens in defocused parallel light ray
- Phase plate: Alters the phase and amplitude of the light



- **Differential interference contrast (DIC)**
  - Use of polarized light
  - Wollaston prism separates the light into two sister beams
  - Sister beams pass the specimens (phase shifts occurs)
  - Second prim fuses the beams and phase shifts were translated into amplitude shift



- Pseudo three-dimensional relief

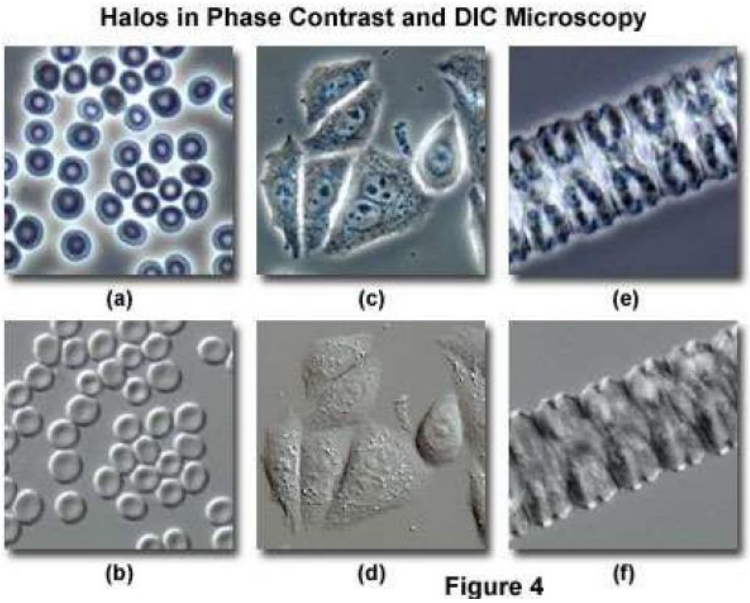


Figure 4

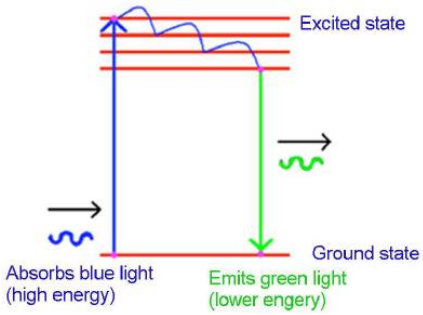
Limitations of light microscopy

- Resolution of light microscopy is limited due to the wavelength of the light source and N.A. of objective and condenser
- Optical contrasting methods resolve limited biological information

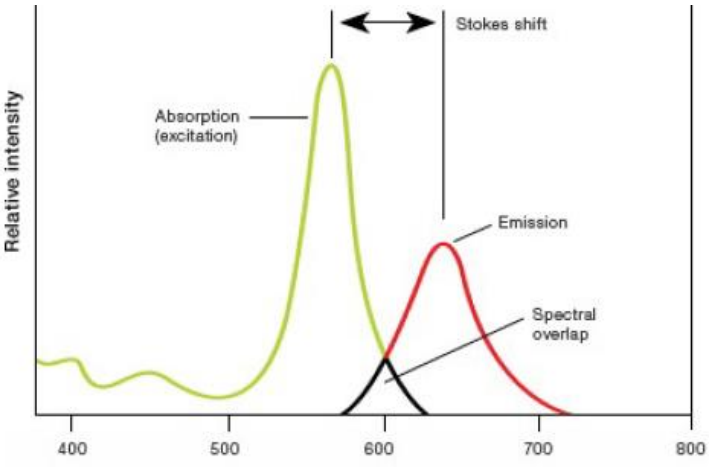
➔ Dyes are needed to visualize specific molecules and structures in biological specimens

Fluorescence Microscopy

Principle



- Luminescent process in which molecules emit light
- Fluorophores absorb light of one wavelength and emit (fluoresce) light of another wavelength
- Fluorescence light is emitted rapidly during relaxation of excited molecules
- Random process
- **“Stokes shift” describes the wavelength shift from excitation maximum to emission maximum**
- Living samples can contain auto fluorescent material
- Absorption and emission spectrum are specific for a fluorophore



Fluorophores

Fluorescence Intensity F:

$$F = \sigma * Q * I$$

Q = Quantum yield
I = Excitation light intensity
σ = Molecular absorption cross section

Fluorophore	Quantum yield	λ_{Ex}	λ_{Em}
GFP	0.6	488	535
YFP	0.61	520	542
DsRed	0.79	558	583
Alexa488	0.81	488	535
DAPI	0.58	358	421

- Alexa 488 was designed to be used with same microscopes that were already used for GFP, …

Fluorescence life-time

- Every fluorescent molecule has an average time that it spends in the excited state followed after excitation
- Decay of fluorescence intensity after excitation can be measured
- The average time is referred as the fluorescent life time τ

Quenching

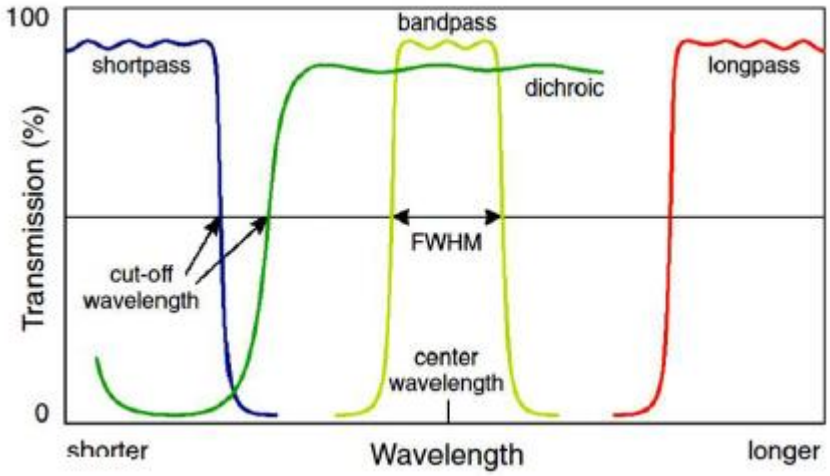
- **Reversible** loss of fluorescence e.g. due to non-radiative energy transfer

Photobleaching

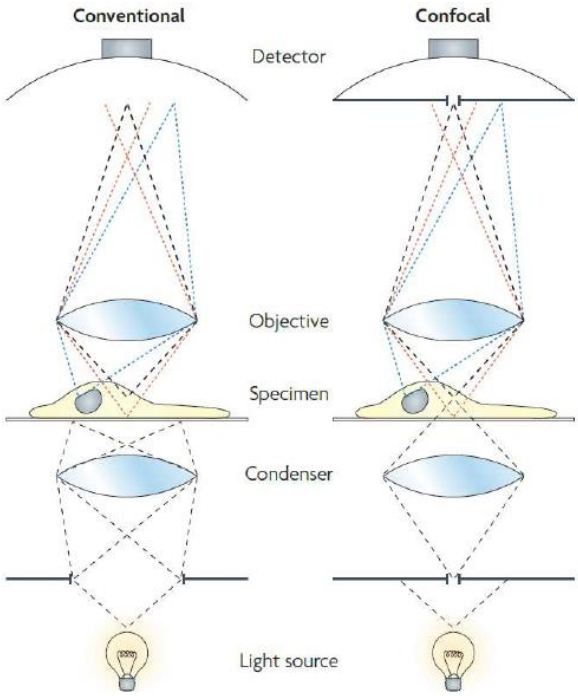
- **Irreversible** reactions that lead to loss of fluorescence
- Oxygen contributes to photobleaching
- Photobleaching is dependent on molecular environment

Microscopes

- Dichroic mirror e.g reflects blue (UV) light; Longer wavelength can pass the mirror
- Light sources:
    - Broad UV/visible light spectrum – Mercury arc lamp, Xenon burner
    - Monochromatic Laser light – Gas lasers, Solid state laser, Diode lasers
- Filters:
    - Shortpass: transmits light only **below certain wavelength**
    - Bandpass: transmits only **light of a specific segment**
    - Longpass: **cuts a defined wavelength**

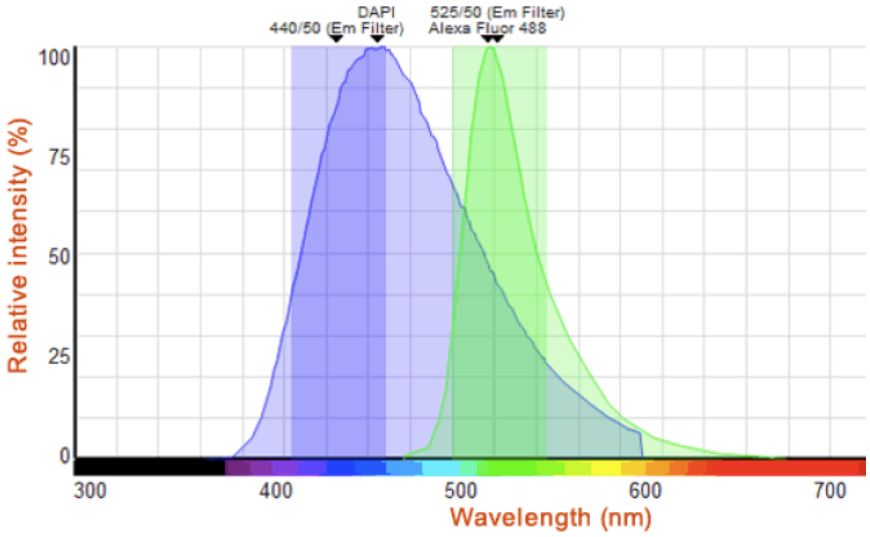


- Conventional microscope: Fluorescent light is detected from all focal planes
- Confocal: Fluorescent light emitted from one focal spot is detected



Simultaneous Fluorescence Imaging

- emission spectra should be clearly distinguishable
- e.g. some of the fluorescence emission from both DAPI and AF488 will pass through the 525/50nm filter
  About 30% of the total signal detected in the second detector will originate from DAPI



How to get fluorescence into the specimens?

- Fluorescent dyes bind to specific cellular structures (nuclei, mitochondria)
- Antibodies conjugated to fluorophores
- Cells / animals are genetically modified to express fluorescent proteins e.g. green fluorescent protein (GFP)
- Use of intrinsic fluorescence (autofluorescence and multiphoton imaging)

Limitations of fluorescence microscopy

Wide-field

- Thickness of specimens
- No lateral resolution
- Information about colocalization is insufficient
- Use of multiple dyes is limited
- Dyes with similar excitation and emission ranges cannot be discriminated
- Bleaching of fluorophores

Confocal

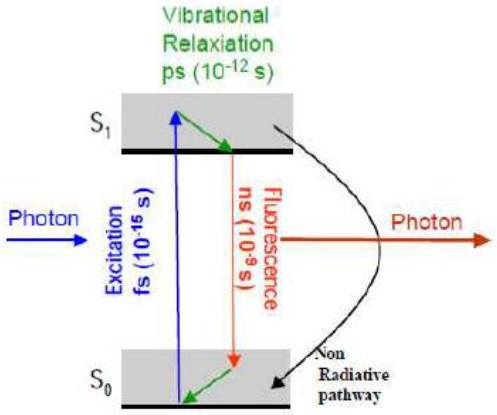
- Dyes with similar excitation and emission ranges cannot be discriminated
- Simultaneous imaging of several fluorescent probes is limited
- Issues due to autofluorescence within the specimens
- Required time for imaging of thick tissue (several minutes)
- Bleaching of fluorophores
- Quantification of fluorescence signal is limited

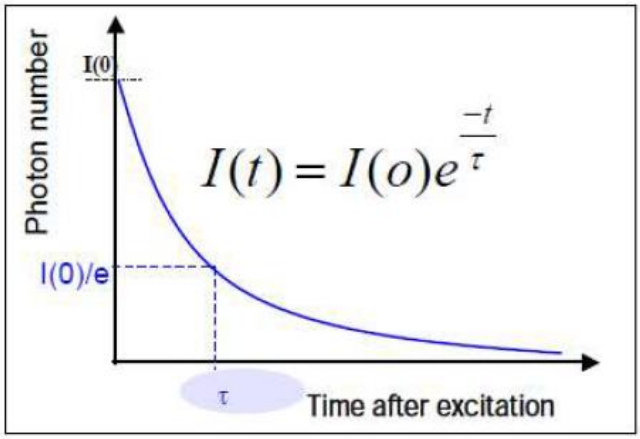
Special fluorescence imaging techniques

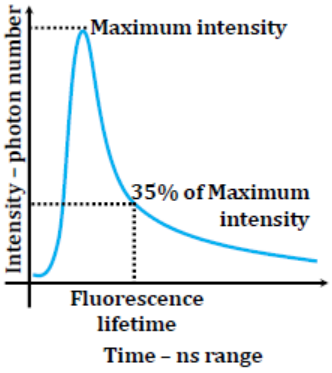
- Fluorescence bleaching
    - Fluorescence recovery after photobleaching (FRAP)
    - Fluorescence loss in photobleaching (FLIP)
    - Fluorescence correlation spectroscopy (FCS)
- Quenching
    - Förster-Resonance Energy Transfer (FRET)
- **Fluorescence life-time imaging (FLIM)**

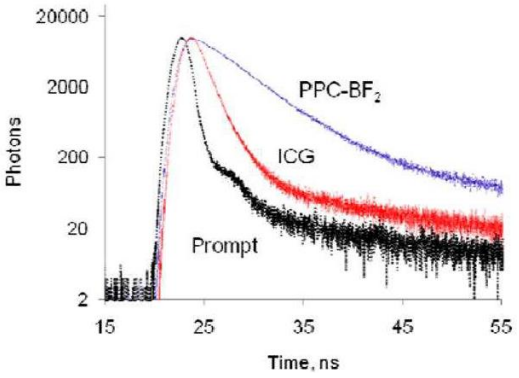
Fluorescence Life-time Imaging (FLIM)

- Every fluorescent molecule has an average time that it spends in the excited state followed after excitation
- This average time is referred as the fluorescent life time τ
- Decay after excitation is an exponential curve
- Fluorescence lifetime imaging uses contrast is based upon the fluorescence lifetimes







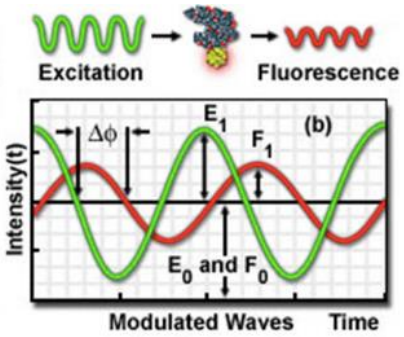


← in reality (prompt = instrument response with no fluorophore present)

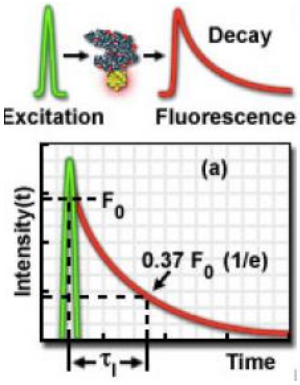
Advantages

- FLIM is insensitive to a variety of intensity-based artifacts:
  - Variations in excitation source intensity
  - Detection gain settings
  - Optical loss in the optical path
  - Sample variation in sample fluorophore concentration
  - Photobleaching
  - Microscope focusing
- τ_f is specific for a fluorophore
- Based on τ_f multiple dyes can be distinguished in one channel at high detector sensitivity
- FLIM can be used for:
  - Ion concentration imaging
  - Chemical microenvironment (pH, protein-binding…)

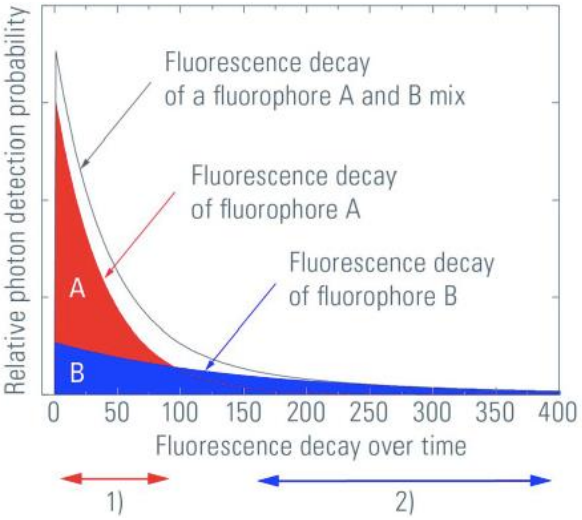
    - Quantitative fluorescence measurements



- **Frequency-domain FLIM**
    - Measurement of the phase shift of fluorescent light with respect to the phase of a modulated excitation source
    - Commonly used with wide-field microscopy
    - Phase-modulated light source
    - Emission is delayed relative to the excitation wave
    - Phase shift Δφ is measured

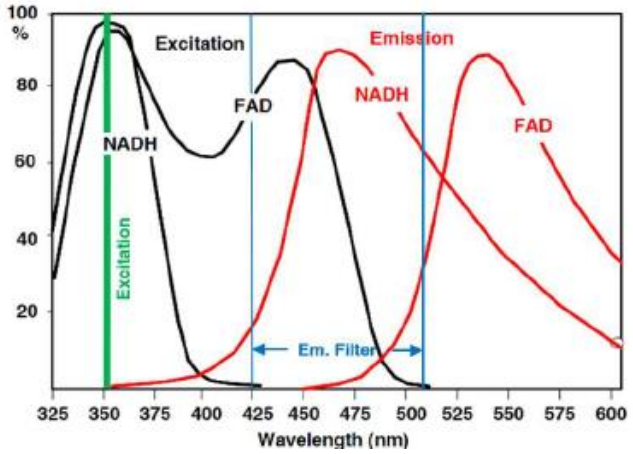


- **Time-domain FLIM**
    - Measurement of the time dependency of fluorescence intensity with respect to a short excitation pulse.
    - Use of a pulsed light source
    - Time correlated single photon counting (TCSPC)
    - Or time gating

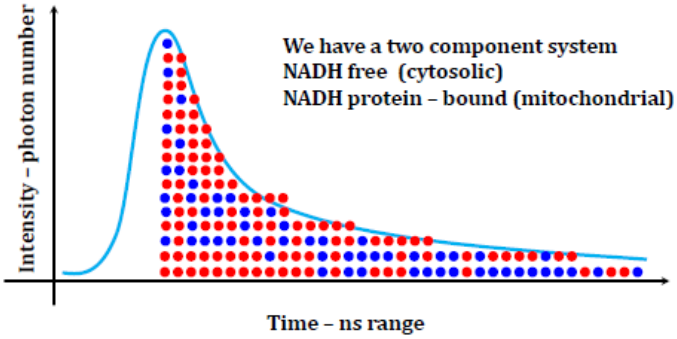


FLIM of NADH – ATP production

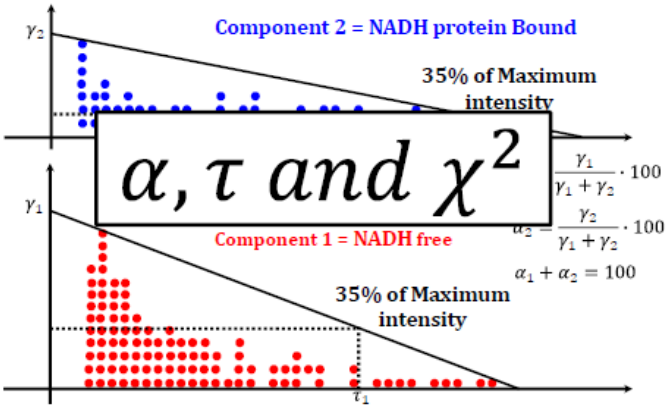
- Reduced Nicotinamide adenine dinucleotide (phosphate)
- Important cofactor in cell metabolism
- **Free NAD(P)H: short lifetime τ1**
- **Protein-bound NAD(P)H: long lifetime** τ2
- Typical values: τ1~ 700 ps and τ2 ~ 2600 ps
- Hypothesis: lifetime and contribution changes with disease



FAD and NADH overlap



Multiexponential decay fitting



Summary FLIM

- Intensity-independent information from fluorescent molecules
- Imaging of protein-bound and free metabolites
  → Label-free Metabolic profiling of cells
- Sensitive to the fluorophores microenvironment
  → new ways for life scientists to detect, visualize, and investigate structure and function in biological systems

Medical information systems and economic aspects of facility planning (Jens Maschmann, 08.07.19)

- Approx. 95% of medicine is information management:
  - History taking
  - Diagnostics (Lab, Radiology, Sono/Echo etc.)
  - Continuous clinical assessment (medical record)
- And only a small proportion is pure physical interaction:
  - Physical examination
  - Operation
  - Procedures (cannulation, intubation, catheterization etc.)

Process management

- Documentation is omnipresent, yet data for process management are scarce
- Heterogeneous documentation (→ mix of paper & IT)
- at least IT-based information should serve as basis for process optimization

Management at UKT

- landscape is rather fragmented, although SAP ish-med is leading medical documentation system
- IT-systems with clinical relevance at UKT:
  1. SAP ish-med
     - patient organizer, results, appointments, DRG documentation etc.
  2. CareVue/ICCA
     - anaesthesiology and ICU – medical record
  3. ViewPoint
     - endoscopy- and sonography pictures, results
  4. Carddas
     - cardiology: heart catheter, ECG, echo
  5. Lorenzo iSoft
     - Radiology information system
  6. Swisslab (laboratory information system)
  7. Paschmann/nexus
     - pathology system
  8. LEP-documentation module
  9. AIS
     - Eye(clinic) information system
  10. Neodat/Medipaed
     - neonatology
  11. Meona (pilot project: general medical record)

- IT-systems with assisting clinical relevance at UKT:
  1. LogBuch (patient and object transportation system)

2. ConSense warehouse logistics (OP warehousing, deliviery, and documentation system)
3. Kenntenich (Central sterilisation unit)
4. Hybase (hygiene/infection monitoring system)

- Collection and preparation of data must be
    - possible from different source systems
    - possible with high volumes of data
    - performing and reliable
    - flexible and adaptable without IT knowledge
    - easy and intuitive for users
- Classic Business Warehouse-Systems
    - are often too rigid and inflexible
    - Cannot be adapted to customers' needs without major effort
    - thus, often need external support and consulting
    - Decision at UKT for software „QlikView" (QlikTech)
- Data warehouse (DWH)
    - Central data pool from IT-systems (SAP etc.) for variety of applications
    - Avoids redundancy
    - Data regularly updated
    - Integrity of data (same data at same time)
    - Data security
    - Principle: central data are stored centrally and provided decentrally

Healthcare IT systems

- Healthcare IT systems in hospitals often have different „names" and different meanings:
    - HIS (hospital information system, German: KIS – Krankenhausinformationssystem)
    - EMR (electronic medical record, German: KAS – klinisches Arbeitsplatzsystem)
    - PDMS (patient data management system bzw. elektronische Patientenakte)
    - RIS (radiology information system)
    - PACS (picture archiving and communication system)
    - LIS (laboratory information system)
    - MIS (medical information system)
    - EPA (elektronische Patientenakte – trans sectoral)
    - HL7 (health level 7: communication standard)
    - DICOM (digital imaging and communications in medicine – standard for viewing and distributing all kind of medical images)
- Healthcare IT systems were initially not designed for clinicians, but for financing and billing
- In hospitals two principles exist:
    - **Heterogeneous** systems
        - often with SAP administrative background (predominantly found at university hospitals)
        - Pro: best of breed integration, flexible

        - Con: Interface problems, administration costly
    - **Monolithic** systems (one application fits all purposes)
        - often found at teaching and provider hospitals
        - Pro: highly integrated, less costly administration
        - Con: limited functionality for medical specialties, dependence on one supplier

the ambulatory sector

- Almost all medical cabinets in Germany are working with electronic patient record systems
- Forced to deliver billing data electronically to Kassenärztliche Vereinigung to get paid
- Market share: CompuGroup is leading supplier (different systems)
- Interaction between these systems and hospital systems scarce and difficult

- Hospital doctors are much less satisfied with the IT systems than doctors in med. Cabinets

Data security in both sectors of very importance

- Germany has high and strict data security regulations
- Difficult in hospitals: emergency situations require access to patient data even for „medical strangers“
- Logging of access data is accepted to monitor violations of patient data use
- Interaction between sectors or even between professionals and patients is limited due to scarce or complicated solutions
- Mobile devices (smart phones, tablets) evoke even higher standards of data security
- Wireless data transmission enhances the risk of unintended use of sensible medical data
- So far no widespread use of mobile devices in hospitals, although everybody talks about it.

- Two gib organizations:
    - German Medical Informatics Initiative
    - Smart Medical Information Technology for Healthcare

What IT-systems can help making medicine safer?

- CIRS (critical incident reporting systems)
- Patient identification with bar code wrist bands
- AMTS (Arzneimitteltherapiesicherheit – medical presciption safety)
- Rule based information about process adherence
- 2% of all patients in hospitals suffer from an avoidable unintended medication error

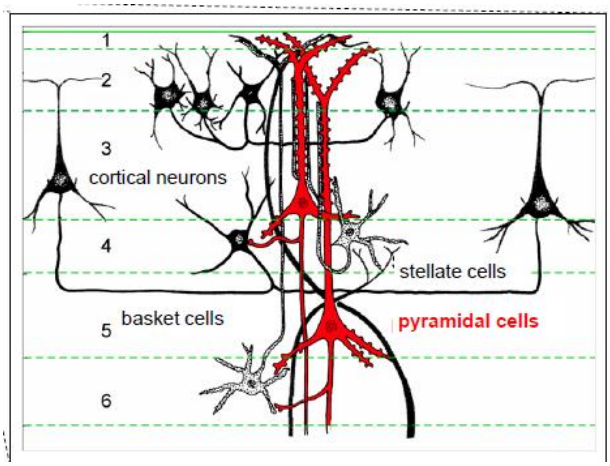
IT within hospitals…

- Always interferes with processes and has to serve and to facilitate them
- Can help in automation and error reduction of medical processes
- Has to have a smart interface with the users
- Is a risk because it has to work 24/7 and shut-downs are critical for patient care
- Never consider an IT-project a mere IT-project, always consider the expertise of clinicians!

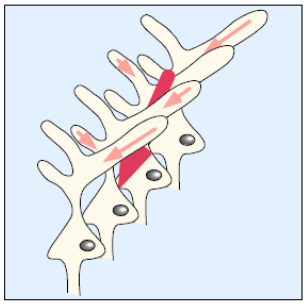
Fetal Magnetoencephalography (fMEG) (22.07.19)

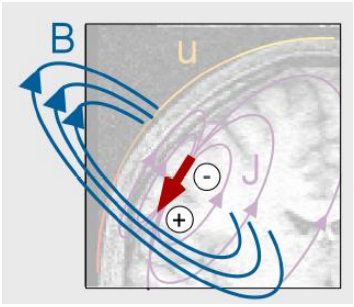
- Magnetoencephalography (MEG) is a non-invasive, imaging method to study brain functions
- records neuronal electro-magnetic activity of the brain
- MEG and Electroencephalography (EEG) can be recorded simultaneously

Measurement of Cerebral Magnetic Fields

- Nerve cells: Pyramidal cells are oriented in a parallel way
- 

- synaptic activity causes shifts in electrical currents (ions go in and out)
- postsynaptic potential emerges
- the polarization can be described physically by a dipole
- simultaneous activation of multiple neurons superimposes
- a prerequisite for the generation of a measurable EEG- or MEG-signal is the coordinated activity of about 10 000 neurons.





- synaptic activity causes an intracellular primary current and a extra-cellular secondary current.
- **MEG** records magnetic fields of the **primary currents** (summated intra-cellular currents →Biot-Savart's law, right hand rule)
- **EEG** measures the voltage changes of the **secondary current** (extra-cellular currents at the head surface → Ohm's law).

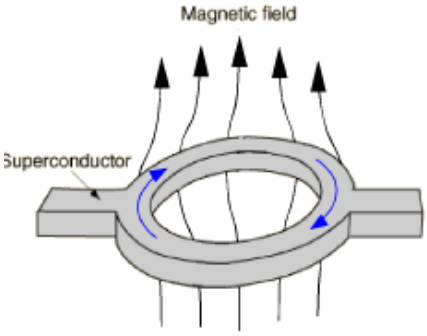
Generation of magnetic fields

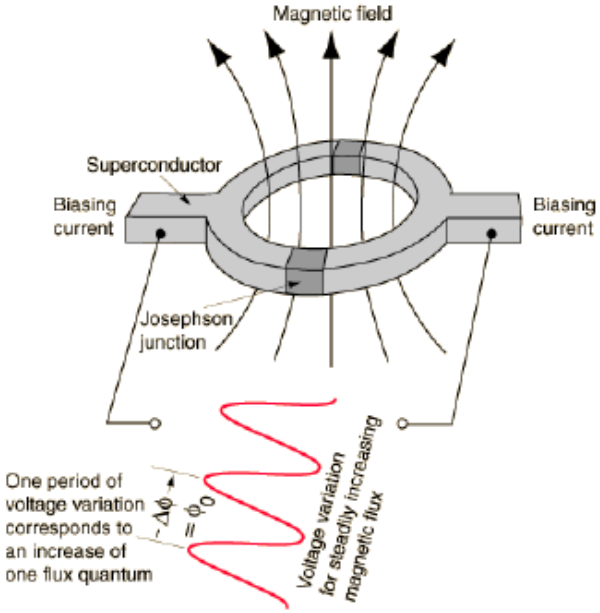
- Depolarization of the dendritic membrane of pyramidal cells generates extra- and intracellular currents.
- fMEG magnetic field in range of 10 fT - pT

SQUID: Superconducting Quantum Interference Device

- In 1971 David Cohen measured the spontaneous alpha rhythm using a SQUID, and the expression magnetoencephalography (MEG) was introduced
- First only one channel, nowadays some hundred channels
- superconducting ring
    - an external magnetic field creates a current in the coil
    - current creates magnetic field opposite to the original field
    - no magnetic field inside the ring
- superconducting ring with Josephson conjunction
    - differences between multiples of flux quanta
    - **only multiples of flux quanta can pass and generate a current**
    - the current causes a voltage drop at the Josephson conjunction



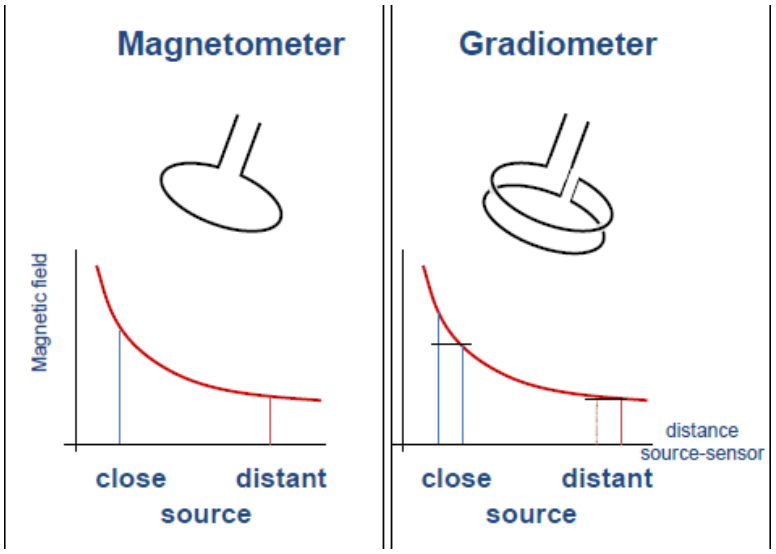




- Superconducting material is niobium or lead alloy with gold/indium
- **Cooled to low temperature with either liquid He (4K)** or N (77K)
    - To make them sensitive for quantum interference

Gradiometer

- Often a gradiometer used
    - Two coils (two magnetometers) with current going in opposite directions
    - Either axial or planar gradiometers
    - Measure difference in magnetic flux
    - Increase SNR

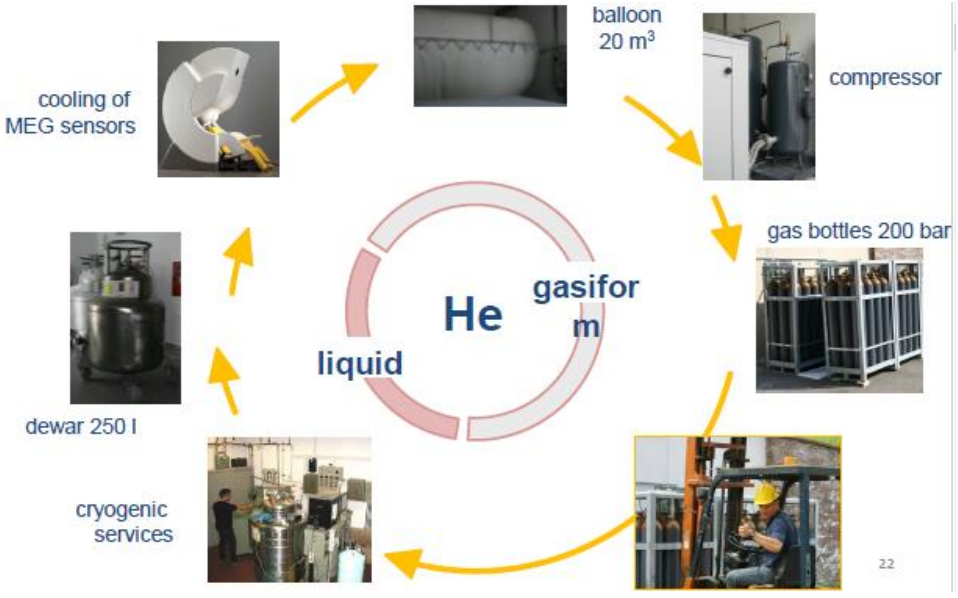


Noise suppression: shielding

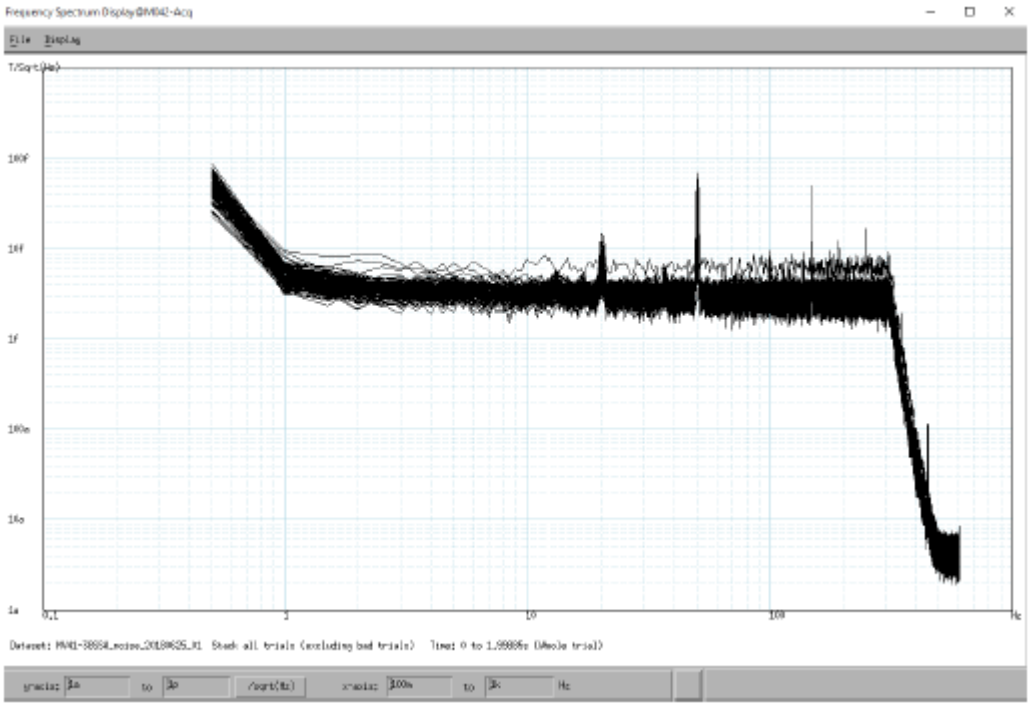
- Magnetic shielded room
  - Basis: aluminum frame
  - Inner layer: copper coated aluminum
  - Middle layer: mu metal with high magnetic susceptibility
  - Outer layer: aluminum
  - passive 7-layer shielding
  - double coil system for
  - active shielding
  - earth field compensation
  - RF-shield

Helium supply and recovery

- Closed loop system
- Operation costs just for helium: 50.000/year



Noise



- High noise for low f, different peaks for environmental influences

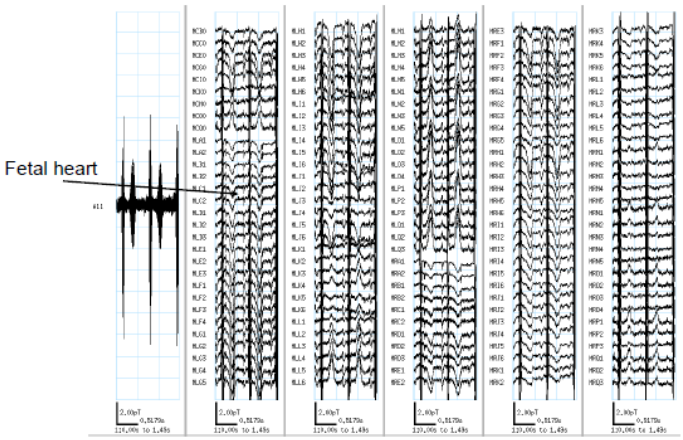
SQUIDS

- UsuallypPick up coils diameter ca. 2 cm
- BabySQUID ® Neonatal Biomagnetometer has smaller pick up coils (6 mm)

Why do we need to measure magnetic fields in the human fetus?

- The fetus is not a preterm
- Prematurity especially for gestational age <32 weeks is an independent risk factor for adverse cognitive development

Requirements to measure magnetic fields in the human fetus



- Highest peak is mother heart and second highest festal heart

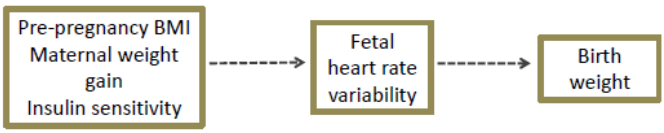
Which neuronal processes are already established in fetal live

- auditory evoked fields can be recorded after 20th week of gestation and the latency decreases over gestation
- fetuses show mismatch like responses to auditory stimulation in the last trimester of gestation
- visual evoked responses can be recorded in the last trimester of gestation and the response latency decreases over gestation
- fetuses show habituation and dishabituation to auditory and visual stimuli
- fetuses show numerosity related mismatch responses
- the fetal behavioral state influences the evoked response latency
- Fetuses and newborns show no habituation to syllables

Fetal state	1F = quiet sleep	2F = active sleep	3F = quiet awake	4F = active awake
Fetal heart rate (FHR) pattern	**FHR pattern A** stable heart rate. small oscillations <10bpm. low number of heart rate accelerations linked to movements	**FHR pattern B** heart rate with large oscillations >10bpm. heart rate accelerations occur during movement	**FHR pattern C** stable heart rate. larger oscillations >10bpm. no accelerations	**FHR pattern D** heart rate with large oscillations >20bpm. strong und long lasting accelerations. baseline can be higher than 160bpm
Body movement	very limited	often and regular	none	continuous and strong
Eye movement	absent	continuous	limited	continuous

The old model of adult degenerative disease was based on the interaction between genes and an adverse environment in adult life. The new model that is developing will include programming by the environment in fetal and infant life.

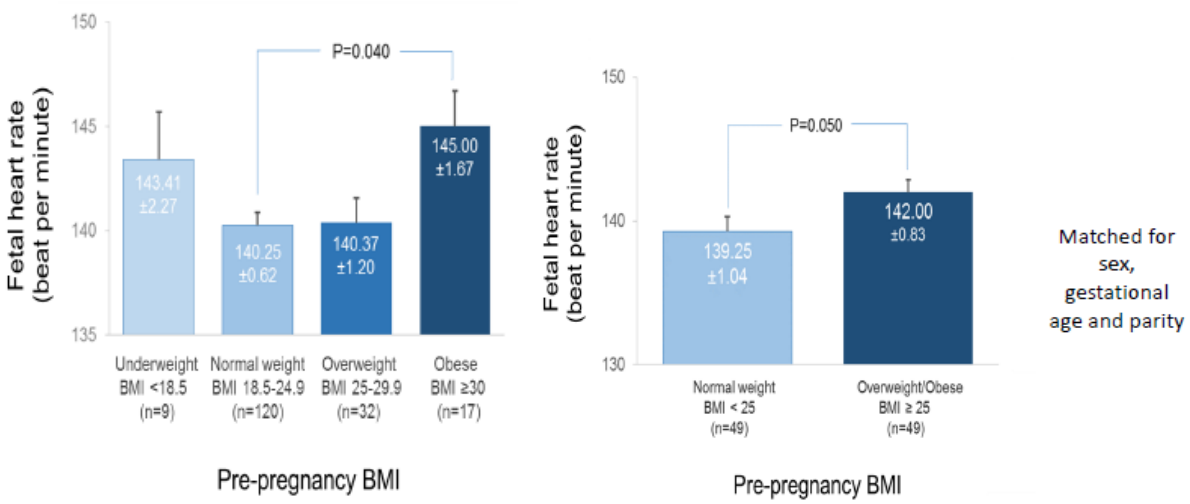
How does intrauterine environment impact autonomic nervous system development and offspring outcome at birth?

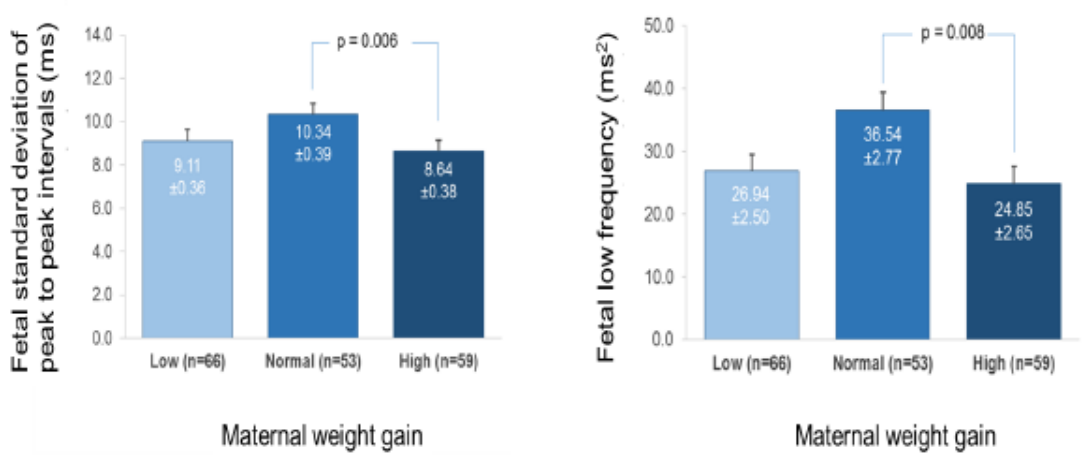


- **Increase in heart rate and decrease in heart rate variability are indications of an adverse effect of autonomic nervous system**

Time Domain	
Mean heart rate (beat per minute)	Sympathetic and parasympathetic tone
Standard deviation of peak to peak intervals (milisecond)	Overall heart rate variability (stability of heart rate)

Frequency Domain	
Low frequency (milisecond^2)	Sympathetic and parasympathetic modulation
High frequency (milisecond^2)	Primarily parasympathetic modulation
Low to high frequency ratio	Sympathovagal balance





Maternal metabolism in the fasting state and fetal heart rate variability

- **Decreased fetal heart rate variability in maternal insulin resistance**
- Maternal obesity → increase of fetal heart rate
- Excessive weight gain → decrease of fetal heart rate variability
- Maternal insulin resistance → decreased of fetal heart rate variability
- adverse effect of the autonomic nervous system
- conclusion:
    - Exposure of fetus to an imbalanced maternal metabolism has an adverse effect on the development of autonomic nervous system
    - Alterations in fetal autonomic nervous system predisposes for a metabolic imbalance and might contribute to the programming of obesity

Gestational diabetes

- during the pregnancy the insulin sensitivity of the mother decreases
- gestational diabetes mellitus (GDM) is defined by increased glucose levels during an oral glucose tolerance test (for screening performed around the 25th week of gestation)

- untreated GDM leads to fetal macrosomia and large for gestational age fetuses
- GDM leads to greater mean body mass index (BMI). fasting glucose and insulin and risk of type 2 diabetes in the offspring
- initial evidence indicates that GDM leads to neurodevelopmental problems in diverse functions
- Knockout of Insulin receptors in the brain cause:
    - Obesity
    - Peripheral Insulin resistance

Fetal Programming?

- Insulin action during hyperglycemia and hyperinsulinemia in a critical time period lead to disruption of metabolic brain networks in the newborn in the mouse
- Human fetal hyperinosemia during the third trimester due to maternal metabolism may prevent the normal development of brain responses.

Other fMEG applications

- Preterm Delivery-Uterus Contraction
    - Finding: Synchronization between sensors increases before delivery
    - Can predict delivery date
- Fetal Arrhythmias and Cardiac Time Intervals

Conclusion

- Fetal MEG provides unique information about the functional development of the human brain
- Fetal magnetocardiography adds additional information to investigate also the interaction of the brain and peripheral nervous system
- Important clinical questions can be addressed by fetal MEG and MCG
- Important basic science questions related to prenatal development can be investigated
- Limited diagnostic applications (arrhythmia, uterine contraction) and limited interventions