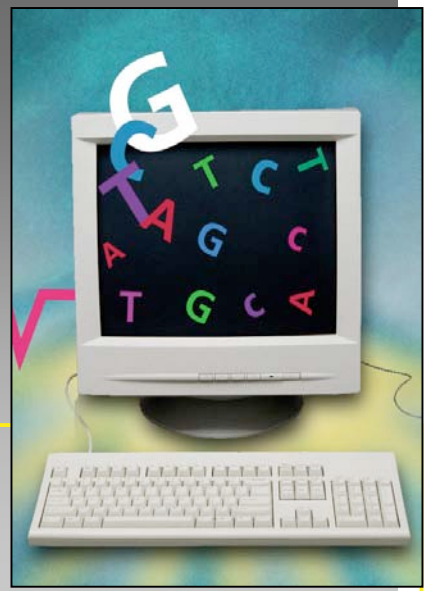
Humanbiologie 4. Semester
1-Tages Praktikum "*Humangenom*"



Funktionelle Einzelzell-Genomik
am Beispiel der dopaminergen Mittelhirn-Neuronen

Institut für Normale und Pathologische Physiologie
AG Molekulare Neurobiologie und Computerraum

B.Liss, A.Alexandru, J.Gründemann

I. Wie kann man methodisch Funktion und Genexpression einzelner Nervenzellen untersuchen ?

(Laser-Mikrodissection, PALM-System)

II. Wie nutzt man dazu
die Informationen des Humangenom-Projektes
und verwandter Internet-Datenbanken / Programme?

(Primer-Design für RT-PCR)

III. Warum ist
Einzelzell-Analyse von Nervenzellen erstrebenswert?

(Theorie soviel wie gewünscht / zeitlich machbar)

Gen-Expressionsanalyse einzelner Zellen mittels RT-PCR

1. Isolation einzelner Zellen / mRNA (z.T. Funktionsanalyse)

(*Laser Mikrodissektion, Patch-clamp Technik*)

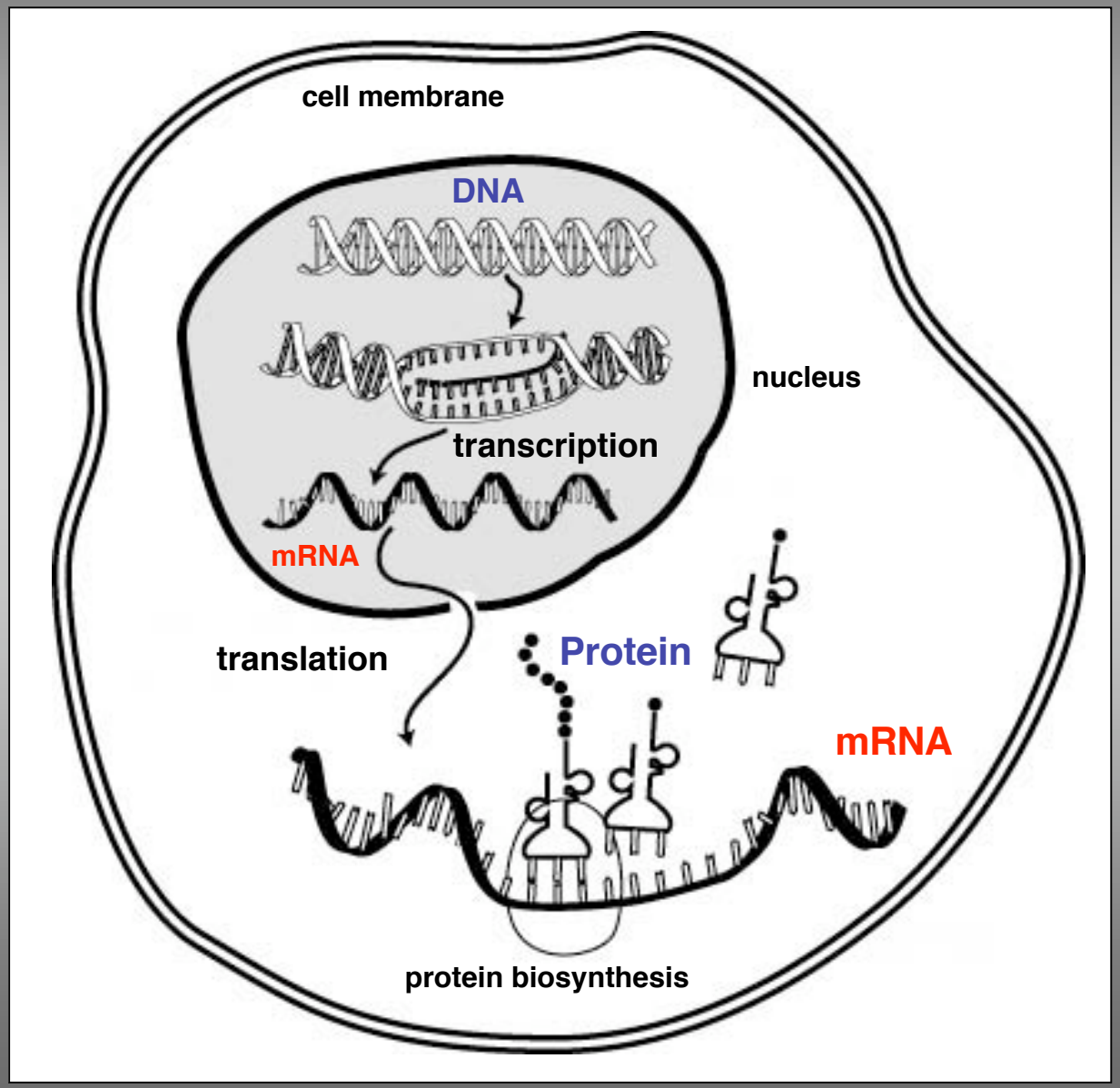
2. cDNA Synthese (Reverse Transkription der mRNA)

(*Reverse Transkription -RT*)

3. PCR Amplifikation der zu untersuchenden Gene/cDNAs

(***Primer-Design**, qualitative PCR, quantitative real-time PCR*)

complementary DNA (cDNA): stabilere Negativkopie der mRNA



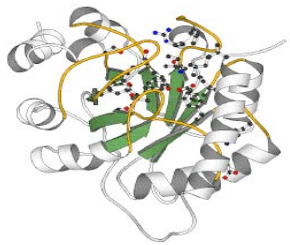
DNA

Transcription

mRNA → cDNA

Translation

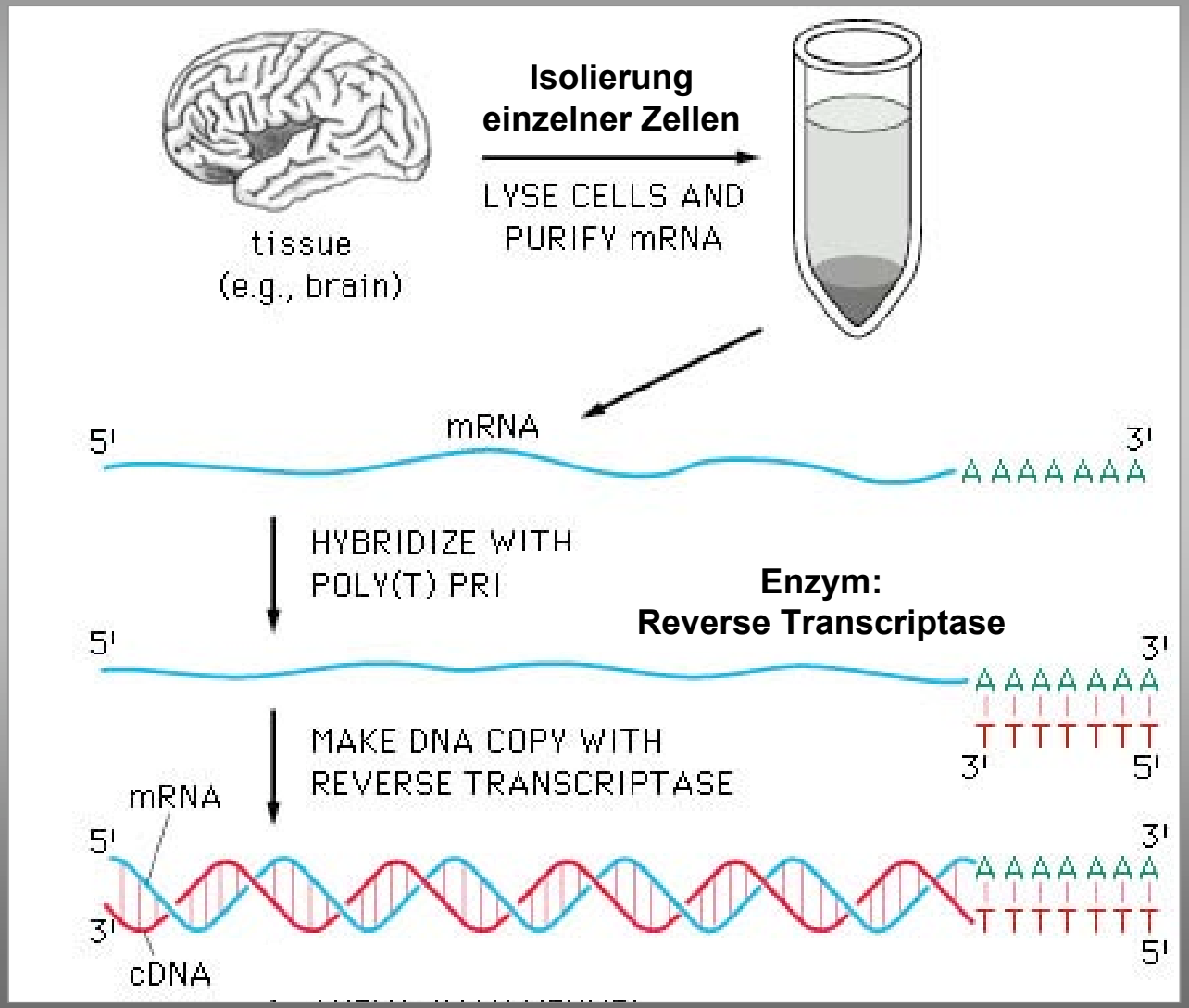
Protein



RNA ist extrem fragil: RNAsen!!

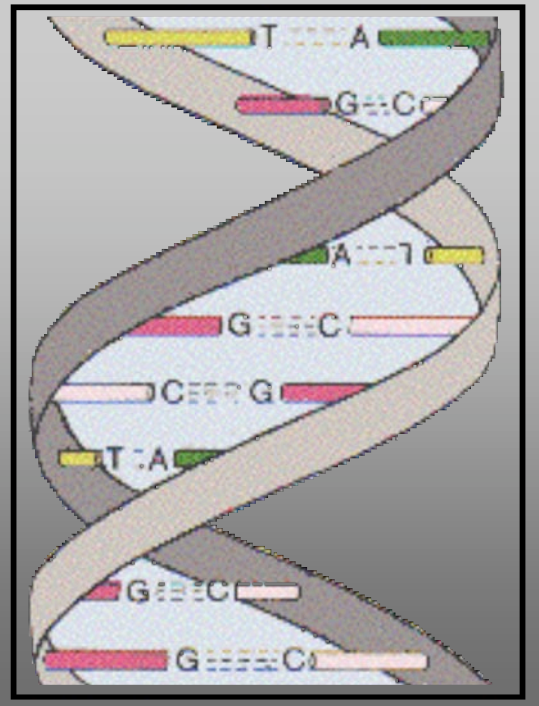
Reverse Transkrition (RT) zur cDNA Synthese:

Umschreiben der mRNA in eine stabilere cDNA Form

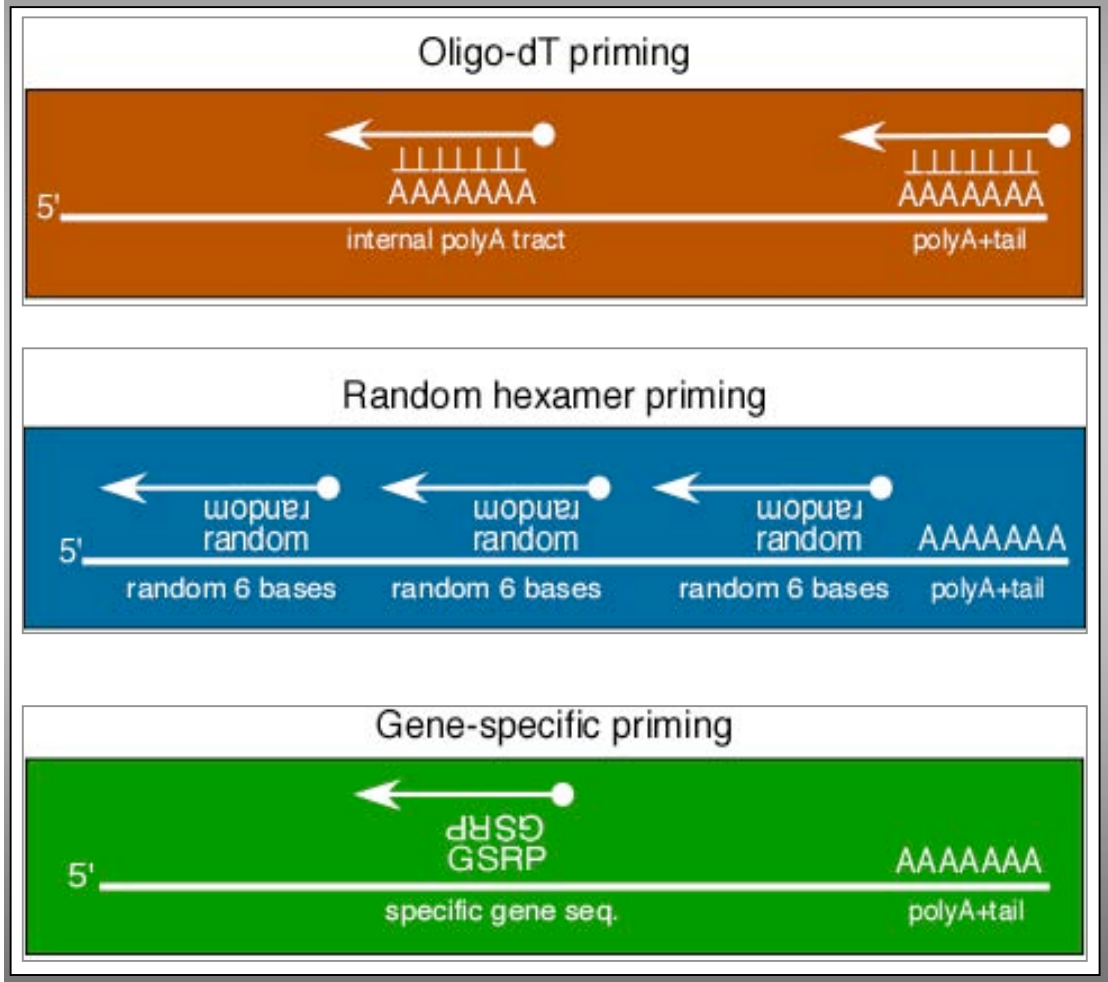


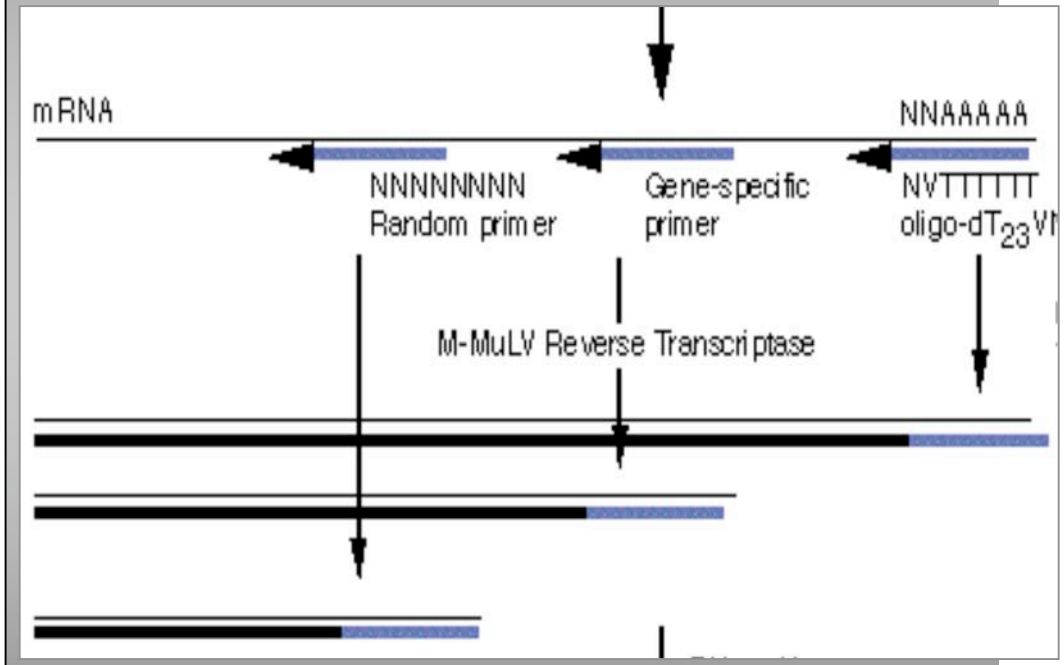
Primer = Oligonukleotide:

Kurze Einzelstrang-DNA (15-50 Basen) die durch Basenpaarung kurze dsDNA Bereiche bilden, die die Enzyme (zB *Reverse Transcriptase*) benötigen



Primerauswahl für cDNA Synthese / RT: 3 verschiedene Primer-Strategien

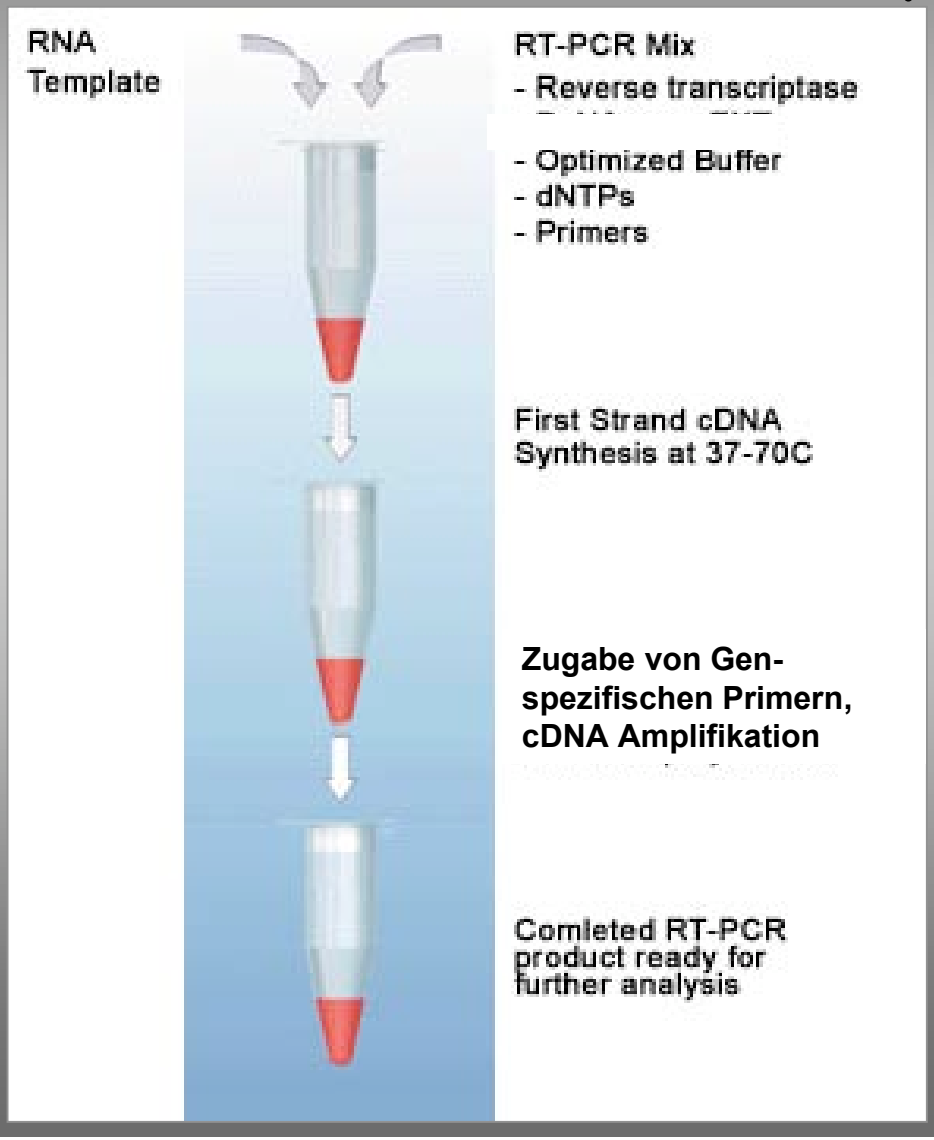


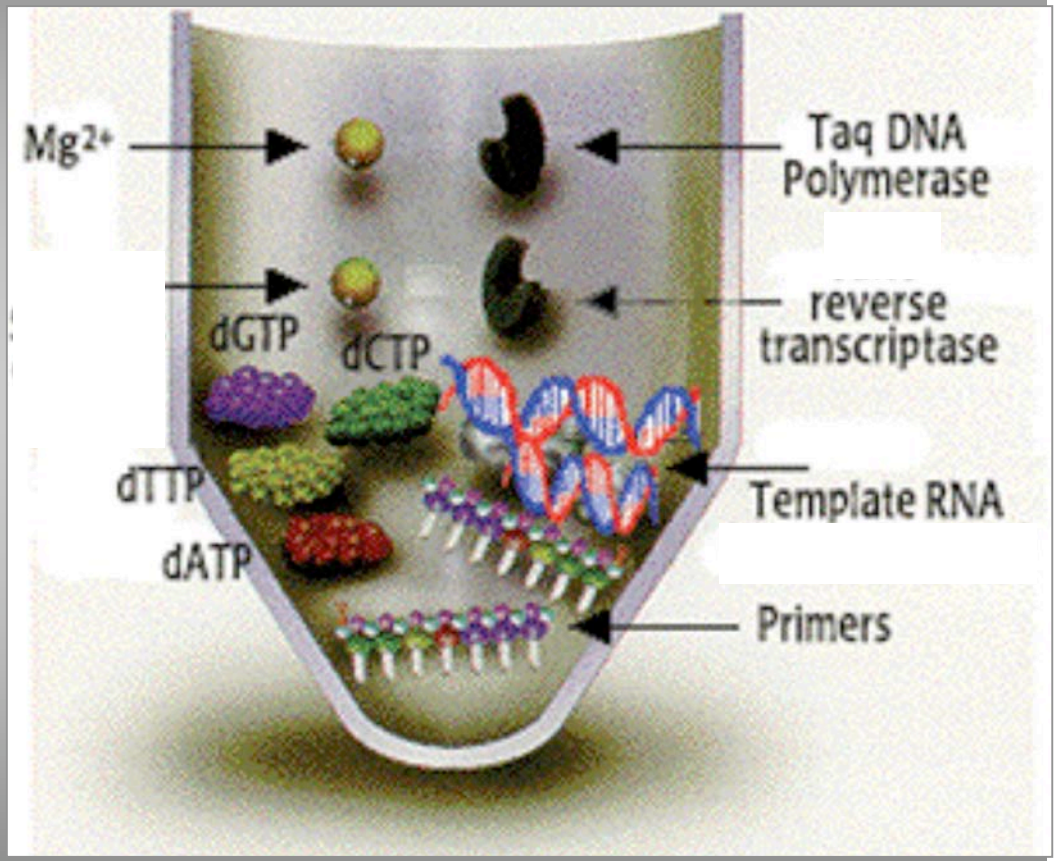


3 verschiedene cDNA Populationen von derselben RNA-Probe möglich: Gen-Expressions Artefakte !

RT-PCR: reverse transcription - polymerase chain reaction:

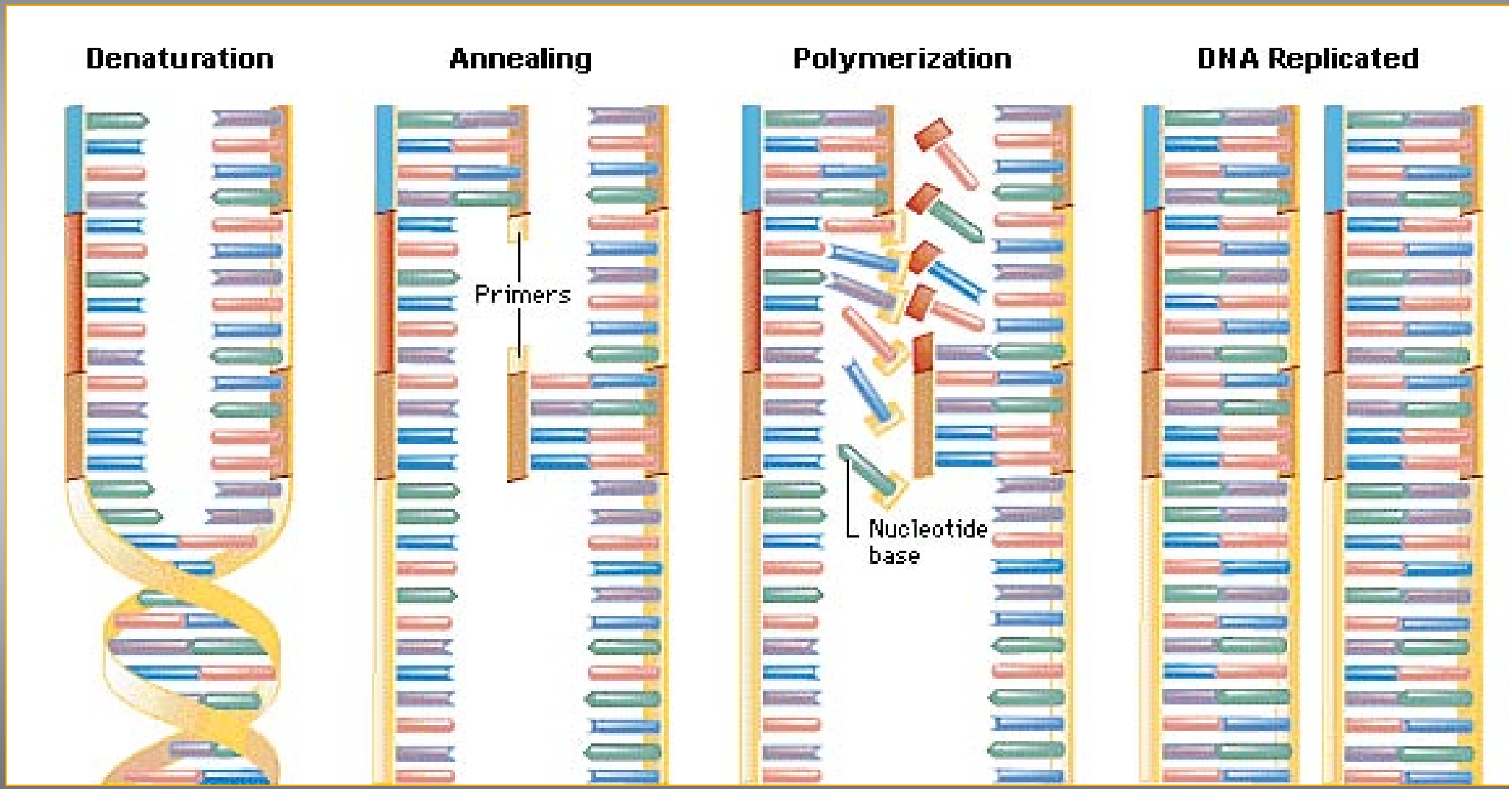
Kombination von cDNA Synthese und PCR-Amplifikation zur Genexpressions-Detektion



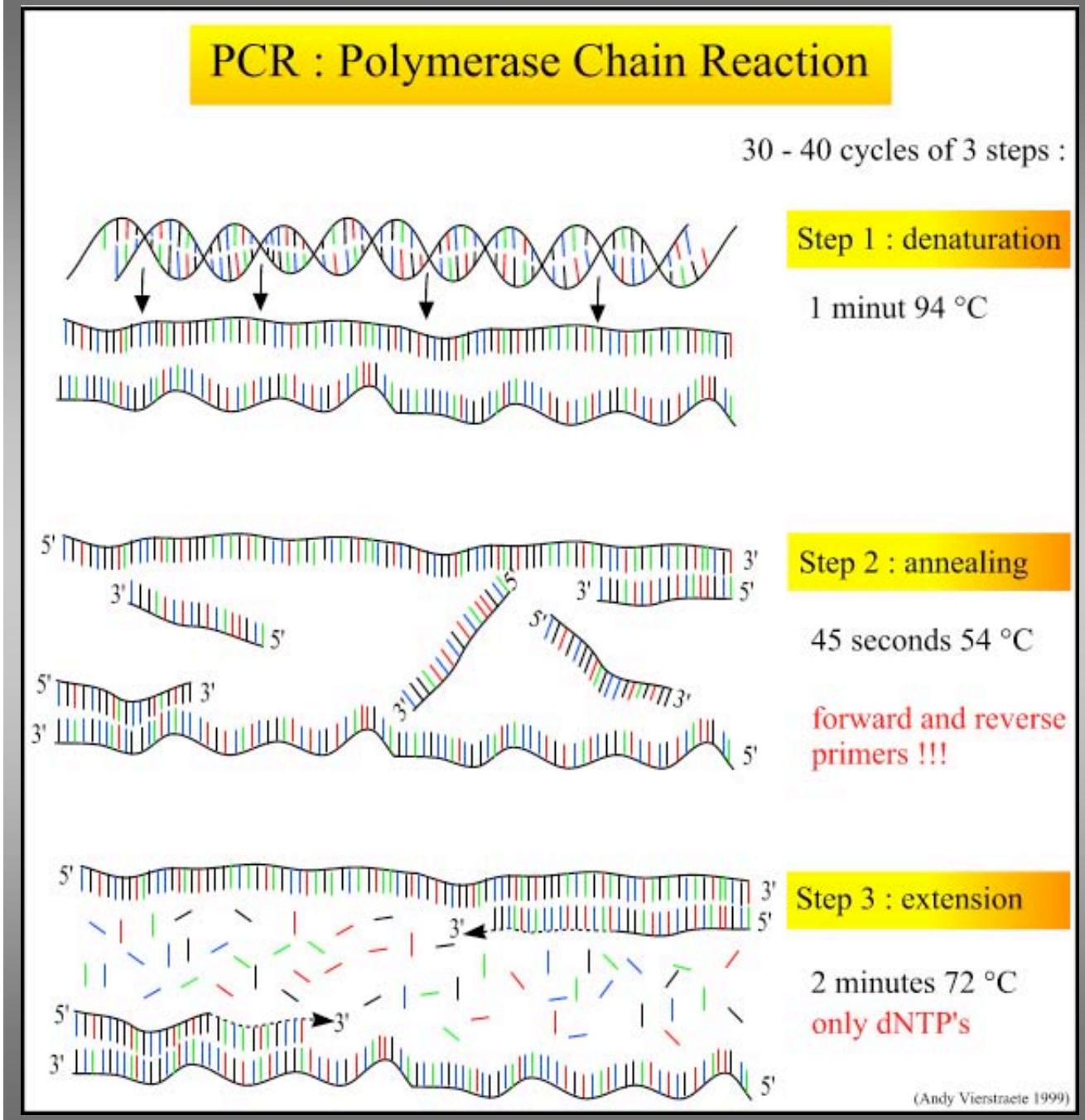


Primerdesign für PCR ist *entscheidend* für RT-PCR Gen-Expressionsanalysen

PCR: polymerase chain reaction:
Wiederholte Abfolge von 3 Temperatur/Inkubationsschritten



94 ° C | 50-70 ° C | 72 ° C | Ende 1. Zyklus



Benötigt werden:

2 PCR-Primer
(Forward / Reverse)

dNTPs + Puffer
Taq-Polimerase
Thermozykler (PCR-Gerät)

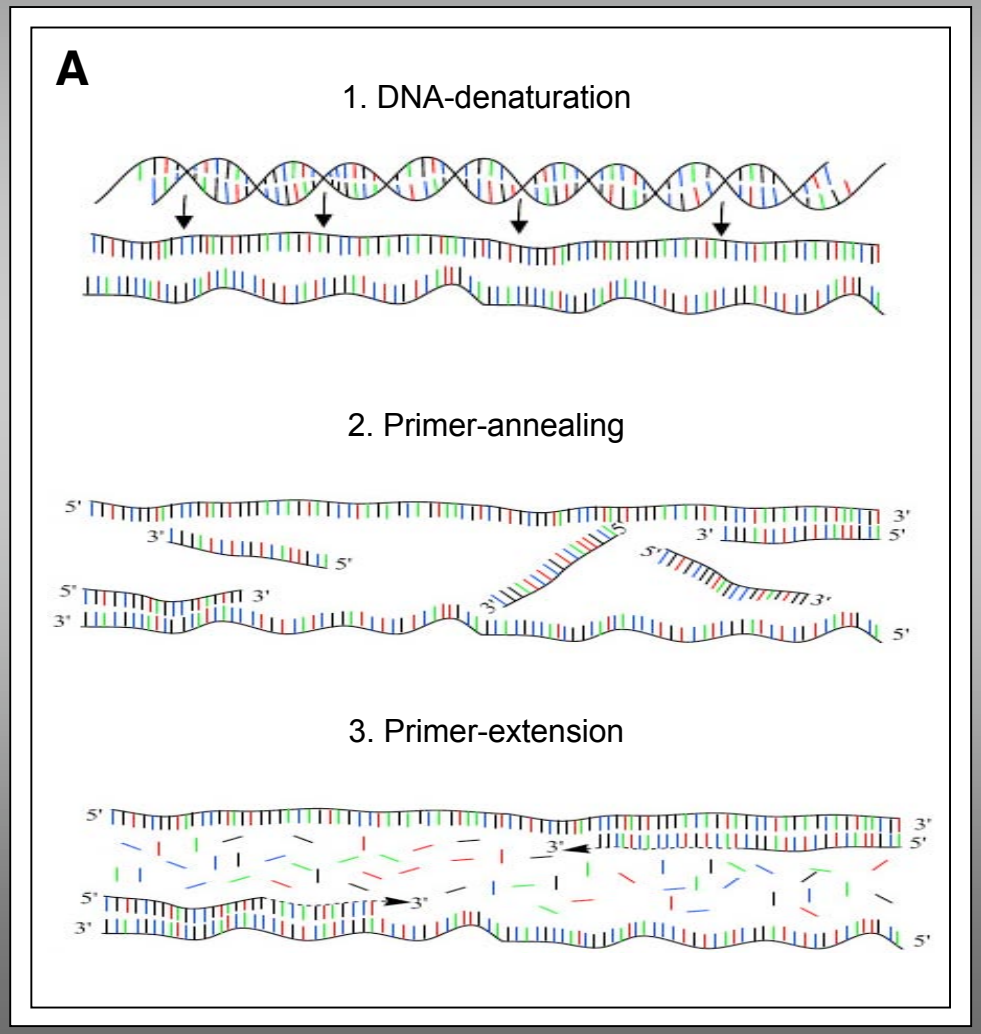
Exponentielle DNA Vermehrung

nach jedem PCR Zyklus
Verdoppelt sich die
Zielmolekül Anzahl:

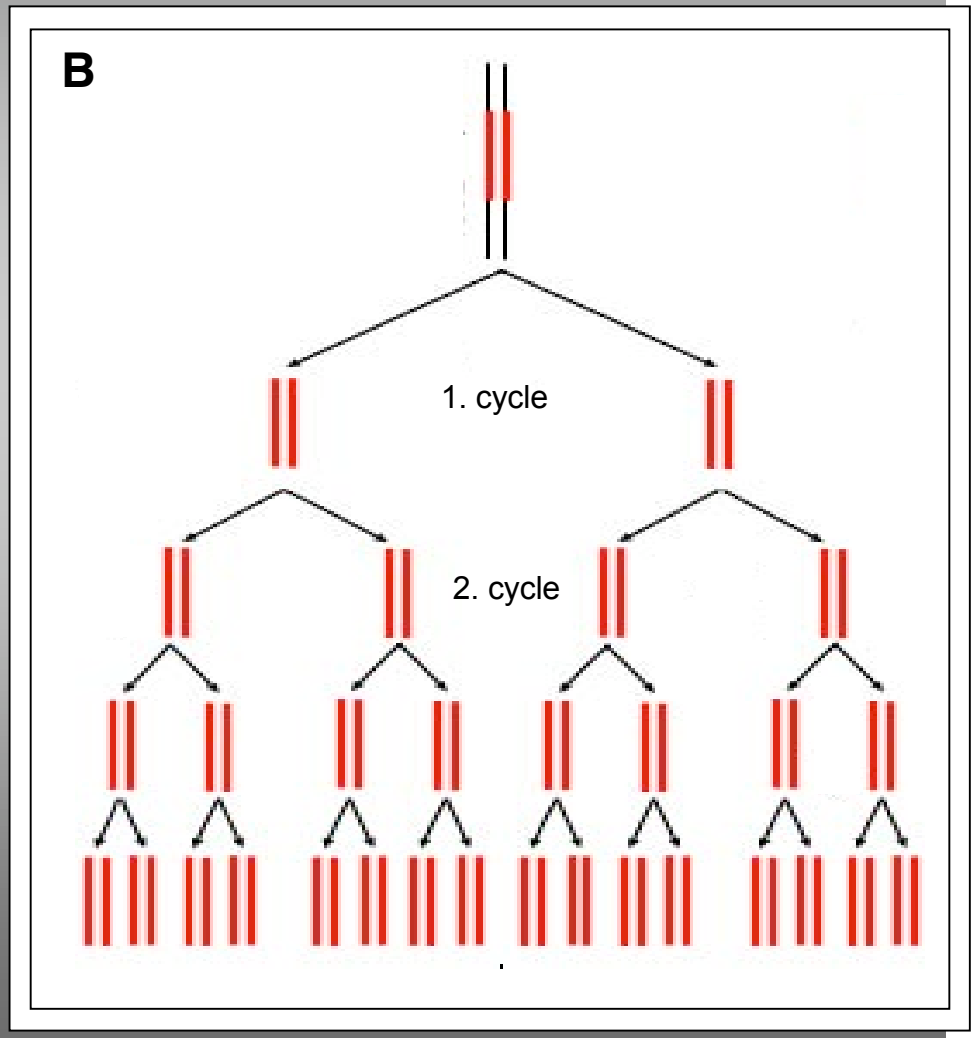
2^n
DNA Moleküle
nach n PCR-Zyklen

Exponentielle DNA-Vermehrung durch PCR

Abfolge von 3 Temperaturstufen



Exponentielle DNA-Vermehrung



Gen-Expressionsanalyse einzelner Zellen mittels RT-PCR

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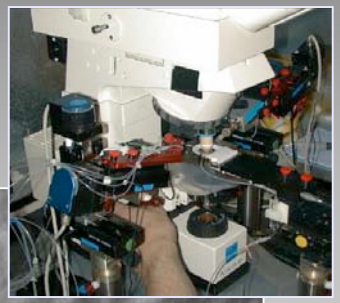
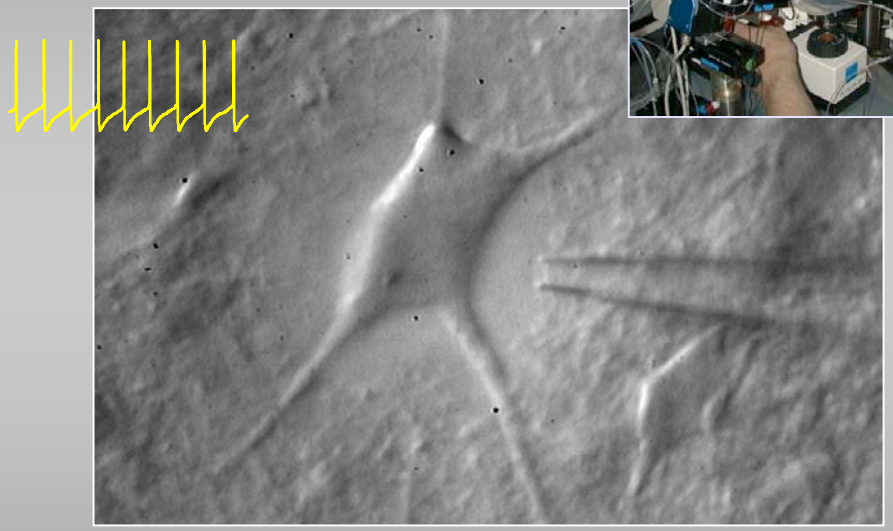
(*Primer-Design*, *qualitative PCR, quantitative real-time PCR*)

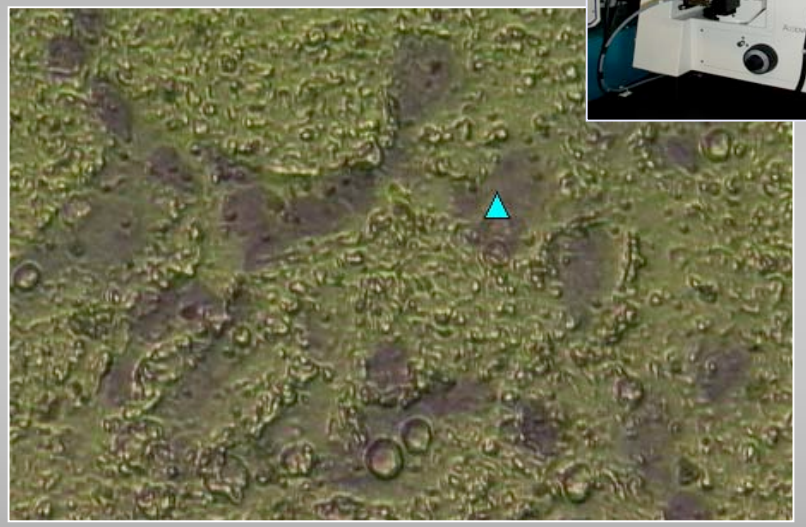
Zwei verschieden Methoden zur Isolierung einzelner Zellen / mRNA aus Gewebe (zB Hirnschnitte)

1. *Living neurons brain-slices*





2. *Post mortem fixed brain-sections*



Zytoplasma-Ernte mittels **Patch-Pipette** (Patch-Clamp Set-up):

Funktion und Genexpression

Kontaktfreie **Laser-Microdissection** / Katapu (PALM-Set-up)

Analyse von post mortem (humanen!) Proben

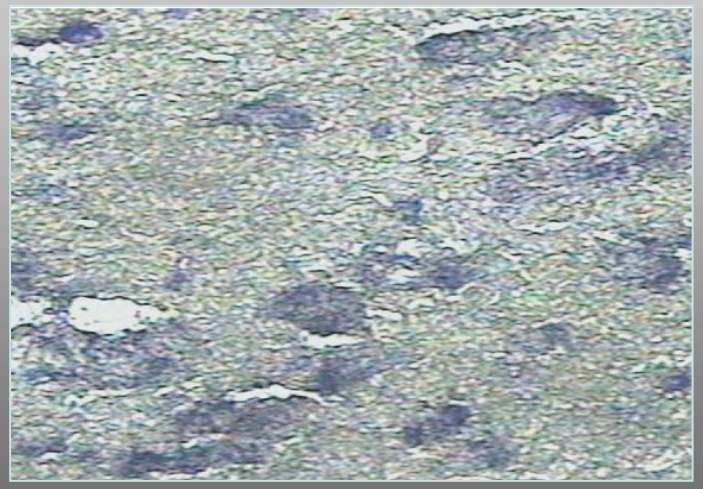
cDNA-Synthese, Gene-expression Profiling (RT-PCR)

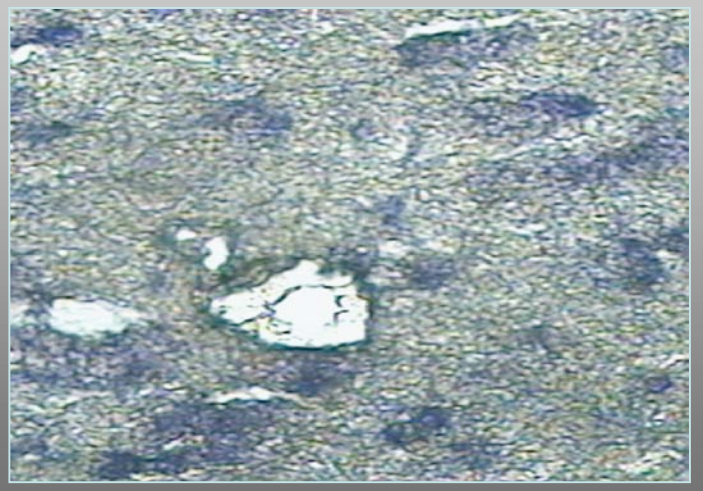
Isolierung einzelner Zellen aus Gewebe (zB Hirnschnitte)

Methode: Laser-Mikrodissection (PALM-System)

Erlaubt schnelle "unkomplizierte" Isolierung einzelner Zellen

Erlaubt Analyse von fixiertem (*post mortem*) Material





PALM: Photo Ablation and Laser Microdissection

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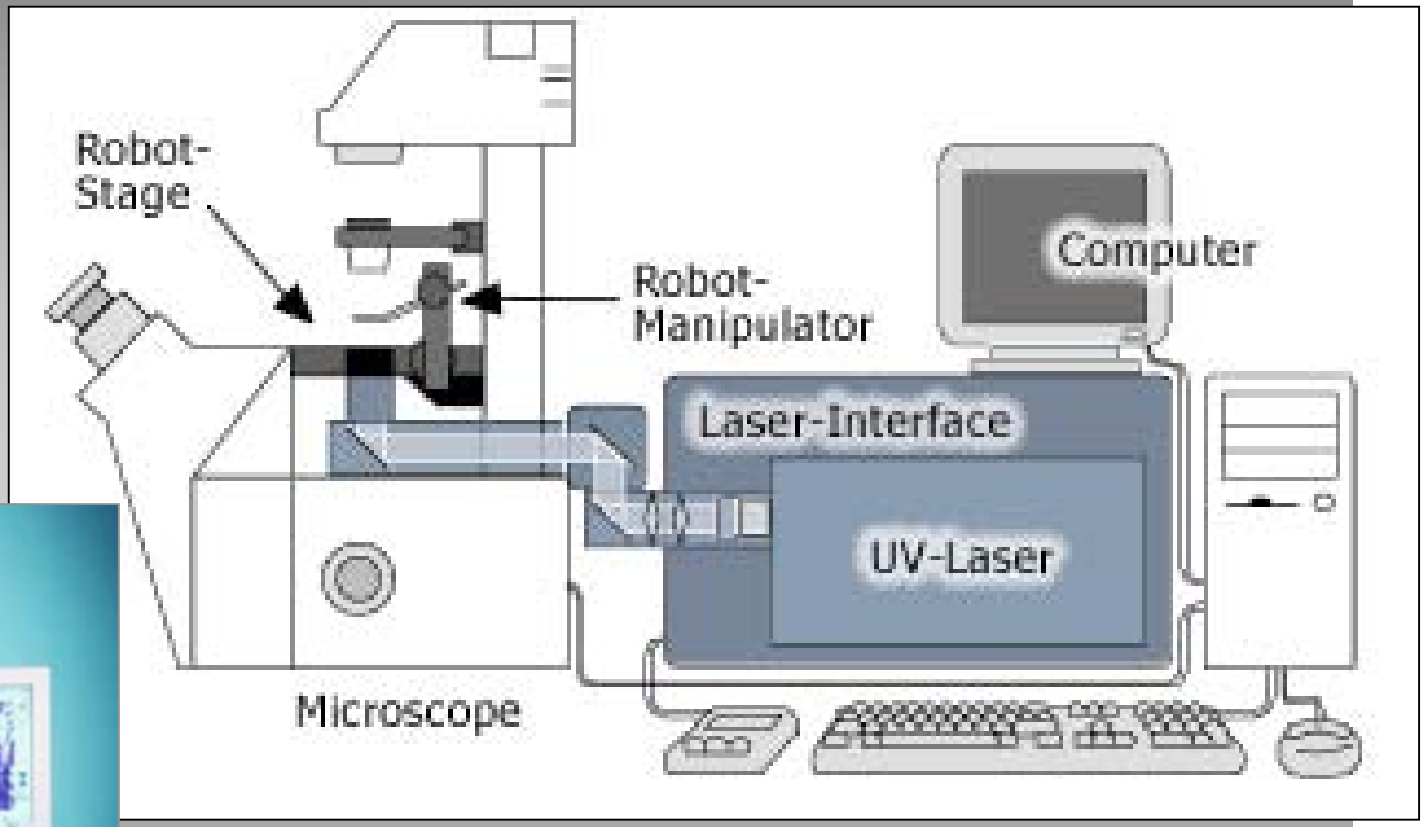
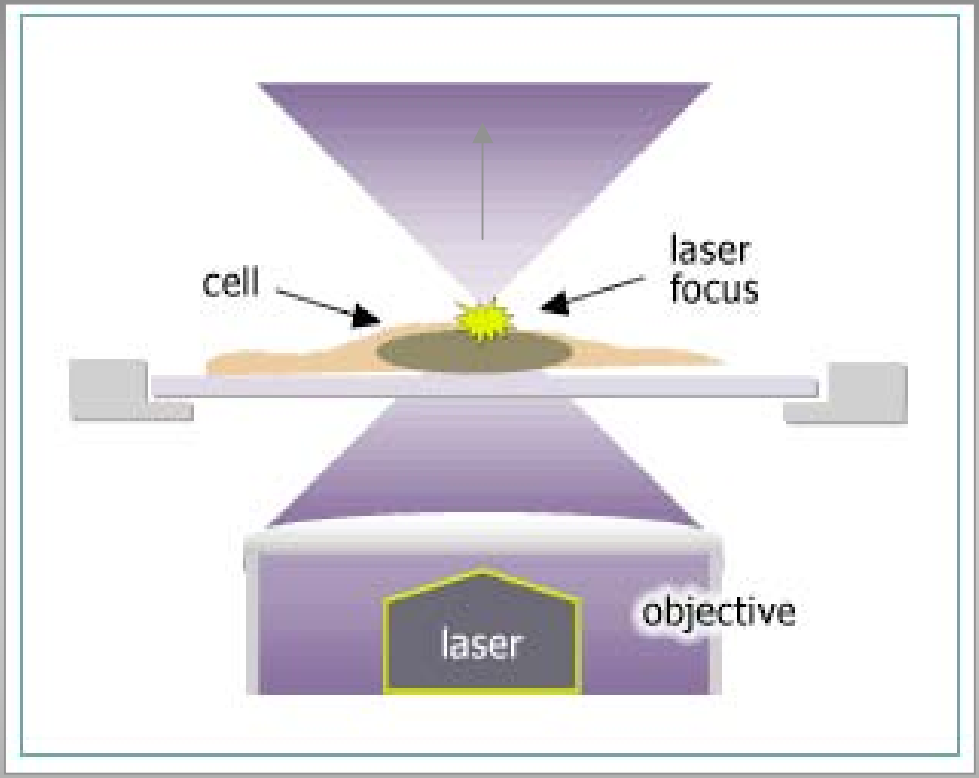
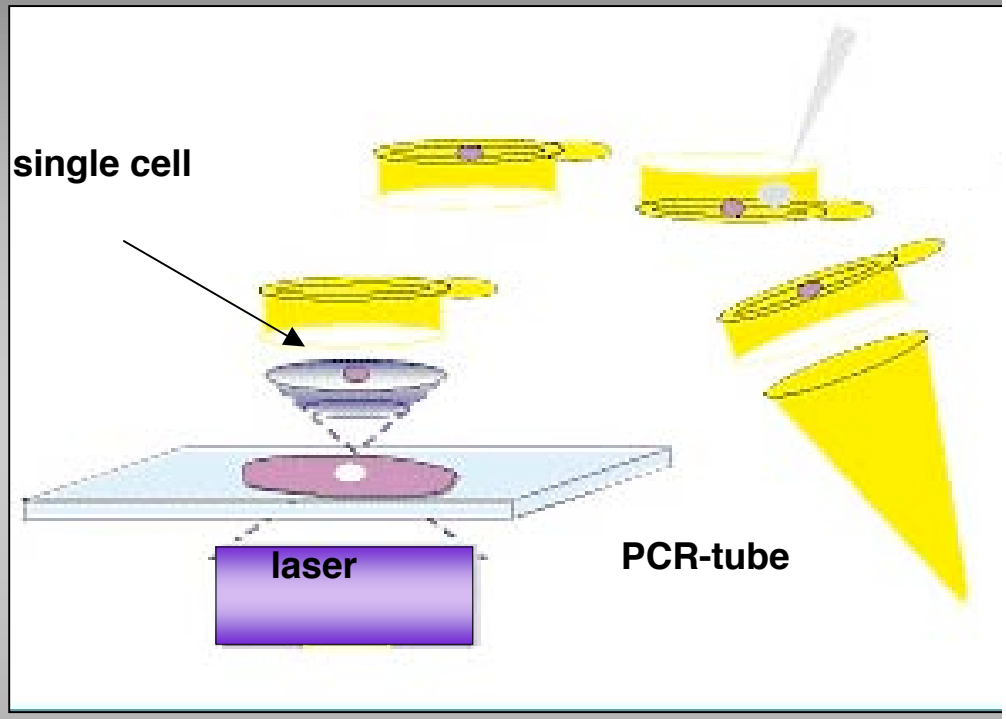


337 nm Nitrogen (UV-A) Laser

Laser Microdissection und Katapulting einzelner Neuronen



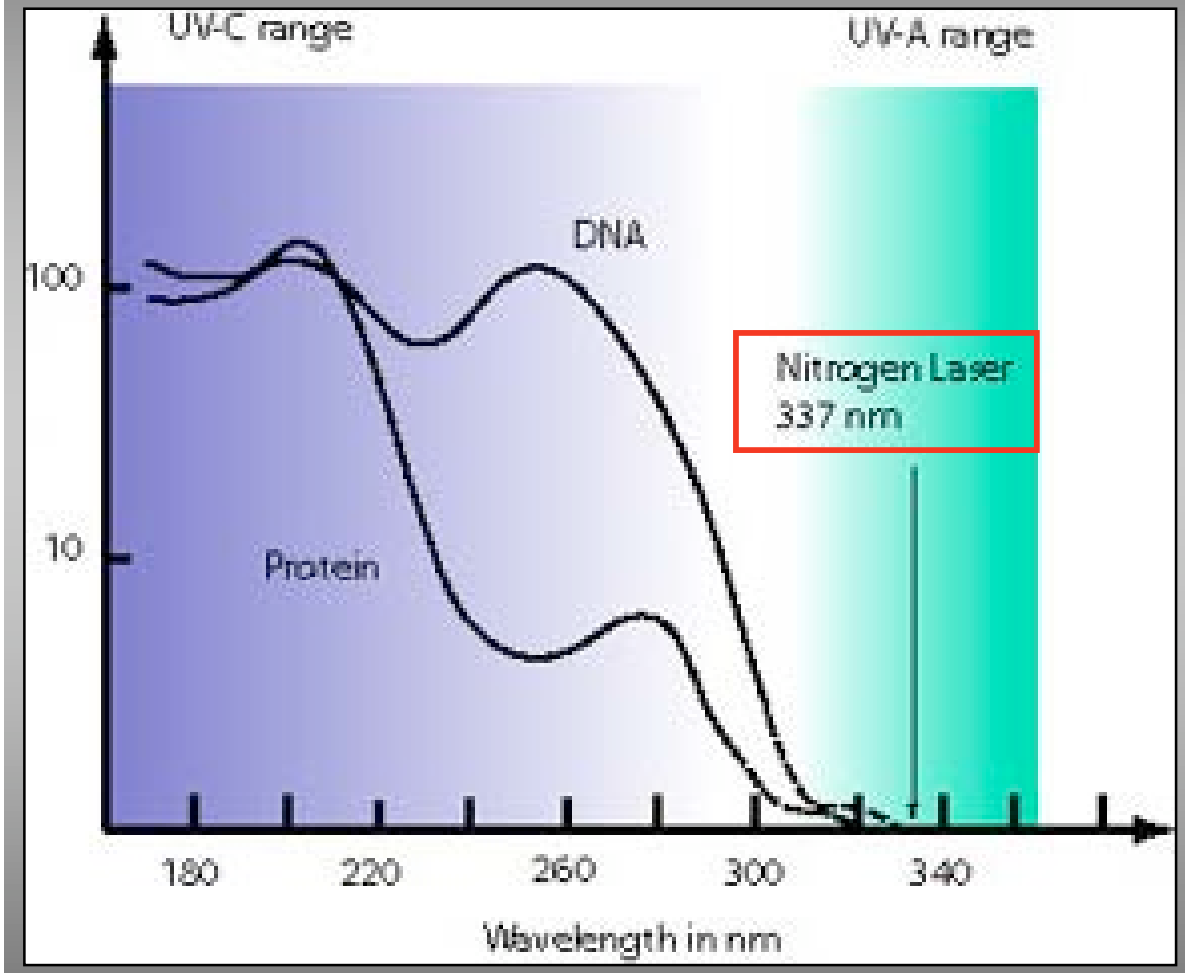


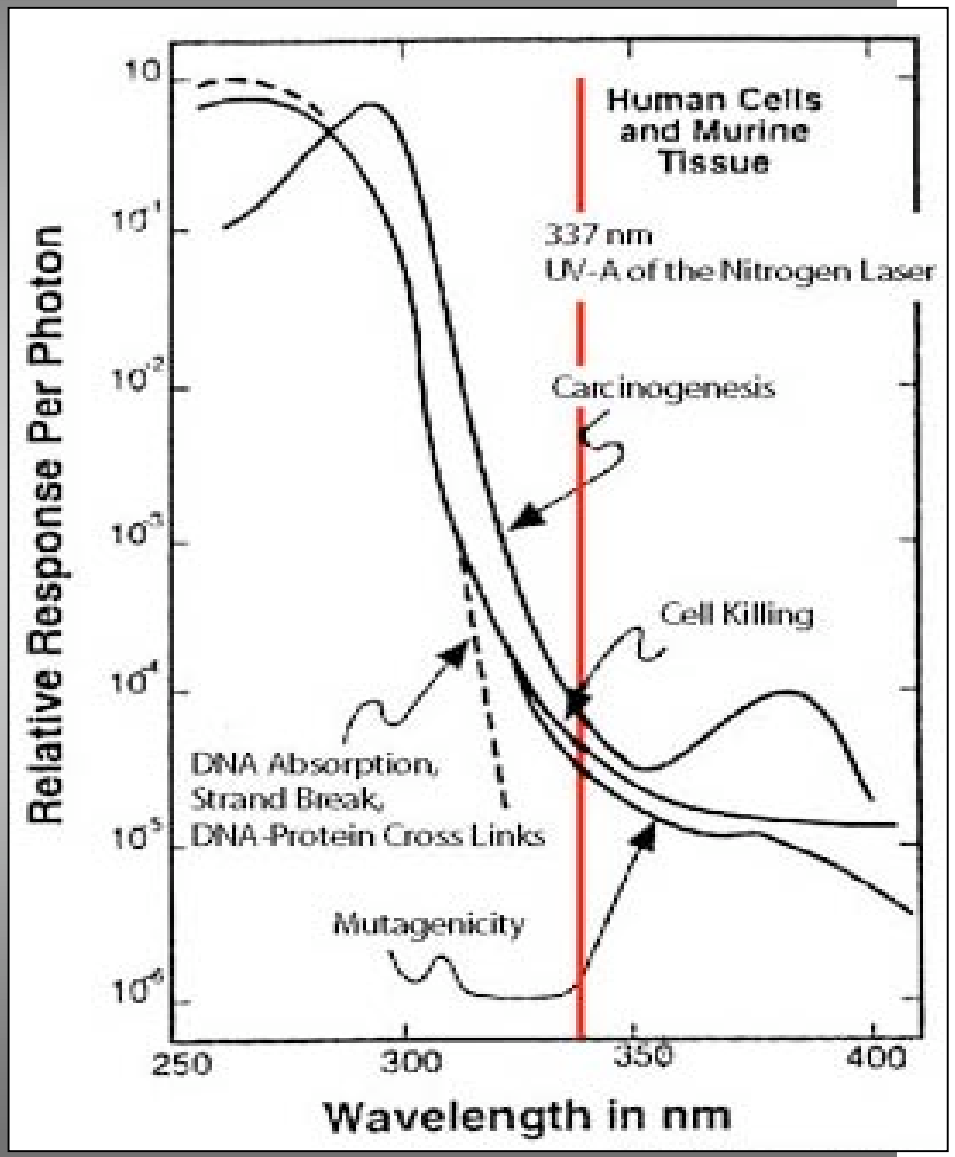
Ablative Photo-Decompensation (APD):

Hohe Photondichte, Energie höher als Molekülbindungsenergie, Microplasma-Formation/Collapsing (nanosec), Expansion, keine Hitze

"*where the laser hits, nothing is left that could be analyzed as biomolecule*"

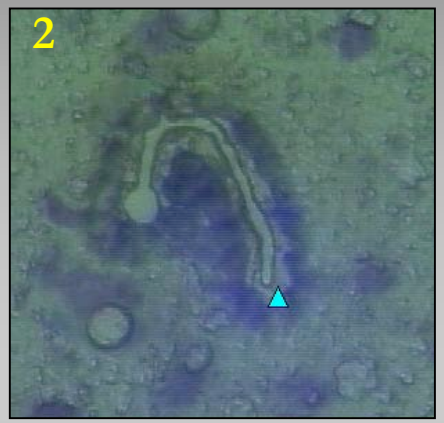
PALM: Photo Ablation and Laser Microdissection

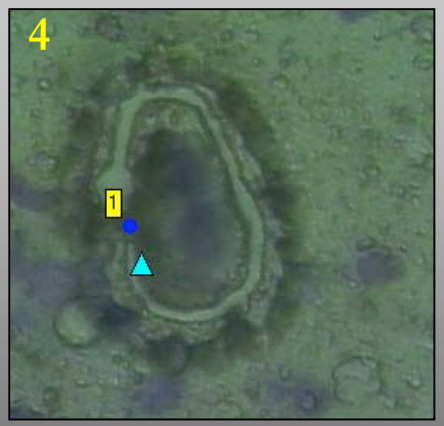




337 nm: keine DNA/RNA/Proteinschädigung

Laser-Microdissection eines dopaminergen Neurons

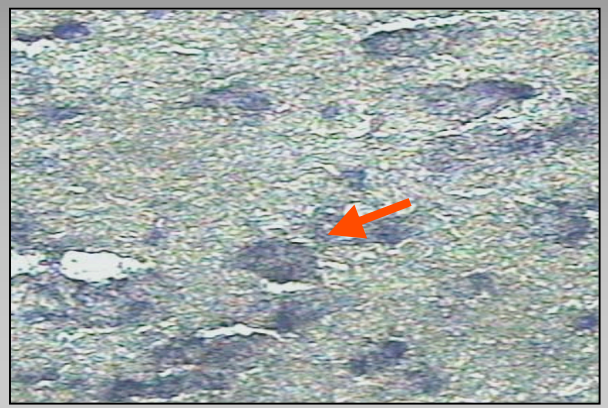




adhesive cap control

Gen-Expressionsanalyse nach Laser-Microdissection

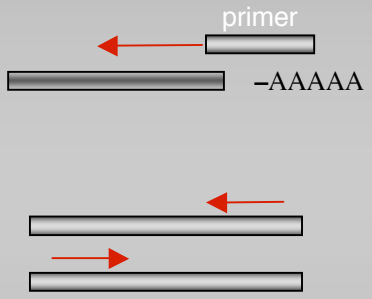
stained mouse brain section (10μm)



after laser dissection and catapulting

RT-PCR amplification of single-cell mRNA

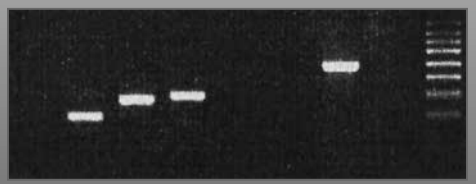
Lysis of captured neurons



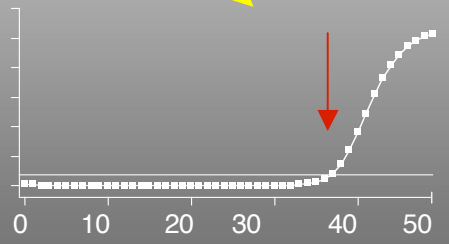
cDNA synthesis

PCR amplification of target cDNAs

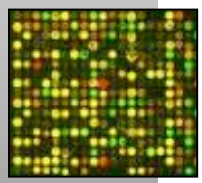
detection of gene expression



qualitative multiplex PCR

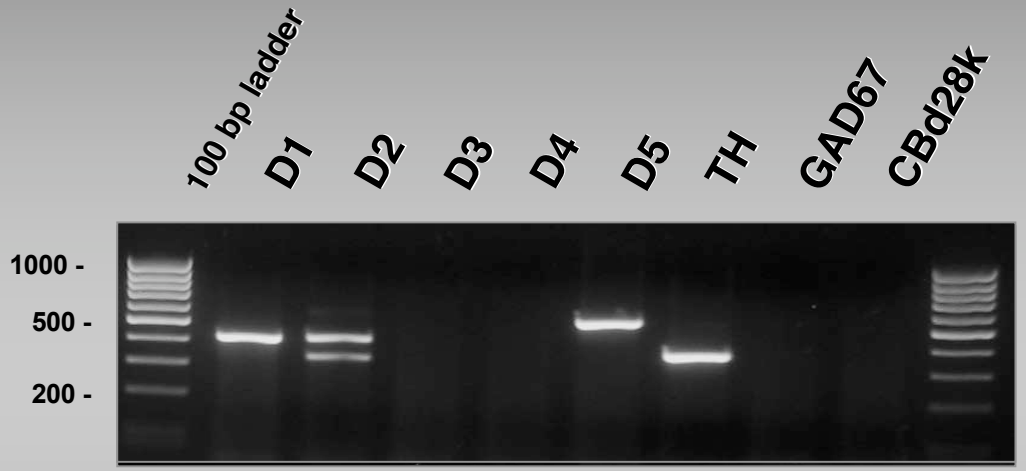


quantitative real-time PCR

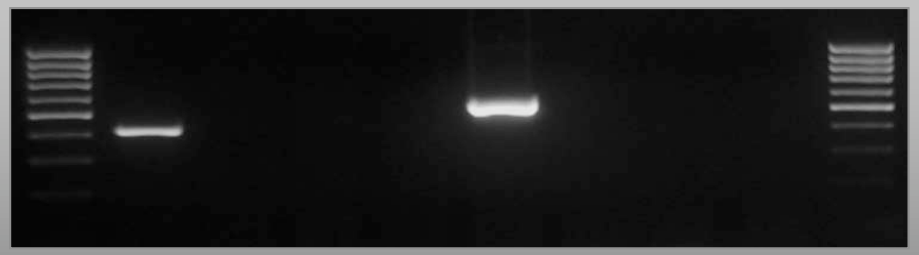


Sensitivität der Einzelzell RT-PCR Protololls: Detektion der genomischen DNA (Zellkern)

Single cell RT-PCR without DNAse digest

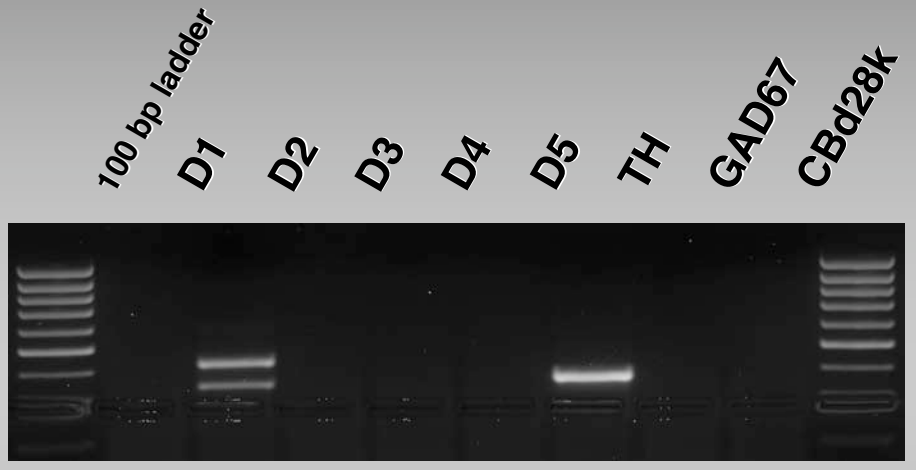


single neuron



single neuron: RT minus control

Single cell DNAse digest prior RT-PCR



single neuron

single neuron: RT minus control

D1 and D5 primers: Intronless PCR product

Protocol robustly detects two copies of genomic DNA from single nucleus

Liss and Roeper (2004

Gen-Expressionsanalyse einzelner Zellen mittels Datenbank-Recherche und RT-PCR

A) Praktischer Teil im Labor: Methode

Laser Microdissektion und Isolation einzelner dopaminerger Neuronen

Jan Gründemann

B) Praktische Teil am Computer: PCR-Theorie

Wie suche ich geeignete Primer für ein RT-PCR Experiment aus?

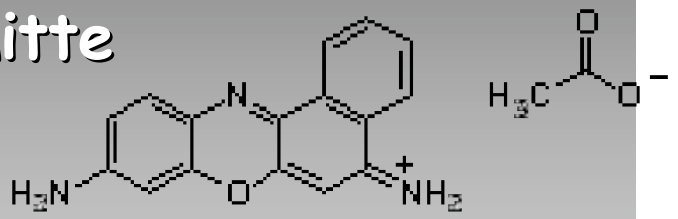
Anca Alexandru

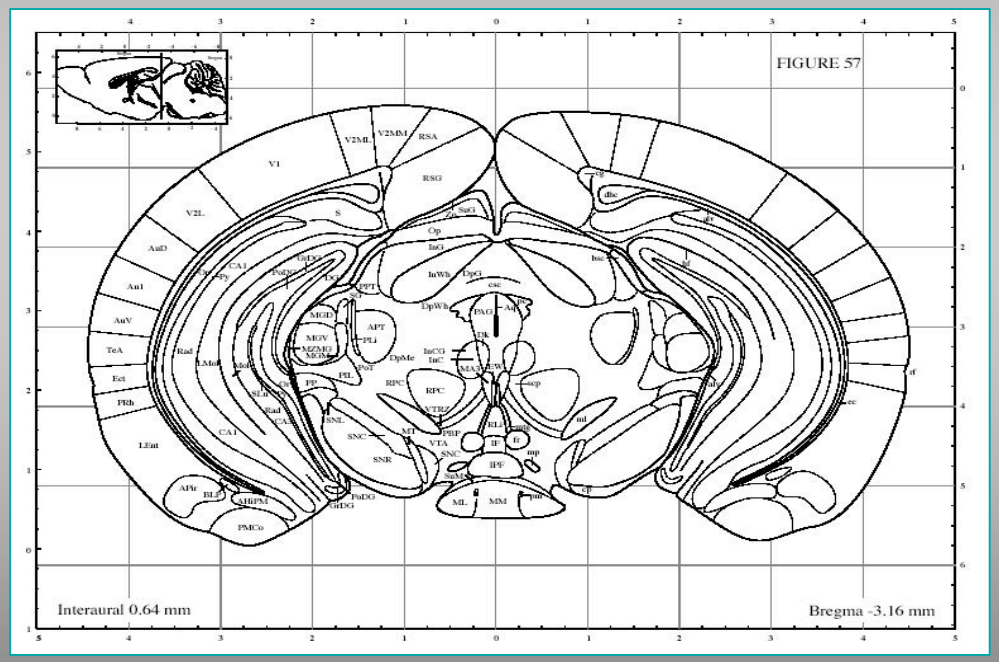
C) Hintergrund (soviel wie gewünscht):

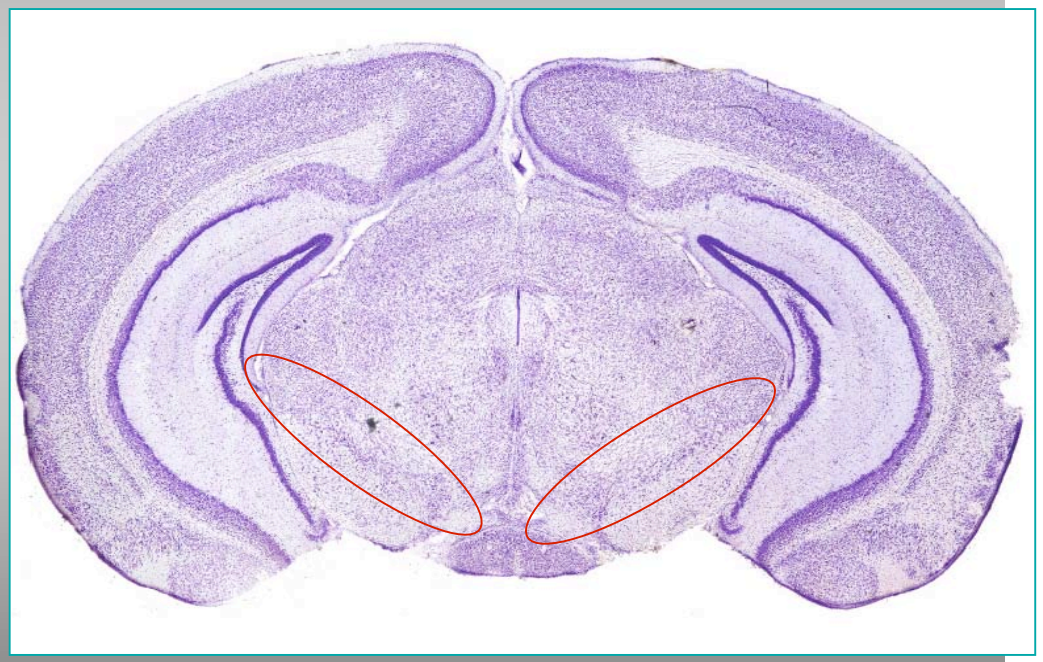
Warum Genexpressions-Analyse von dopaminerge Neuronen?

A) Auffinden der dopaminergen Mittelhirn-Neurone

Maus: koronale Mittelhirnschnitte



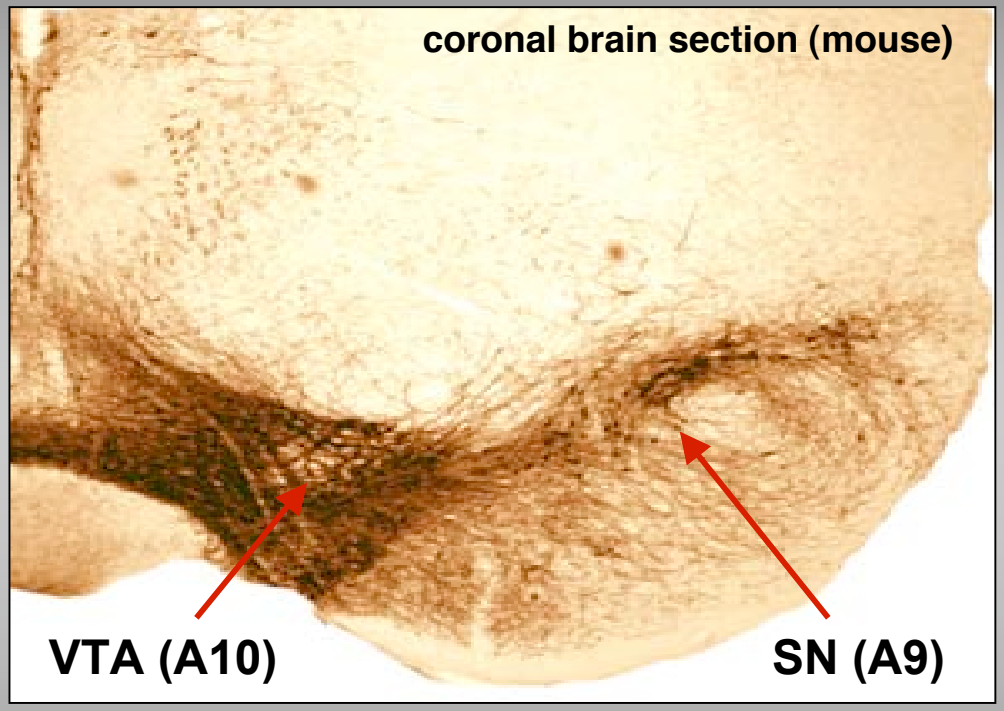




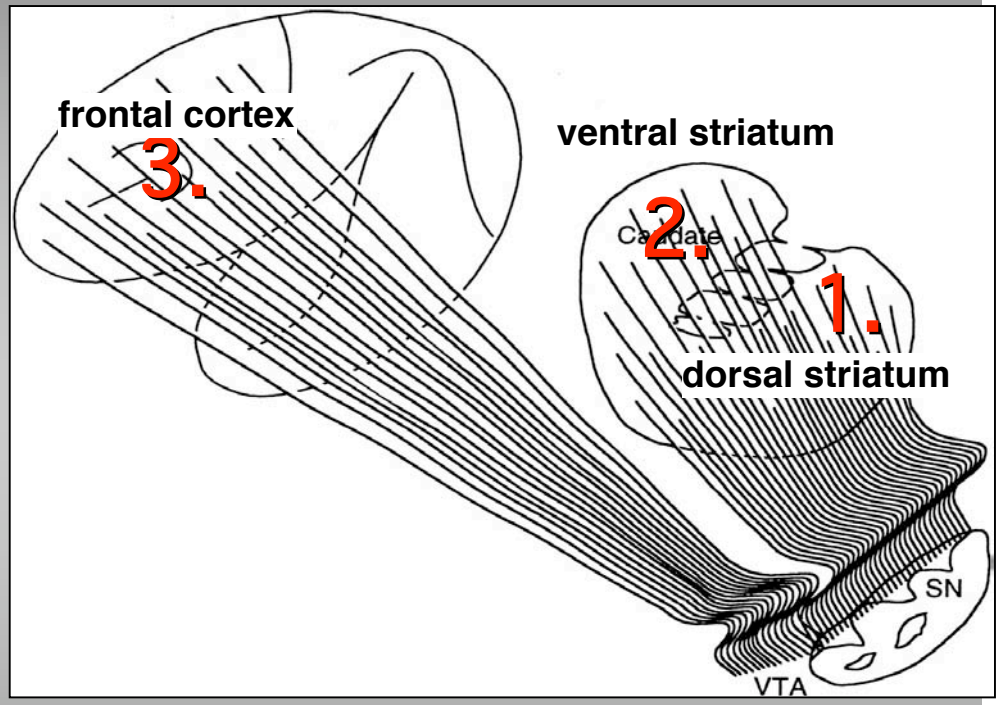
mouse brain atlas

Zellfärbung (Nissl / Cresylviolett)

Drei verschiedene Typen von dopaminergen Neuronen: Drei verschiedene Projektionsgebiete



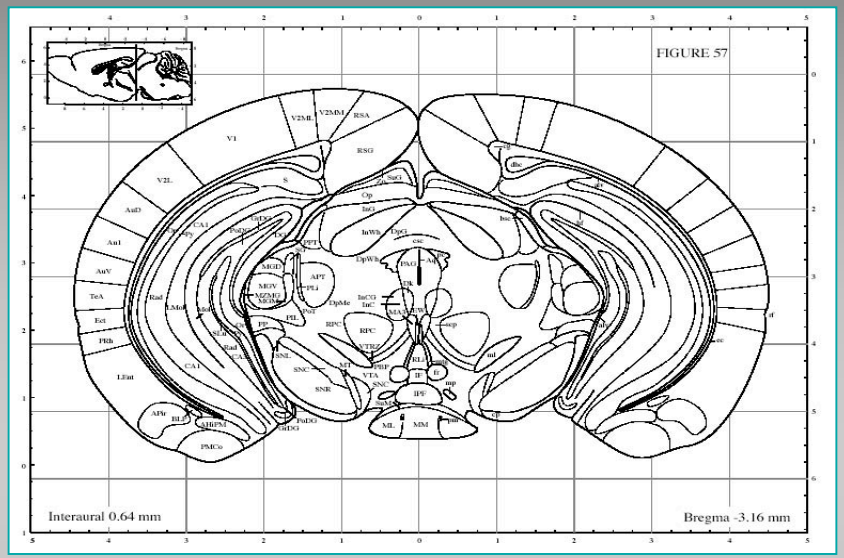
Tyrosine-hydroxylase (TH) immunostain

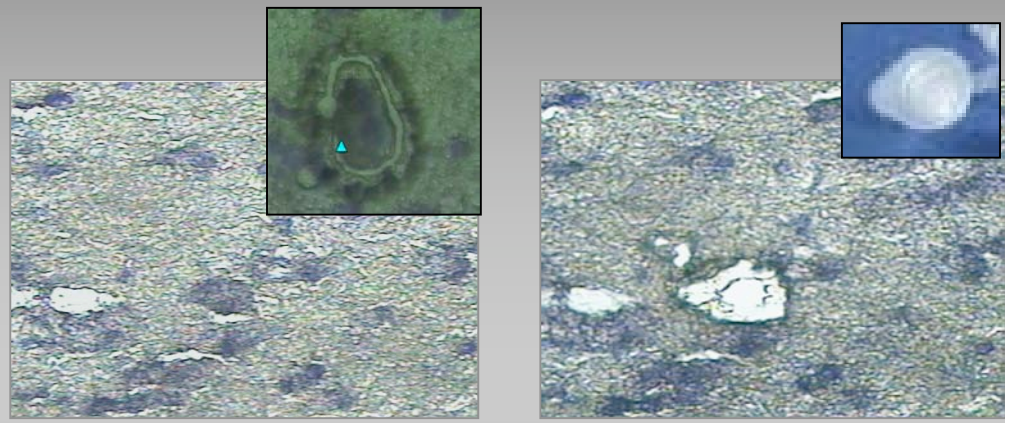


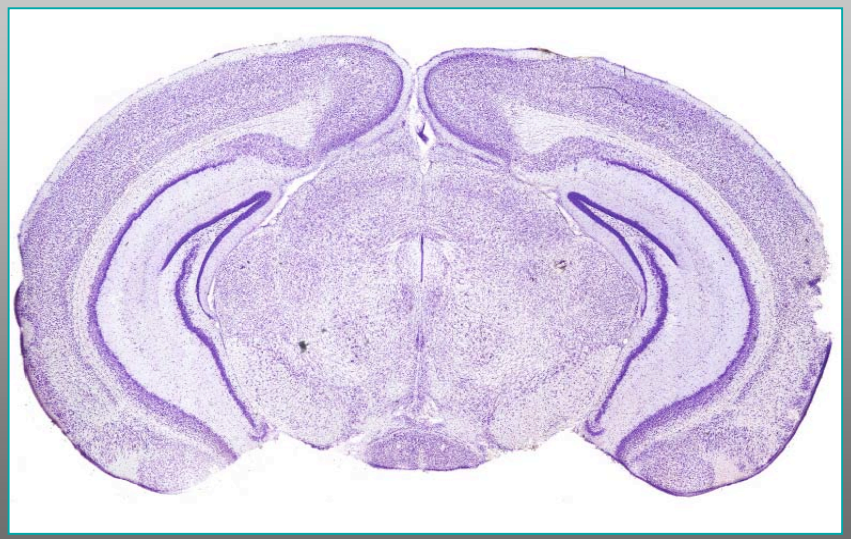
dopaminergic projections

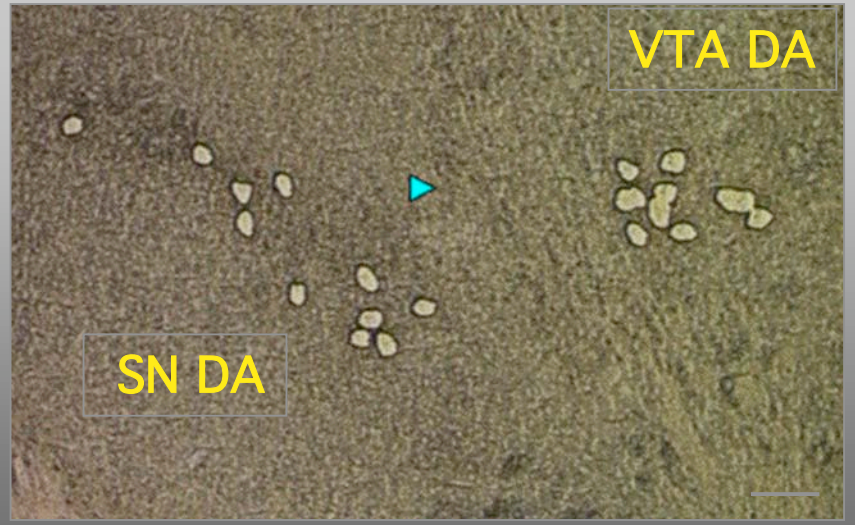
1.	SN:	mesostriatal	voluntary movement	Parkinson's disease
2.	VTA:	mesolimbic	motivation (reward)	Schizophrenia (+) / drug addiction
3.	VTA:	mesocortical	cognition (working memory)	Schizophrenia (-)

A) Laser-Mikrodissection einzelner Mittelhirn-Neurone









B) Praktische Teil am Computer: PCR-Theorie:

Wie suche ich Primer für ein RT-PCR Experiment aus ?

- Der Weg ist das (Praktikums) Ziel ! -

Aktualisierte Praktikumsunterlagen / Einführungsdias
auf meiner Homepage (unter Lehre Info):

http://www.med.uni-marburg.de/d-einrichtungen/molneurophys/lehreinfo/1259/

Design geeigneter Primer für RT-PCR Genexpressions-Analysen

1. Was weiss man über das zu untersuchende Gen bzw die Funktion des Genprodukt?

- Gibt es weitere Gen-Familienmitglieder oder Homologe oder Pseudogene ? (Primer-Spezifität)
- Datenbanken-Search: *NCBI-OMIM, Gencards, Pubmed* (zB Rewievs)

2. Auffinden der cDNA Sequenz (*NCBI Nucleotide*):

- Entscheidung für eine Sequenz, da meistens mehrere Genbankeinträge für ein Gen/Genprodukt:
- Aufschreiben der *Accession number* (einzigartige Identifikationsnummer "Personalausweis")
- Ausdrucken der cDNA Sequenz im "*Genbank-Format*" und im "*Fasta-Format*"

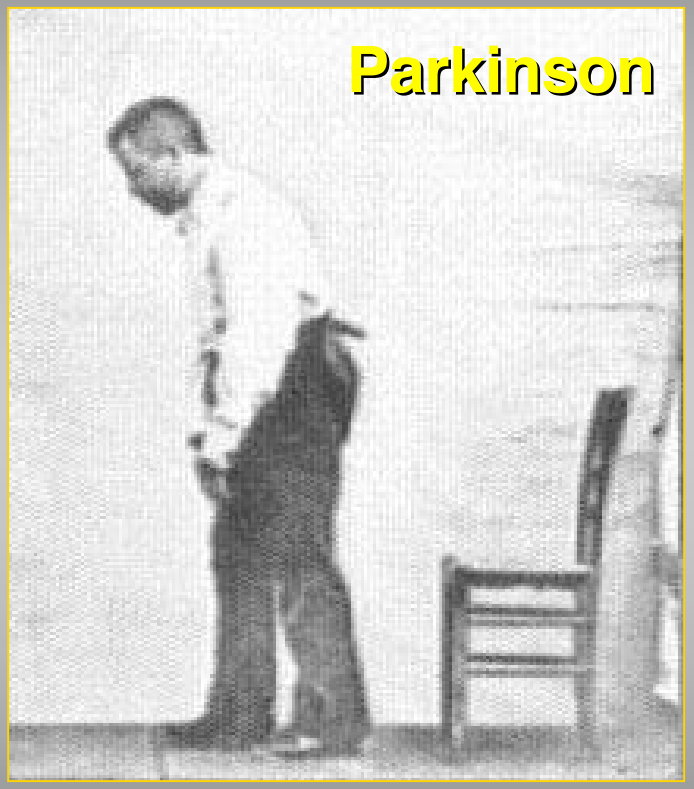
3. Auffinden der Genstruktur bzw der Intron/Exon Übergänge (*Ensemble*):

- Primer / PCR-Produkt sollte mindestens ein Intron-Exon Übergang überspannen (warum?)
- Datenbank "*Ensemble*" ist hierfür am besten geeignet (Achtung: andere Gen-Einträge als NCBI!)

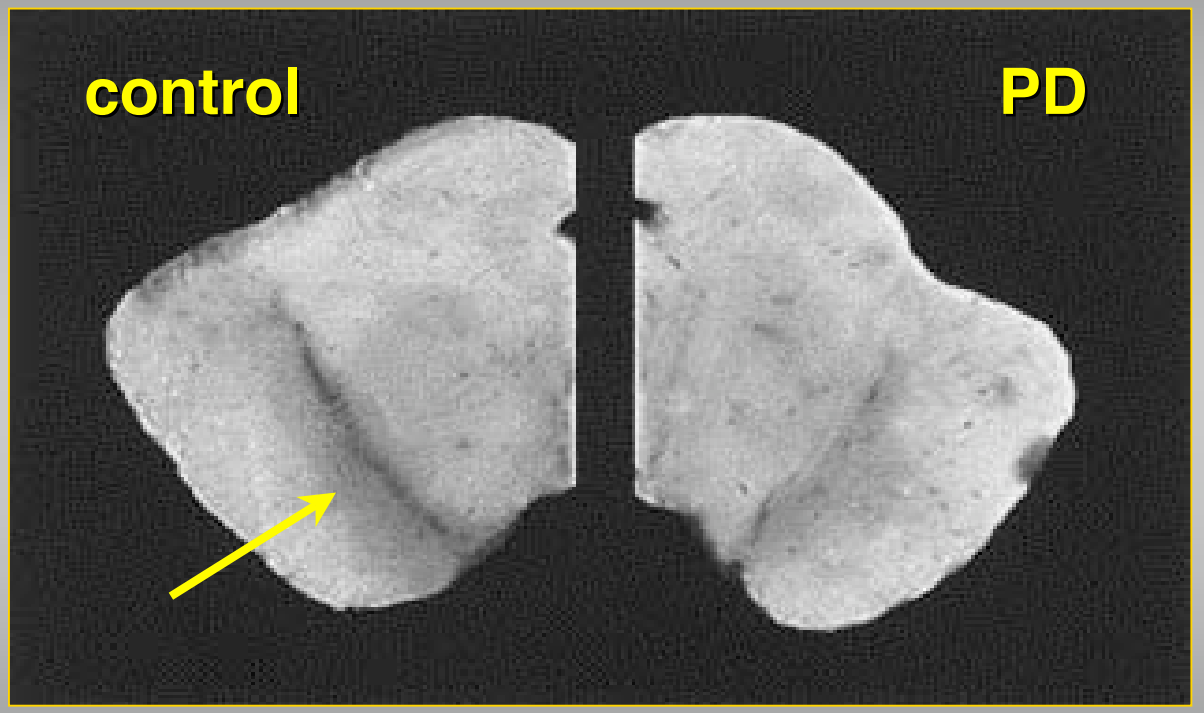
4. Auswahl "geeigneter", spezifischer Primer:

- Einlesen der cDNA Sequenz in ein *Primer-Design Programm* (Länge, Annealing-Temp, GC-Gehalt)
- **Auswahl je eines geeigneten Forward (sense) und Reverse (antisense) Primers:**
  Primer sollen nur Zielgen binden, mind. ein Intron überspannen, PCR Fragment ca 150- max.1000 bp
- **Auffinden/Eintragen der Primersequenzen in der jeweiligen cDNA *Genbank*-Sequenz:**
  Beachte: Reverse Primer idR in 5' - 3' angegeben, müssen also reverse complement gelesen werden
- **Überprüfen der Spezifität der ausgewählten Primer ("*BLAST short sequence search*")**

Selective loss of dopaminergic neurons in Parkinson's disease



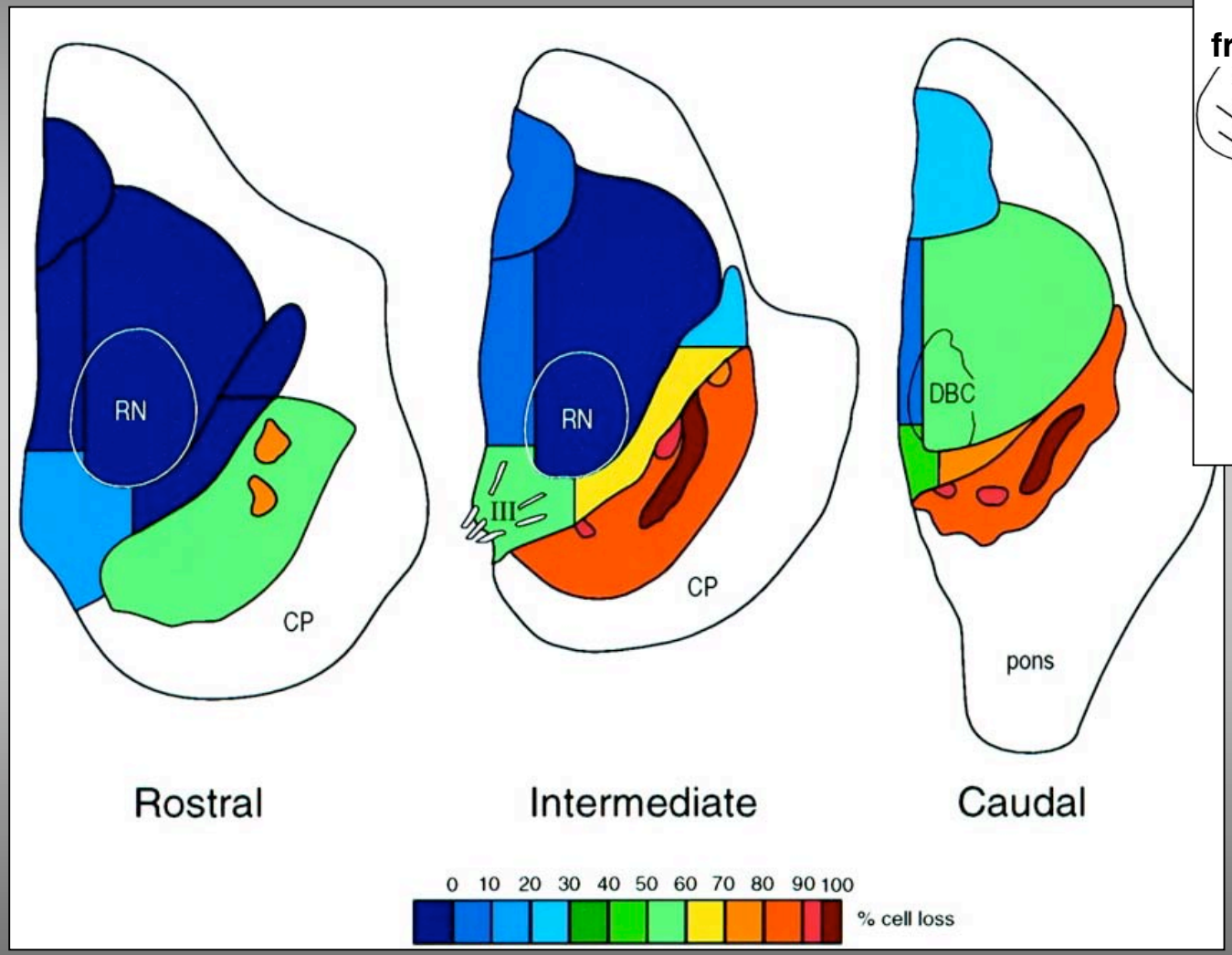
rigor, tremor, akinesia
posturale instability



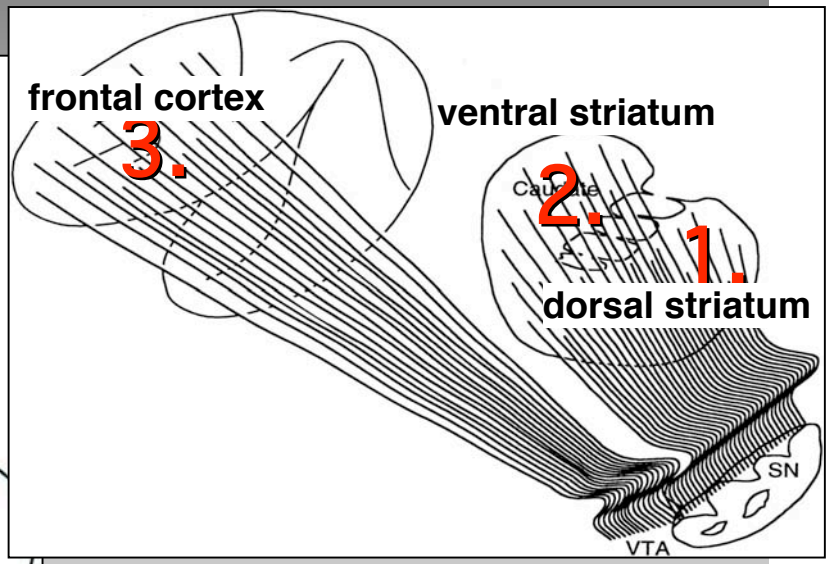
cause : unknown

(mitochondrial (CX I) and proteasome dysfunction as candidate mechanism for PD)

Differential vulnerability of DA neurons in Parkinson's Disease



Damier et al. (1999a/b)



dopaminergic projections

SN (A9) >> VTA (A10)

caudal >> rostral
nigrosomes >> matrix
CB neg >> CB pos

Forschungsschwerpunkt AG Liss:
Molekulare Mechanismen der differentiellen Vulnerabilität ?

1. Isolation der Einzelzell mRNA (Funktionsanalyse)

(*Laser Mikrodissektion, Patch-clamp Technik*)

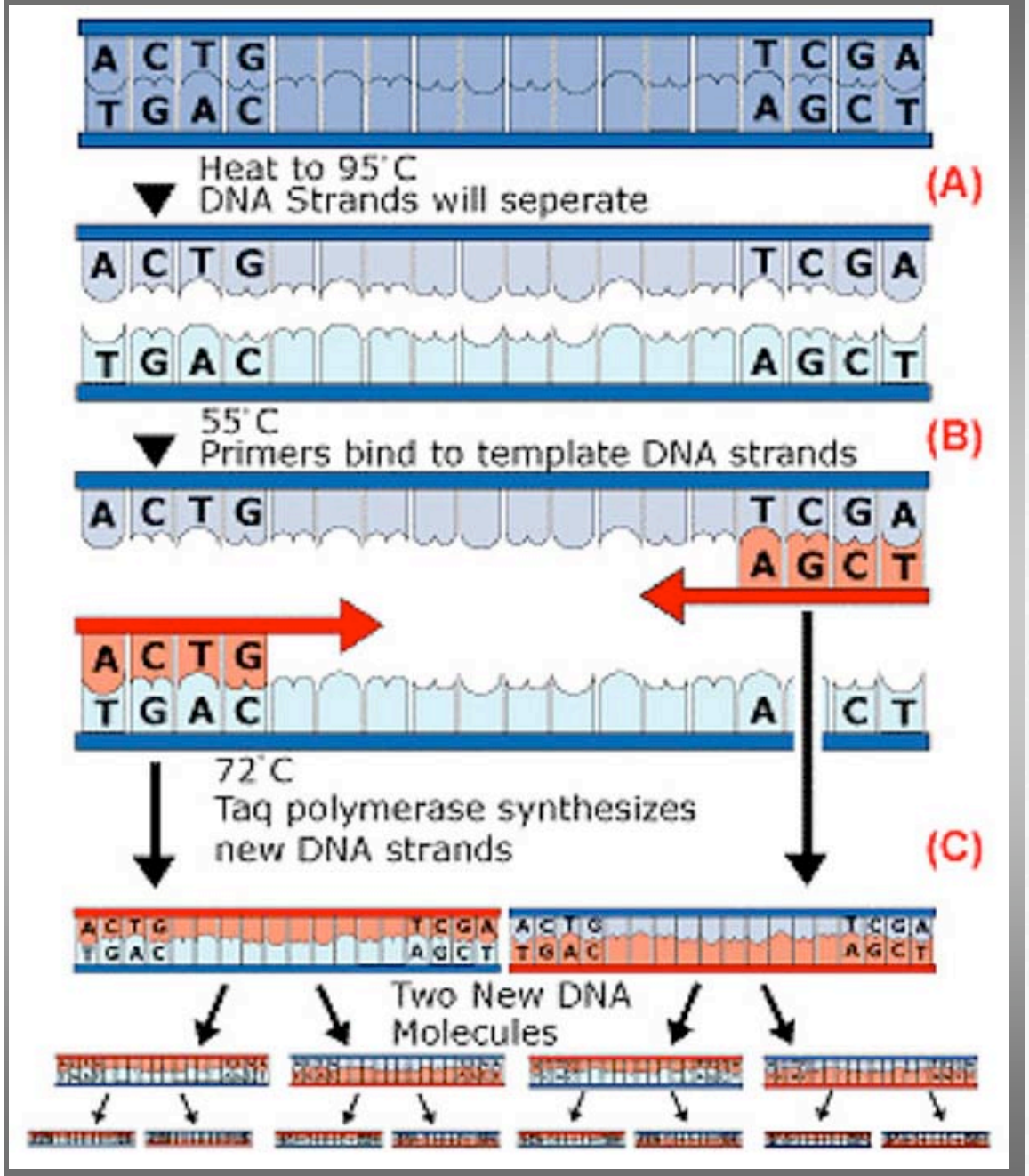
2. cDNA Synthese (Reverse Transkription der mRNA)

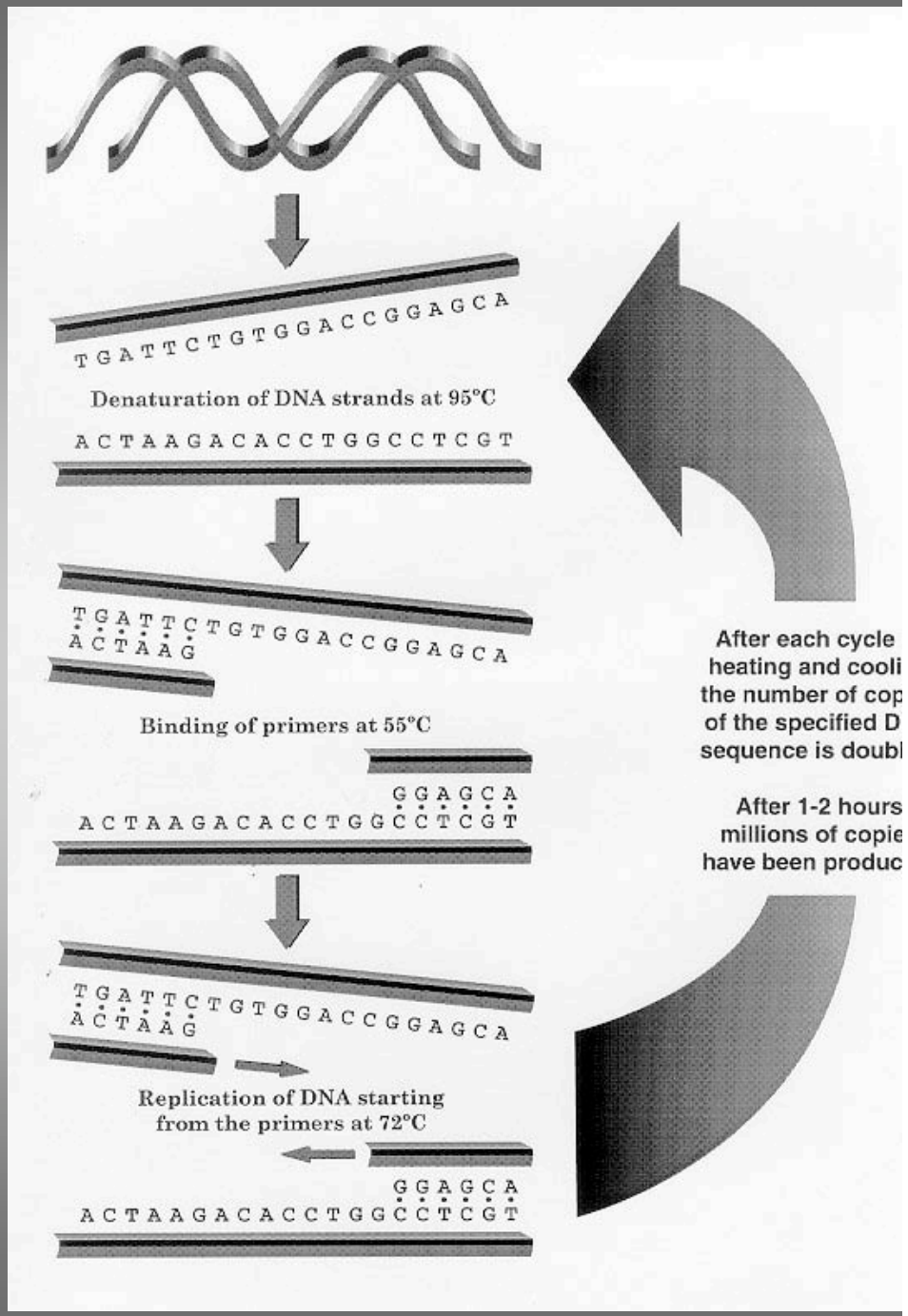
(*Reverse Transkription -RT*)

3. PCR Amplifikation der zu untersuchenden Gene/cDNAs

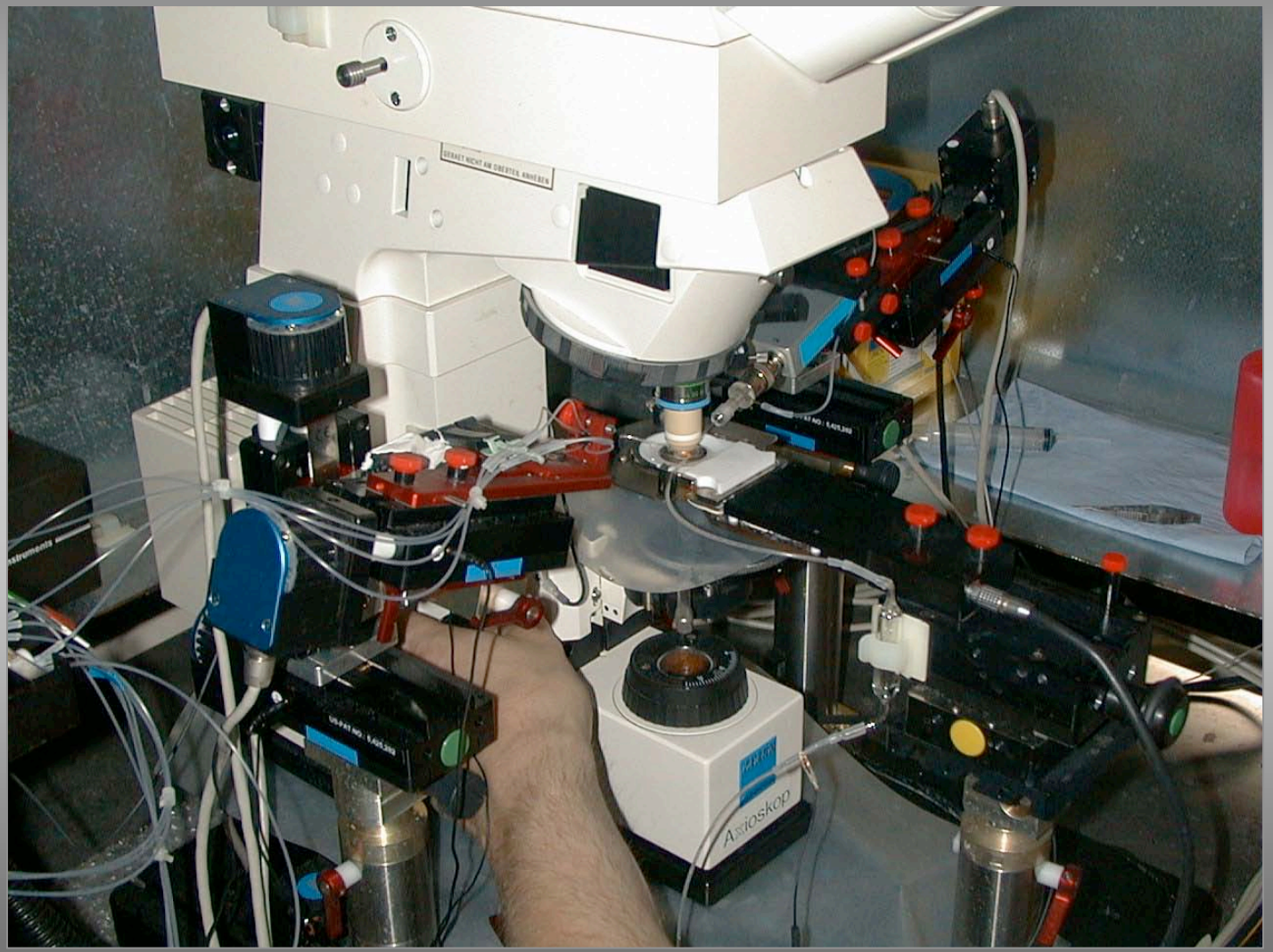
(*qualitative PCR, quantitative real-time PCR*)

Vergeich der Genexpression
der hoch-empfindlichen und der resistenten DA Neuronen


A C T G
T G A C
T C G A
A G C T
Heat to 95°C
DNA Strands will seperate
(A)
55°C
Primers bind to template DNA strands
(B)
72°C
Taq polymerase synthesizes
new DNA strands
(C)
Two New DNA
Molecules


T G A T T C T G T G G A C C G G A G C A
Denaturation of DNA strands at 95°C
A C T A A G A C A C C T G G C C T C G T
Binding of primers at 55°C
G G A G C A
Replication of DNA starting
from the primers at 72°C
After each cycle
heating and cooli
the number of cop
of the specified D
sequence is doubl
After 1-2 hours
millions of copie
have been produc

Hirnschnitt Patch-clamp Technik



Patch-clamp technique - principle

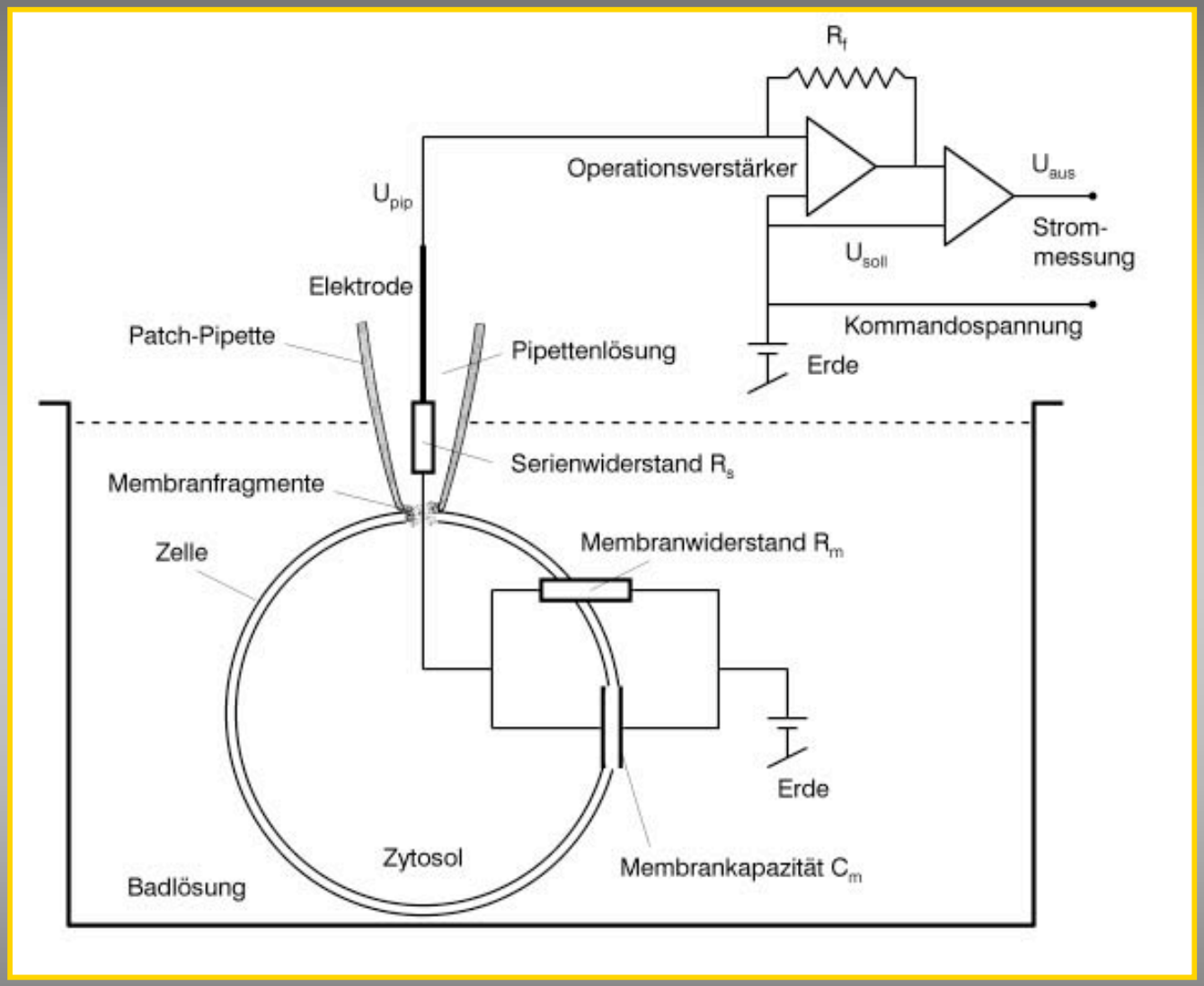
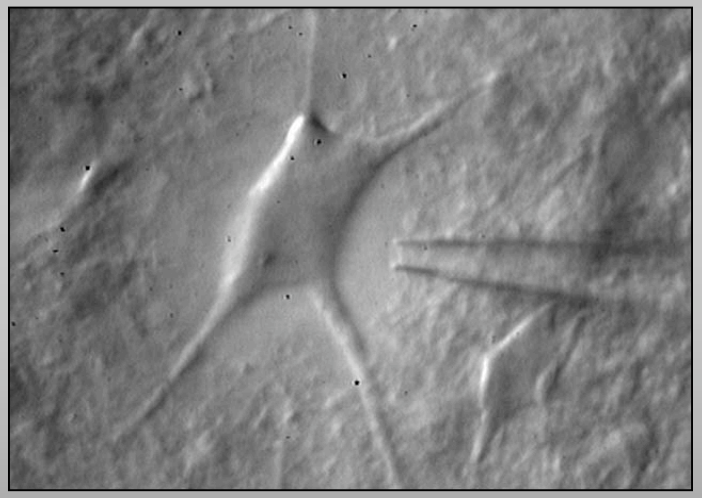
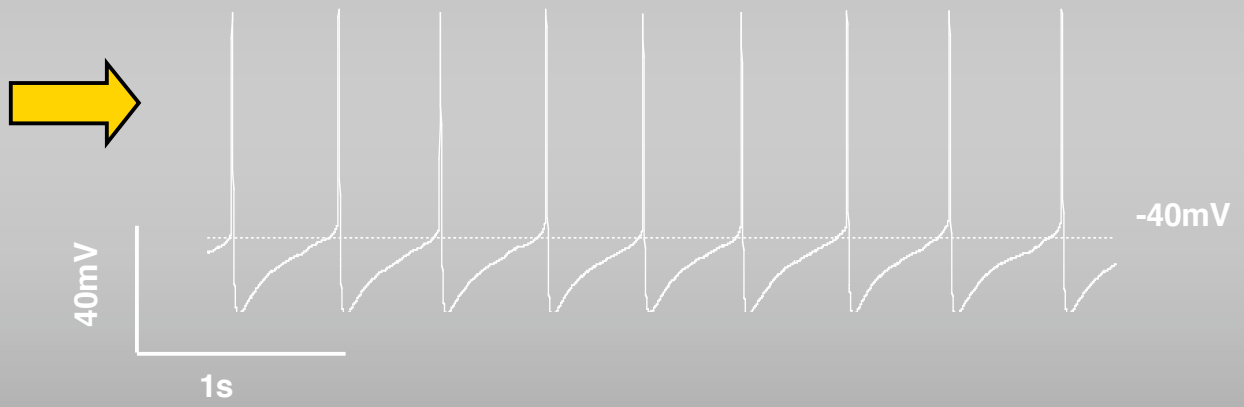


Fig. 1: Simplified circuit of a patch-clamp amplifier and substitute-circuit of the whole-cell-configuration: Rf1: Feedbac resistance, Usoll,: Nominal - or debit-potential, Upip,: Pipette-potential, Uaus,: Exit-potential proportionally to the current. T circuit of the amplfier, that represents a so-called current-potential transformer, is accommodated near the pipette in a sm box (the preamplifier). After breaking through the membrane, the so called whole-cell configuration is reached. Membran fragments and other cell-material are sucked near the tip of the patch-pipette increasing the series-resistance Rs.

Untersuchung der spontanen Aktivitaet

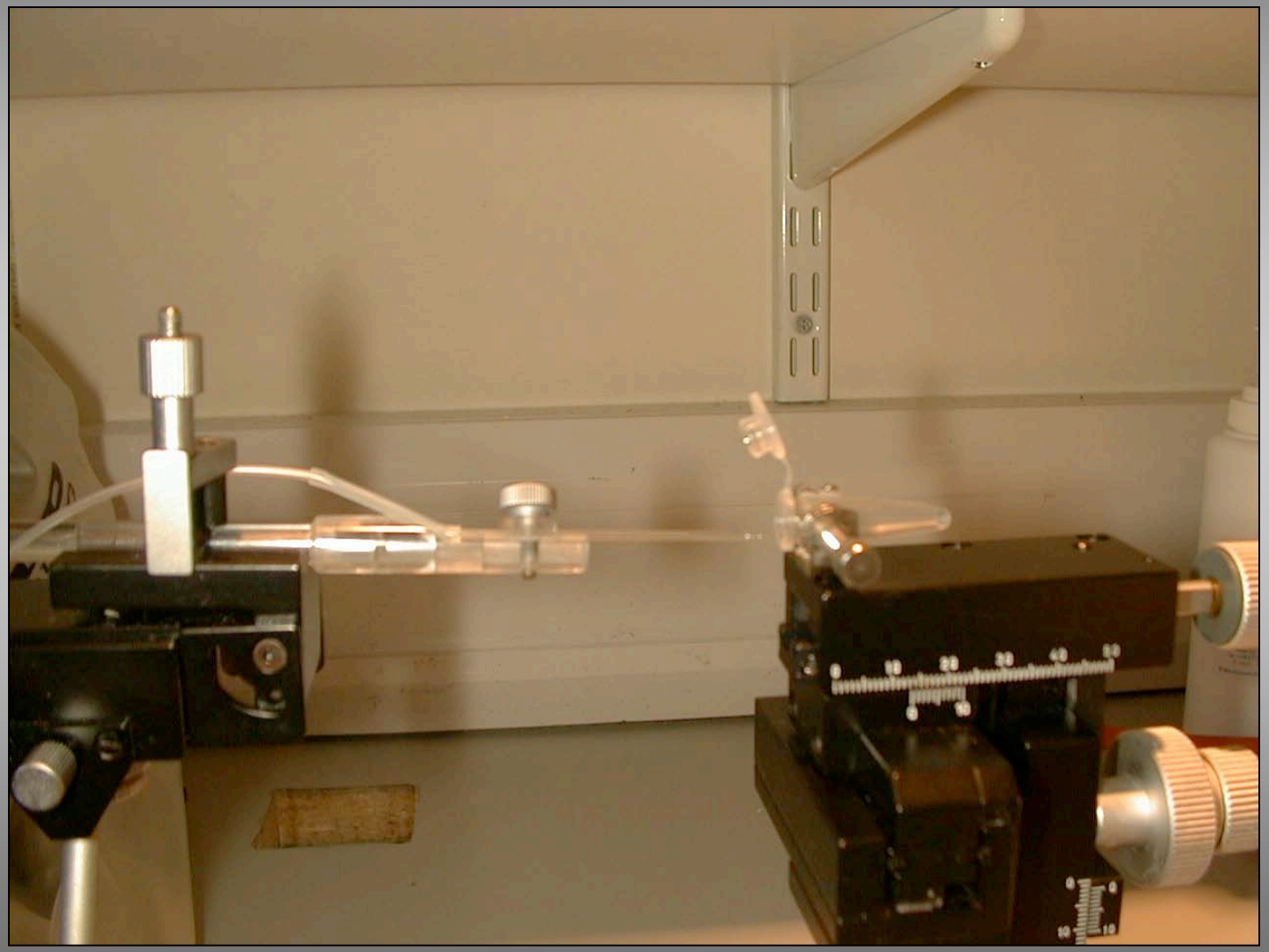
brain-slice whole cell current clamp recording





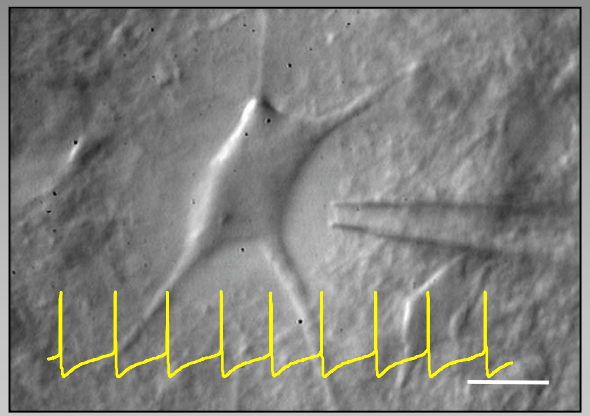
1.3 ± 1.1 Hz

Ueberfuehren des Einzelzell Zytoplasmas in ein PCR Tube



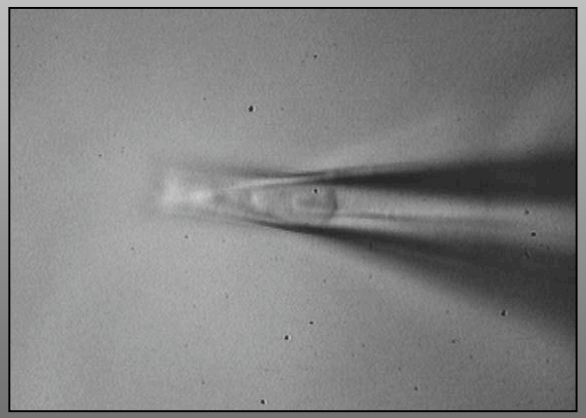
Einzelzell RT-PCR nach Patch-clamp Analyse

electrophysiological characterisation

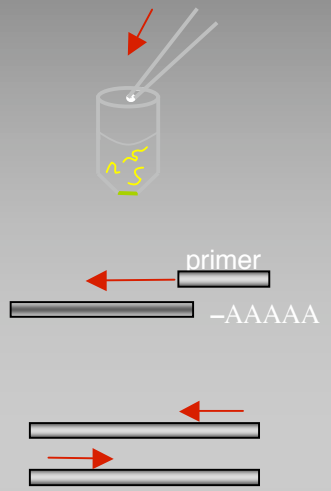


brain-slice patch-clamp technique

harvesting of single-cell cytoplasm



RT-PCR amplification of single-cell mRNA

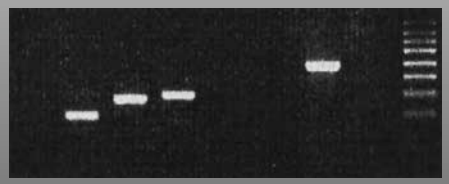


expelling of pipette contents

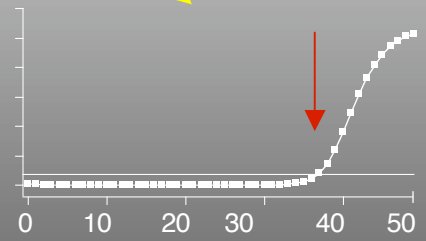
cDNA synthesis

PCR amplification of target cDNAs

detection of gene expression

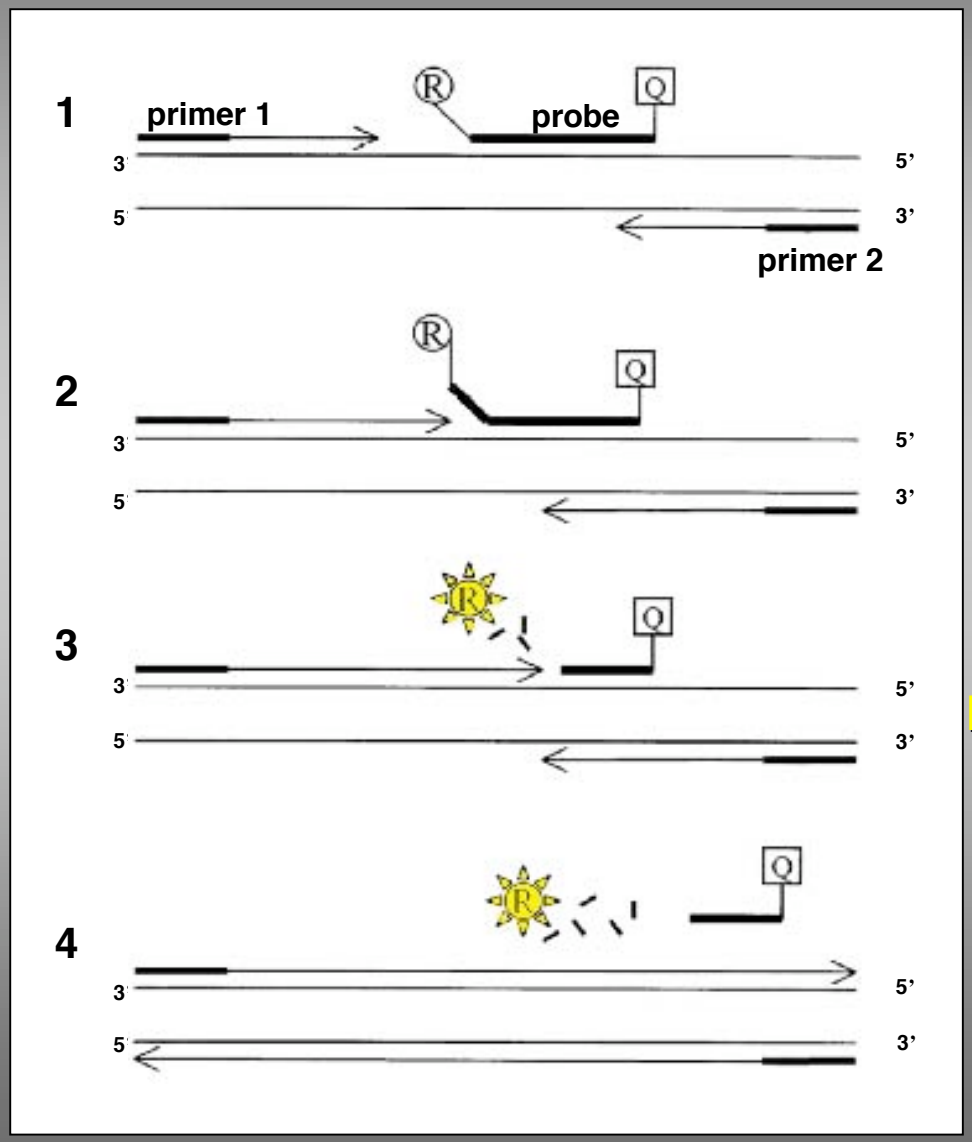


qualitative multiplex PCR



quantitative real-time PC

Quantitative real-time PCR:
TaqMan primer / hybridisation-probe PCR assay



annealing of primer and hybridisation-probe
(5'-reporter / 3'-quencher)

↓

primer-extension

↓

5' exonuclease activity of Taq Polymerase:
Hydrolysis of the probe, release of fluorescence

↓

detection of fluorecence increase