Quantitative single-cell RT-PCR and calcium imaging in acute brain slices

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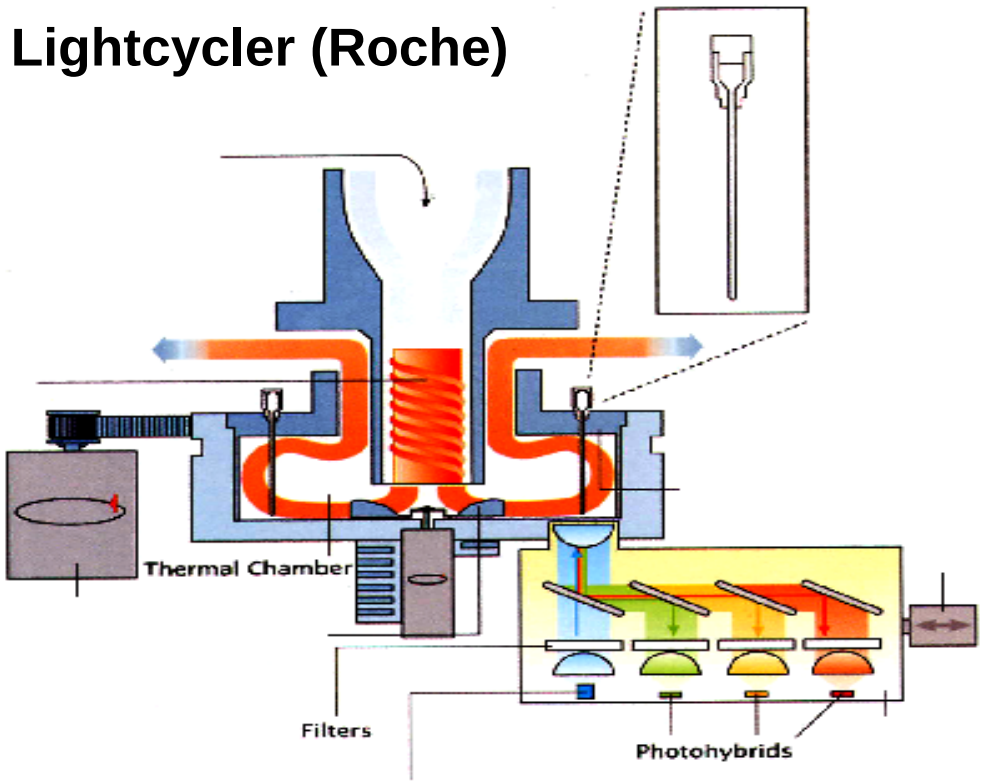
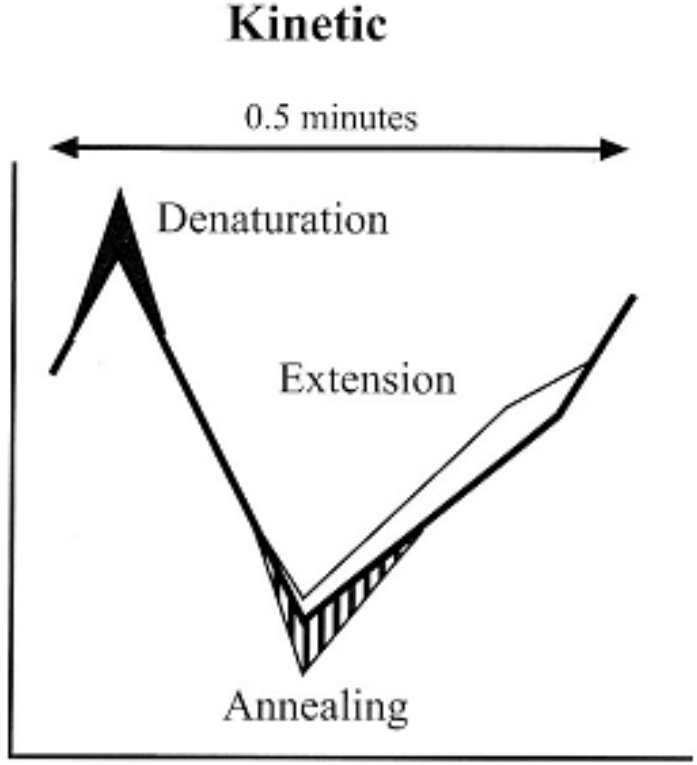
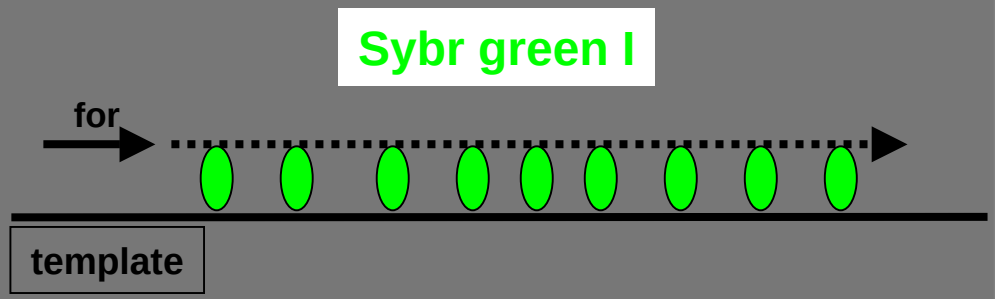
Quantitative rapid cycle real time RT-PCR

Lightcycler (Roche)

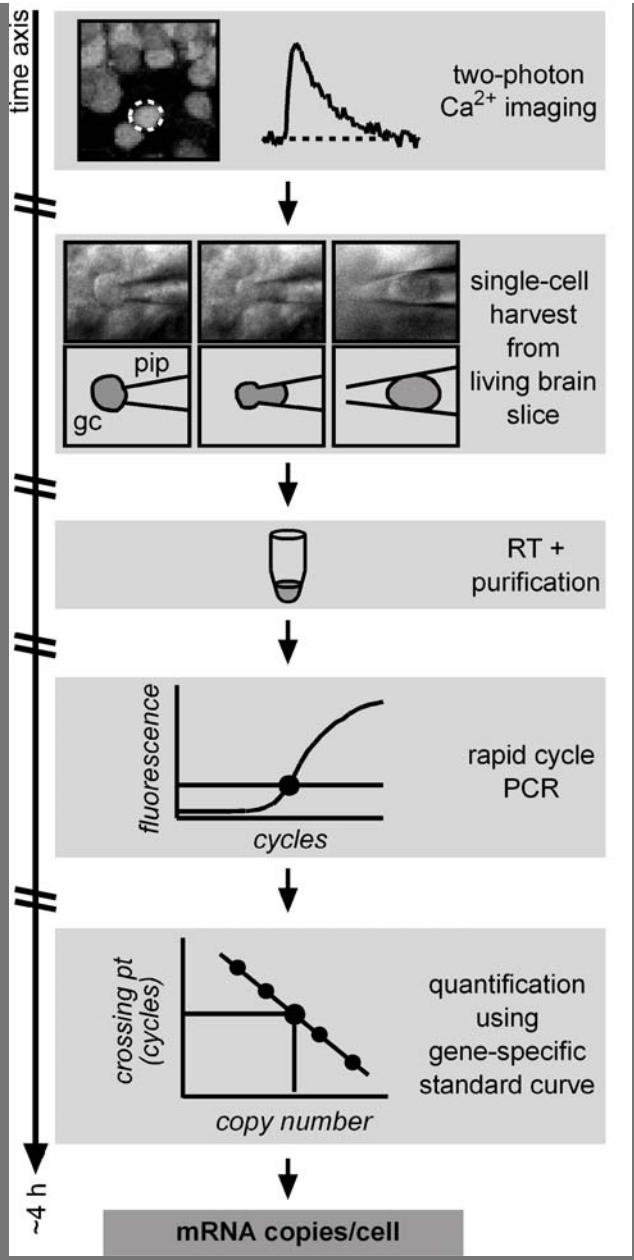






LC-DNA-master SYBRgreenI

Single-cell quantitative RT-PCR -Development of an approach



Alternative title:

Quantitative detection of 2-20 cDNA copies from single-cell RT reactions

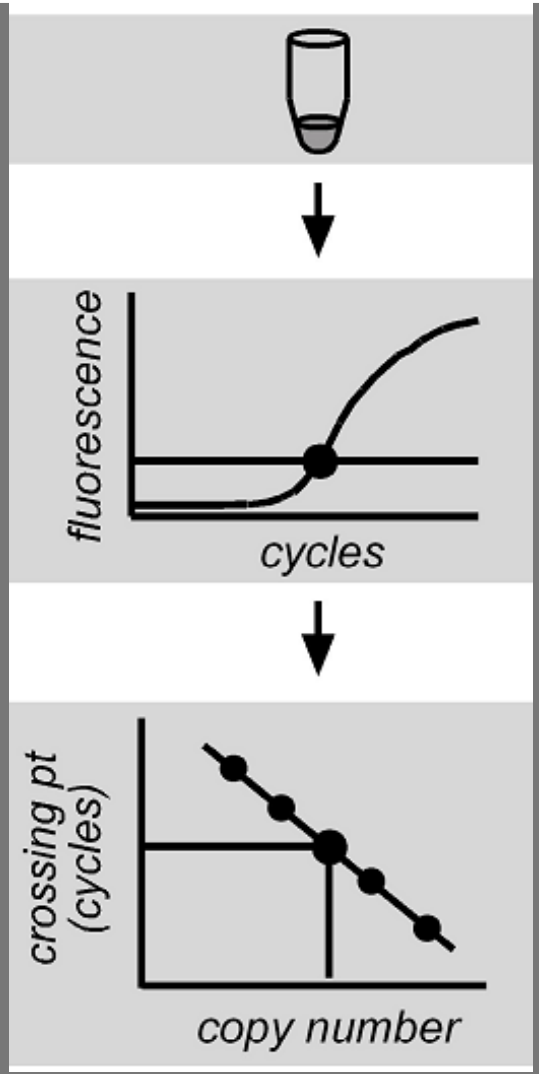
Let‘s talk about arithmetics and random events

cycle	copy no.(eff.=2)	copy no.(eff.=1.8)
1	1+1	1+1
2	4	4 (3.6 either 3 or 4)
3	8	7
4	16	13
5	32	23
10	1024	
15	32768	
20	1.04×10^6	1.27×10^5
25	3.35×10^7	
30	1.07×10^9	
35	3.44×10^{10}	8.06×10^8
40	1.10×10^{12}	1.62×10^{10}

PCR amplification: $T_{\text{copies at cycle (n)}} = T_{\text{initial copies}}(\text{effiency})^{\text{cycle}}$

1 dsDNA copy of 297 bp ($M_w = 3.05 \times 10^{-19}$ g) // 152 ng = 10^{12} dsDNA copies
quantitative detection at: ~10^{11} copies

Optimization of real-time RT- PCR

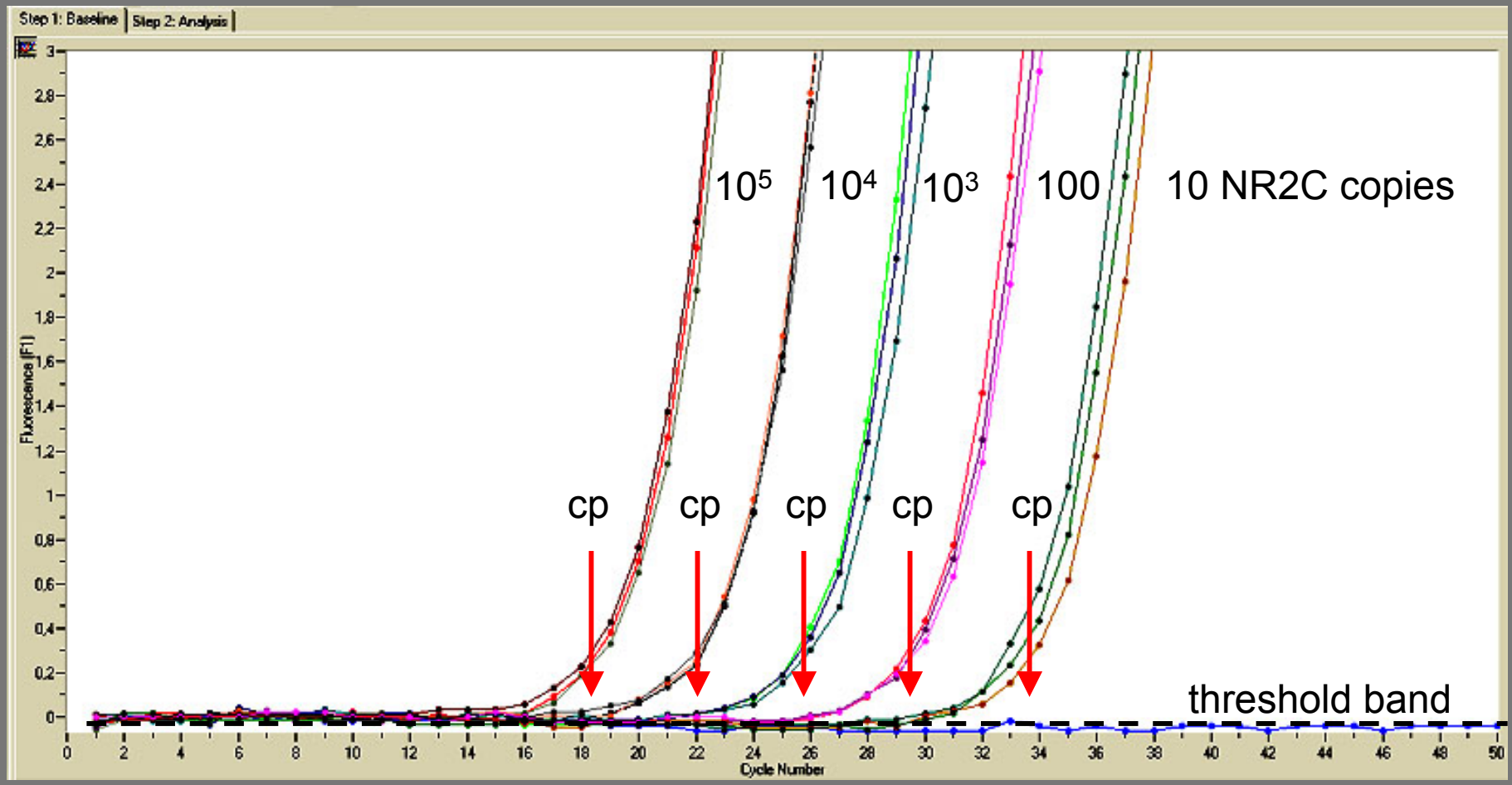


Reverse Transcription:
- random hexamers or dT primers
- MMLV or Superscript
- **quantitative purification of cDNA**

cycle conditions:
- primer sequence
- **sample amount (not more than 12)**
- magnesium concentration
- primer concentration
- annealing temperature
- **hot start protocol (Taq start Ab)**

cycle specific parameters:
- **quantification limit**
- **high versus low DNA amounts**
- **resolution**

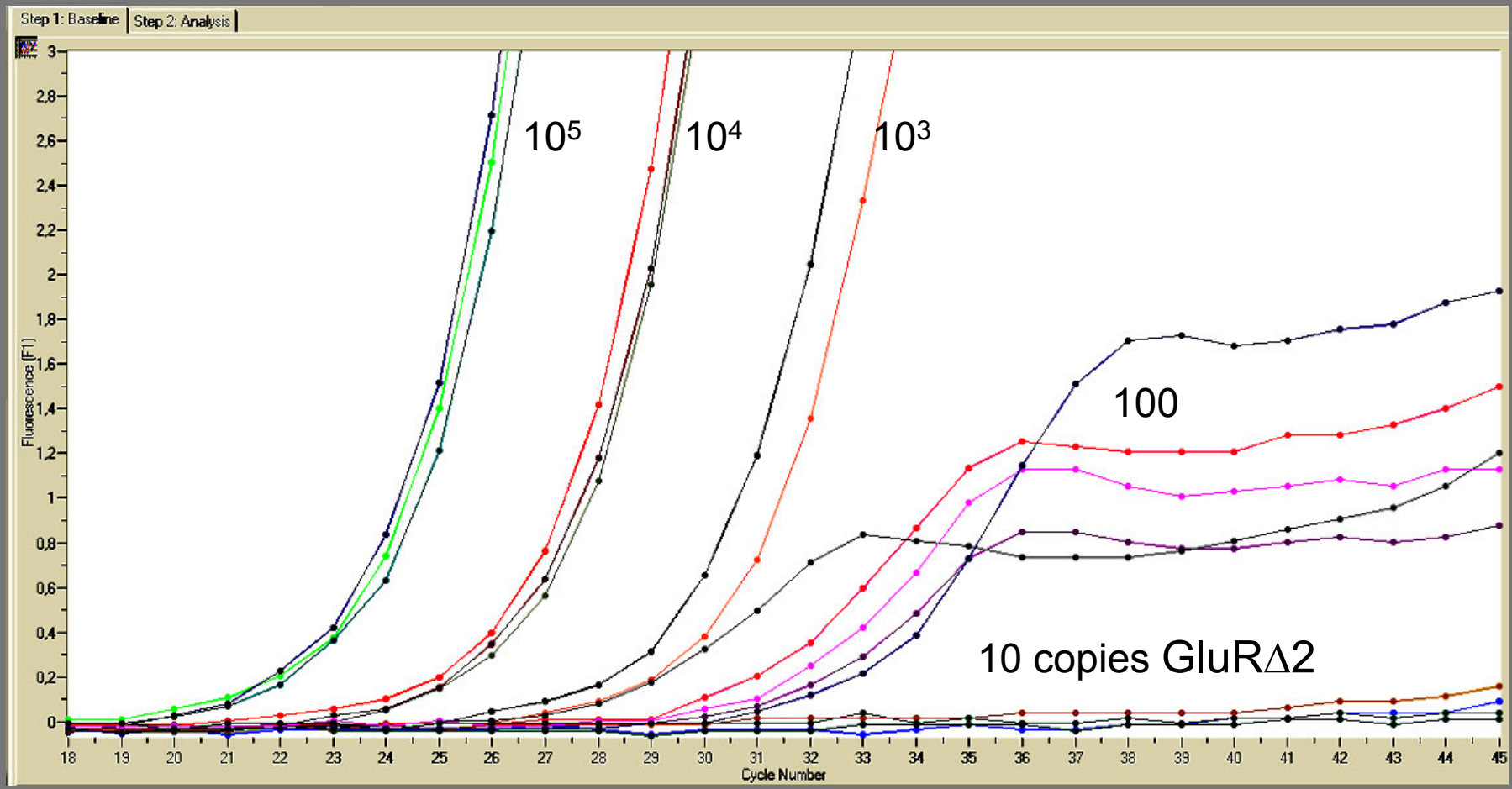
A standard curve, as it should be (here NR2C)



➲ dilutions series: PCR efficiency can be calculated

➲ but: no information about the efficiency of the reaction in the range of >1 to 100 copies!

A standard curve, as it should not be (here GluRΔ2)



- primer dimers (💧) act as competitive inhibitors
- high copy numbers are useless to standardize low copy „unknowns“

High-resolution external standard curves

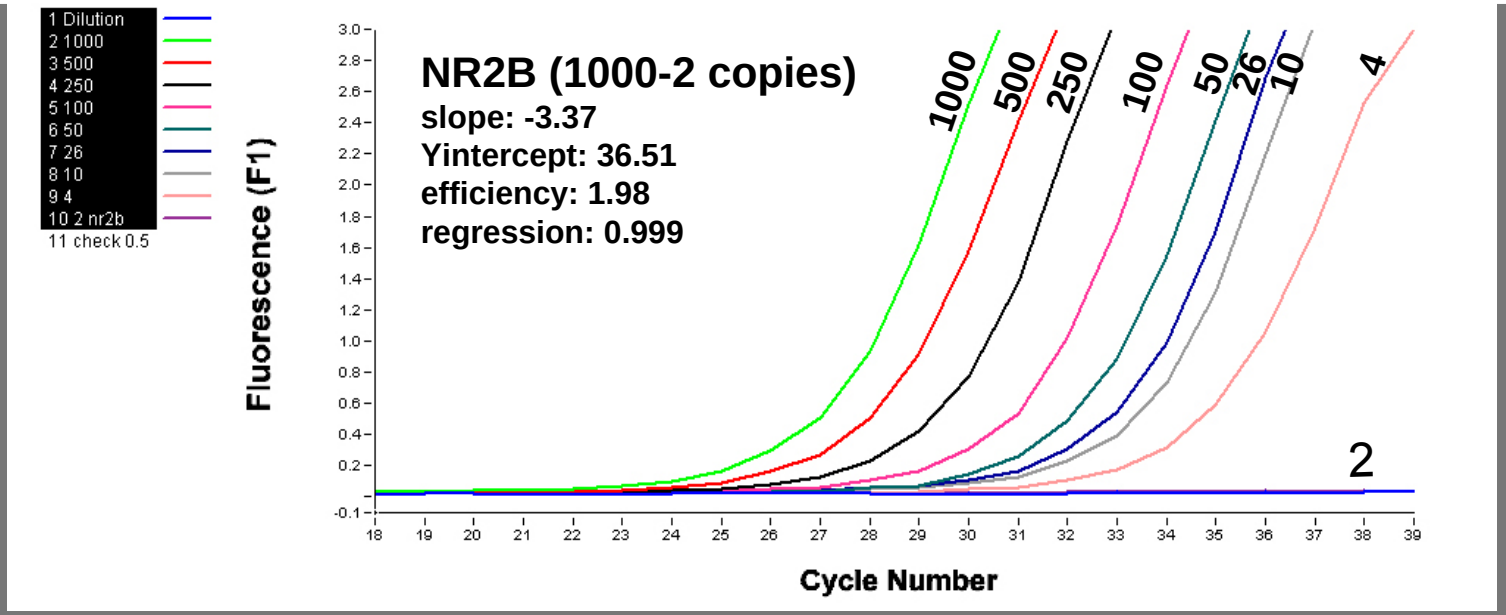
- amplicon-rescue (Qiagen, Machery-Nagl kits)
- OD_{260} (Gene Quant, Amersham-Pharmacia)
- calculation of the molecular weight (Nucweight algorithm)
- Dilution series: (1ds molecule = 2 ss copies)

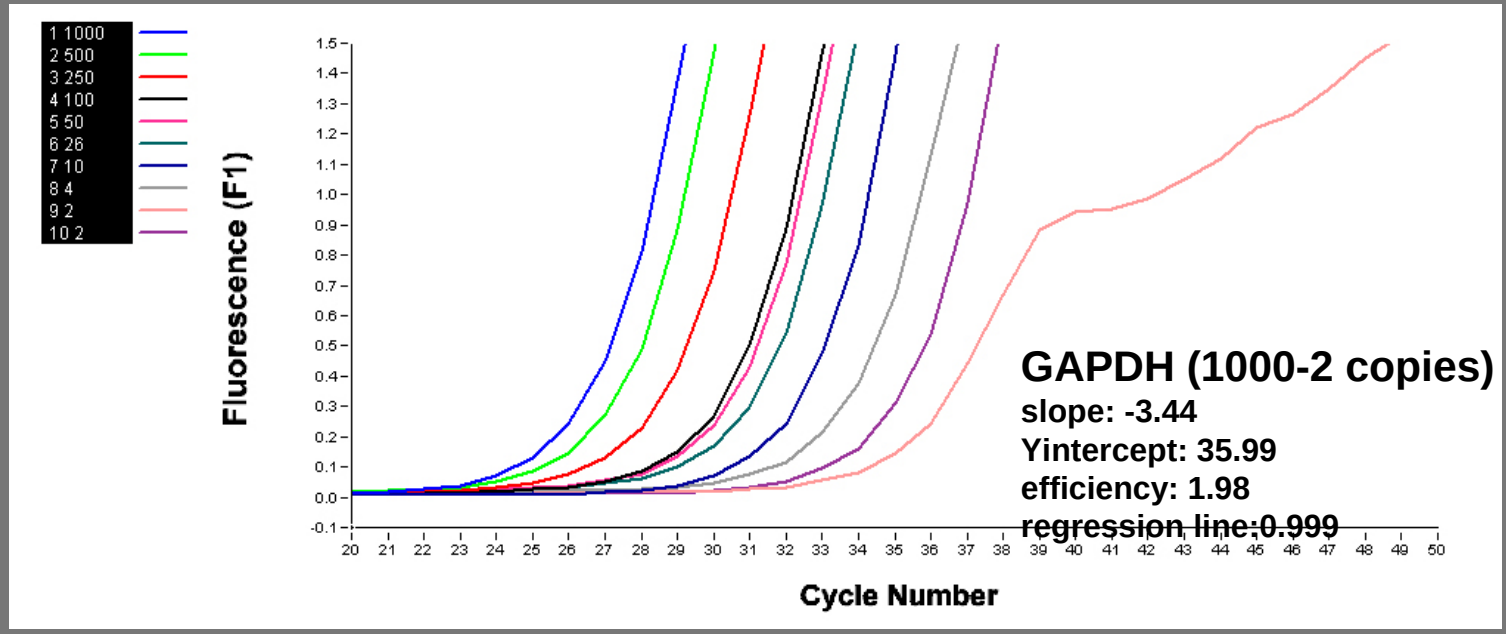
$10^{11} \rightarrow 10^{10} \rightarrow 10^{9} \rightarrow 10^{8} \rightarrow 10^{7} \rightarrow 10^{6} \rightarrow 10^{5} \rightarrow 10^{4} \rightarrow 10^{3}$

$\rightarrow 500 \rightarrow 250 \rightarrow 100 \rightarrow 50 \rightarrow 26 \rightarrow 10 \rightarrow 4 \rightarrow 2 \rightarrow 0.2$

PLEASE NOTE: 2 copies = 1 ds copy

High-resolution external standard curves





Quantification limit

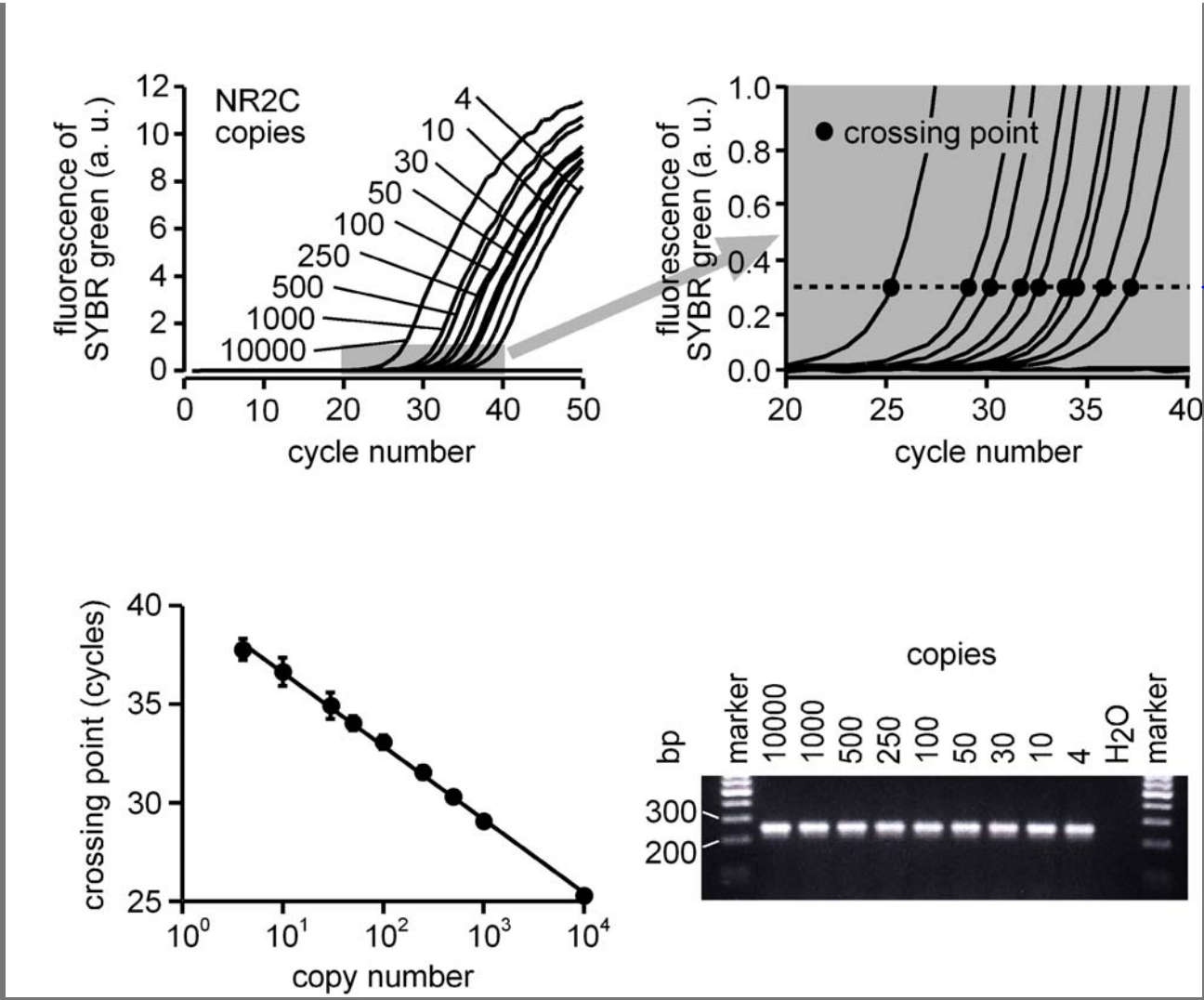
Optimization: QUANTIFICATION LIMIT

- 10 runs
- mean values form the external high resolution standard curve
- definition of the cycle-specific quantification limit

  90%: of all reactions positive for 10 ss copies

  50%: positive for 4 ss copies

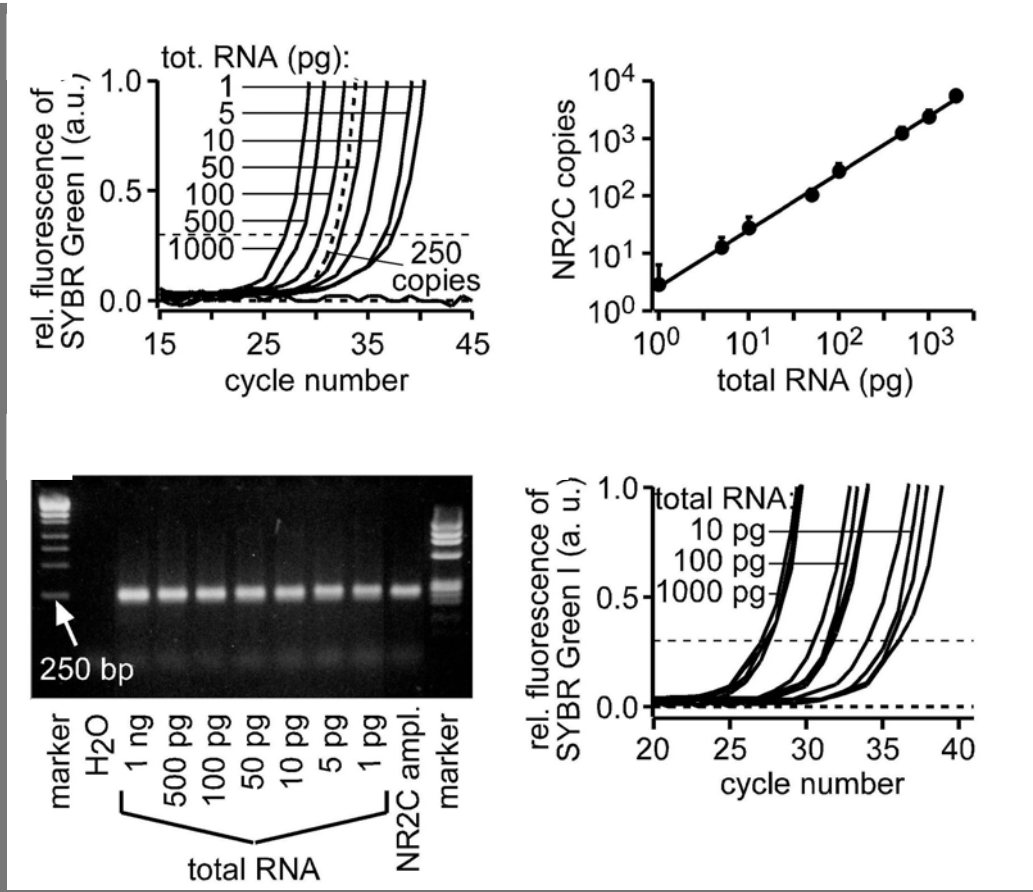
Construction of high-resolution external standard curves



mean slope: -3.72; eff=1.86

detection limit: 1 ds-copy; quantification limit: 4 ss-copies (=50% limit)

Quantification of RNA using external standard curves



- One copy NR2C corresponds to 500 fg brain RNA
- NR2C was detected in 25% of all experiments in 500 fg brain RNA
- Low RNA and higher RNA amounts show similar behavior in RT reactions
- The quantification of one molecule: the theoretical and practical limit of PCR

Conditions necessary for the quantification of low copy numbers

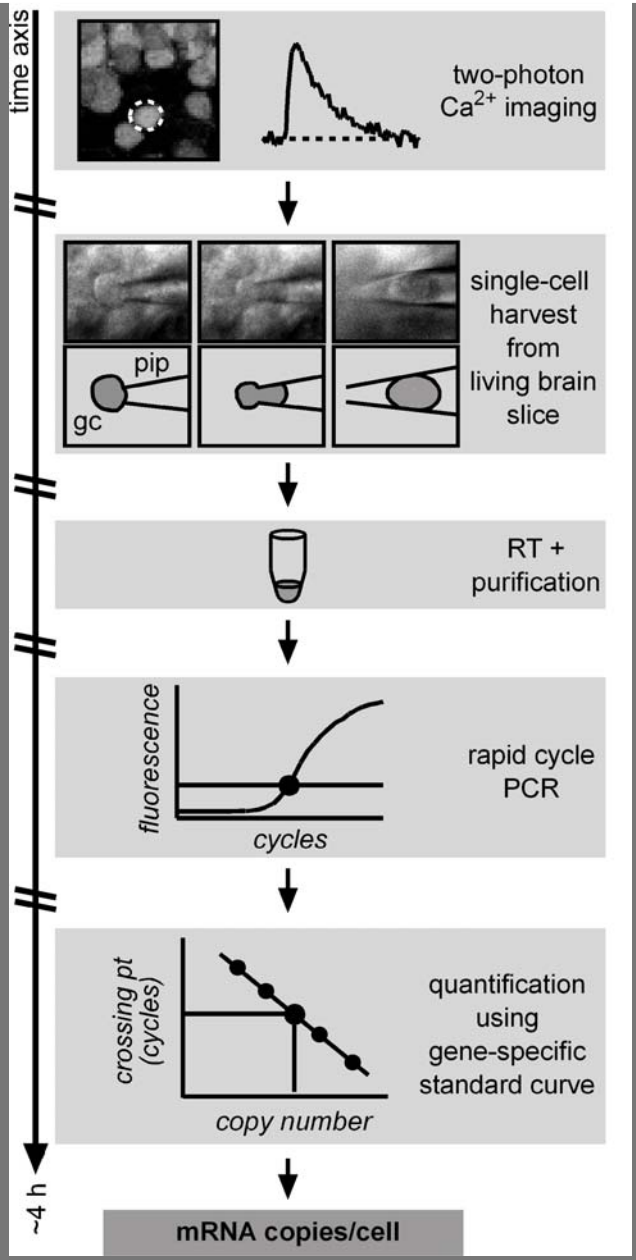
- primers, Primers, PRIMERS

  intron-spanning primers
  primer length: 17-21 bp
  3'-terminal dimer formation: <(-3.0) kcal/mol
  optimal annealing temperature: 58°C - 65°C
  amplicon length: 100bp - 250 bp
  product melting temperature: 85°C – 91°C
  G/C-content: 45-60%
  high internal stability at the 5'-end
  medium/low internal stability at the 3'-priming site
  minimal acceptable loop (hairpin formation) at the 3'-end: 0.0 kcal/mol
  maximum length of acceptable dimers (5'-end / internal region): 4 bp.

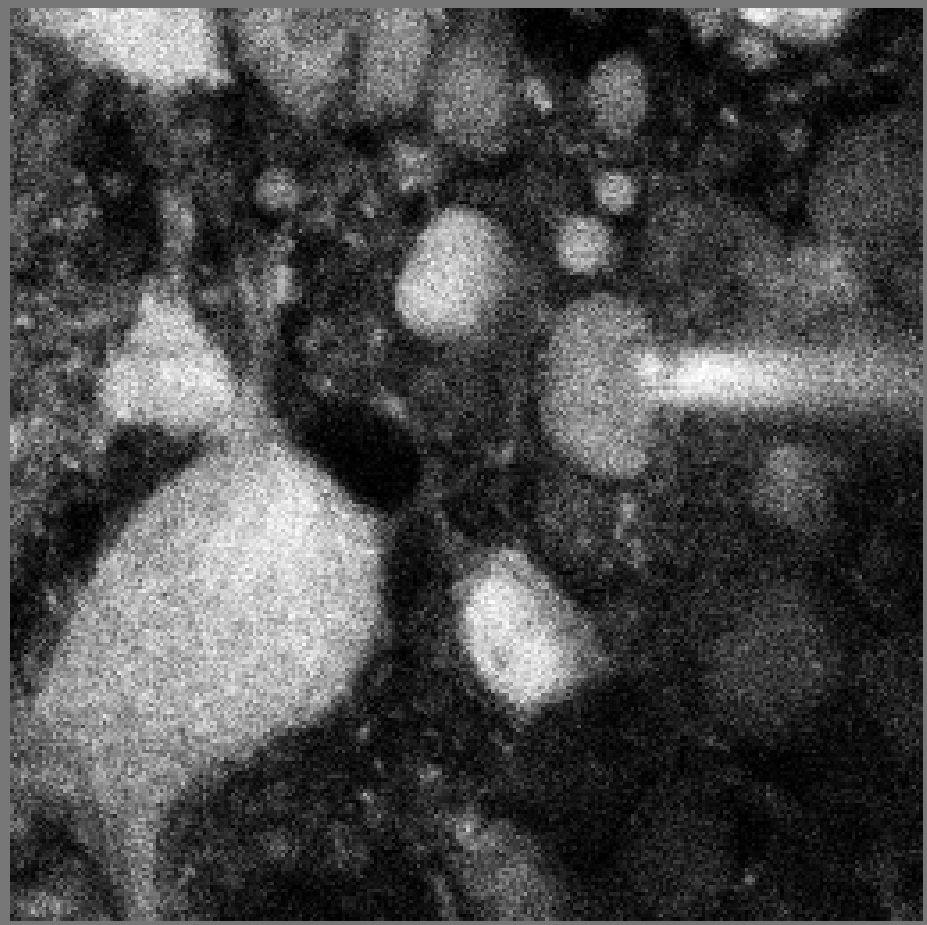
- cDNA purity
- Hot-start protocols (anti Taq-antibody)
- !!! USER !!!

Quantitative single-cell RT-PCR and calcium imaging
-Development of an approach-

Single-cell quantitative RT-PCR

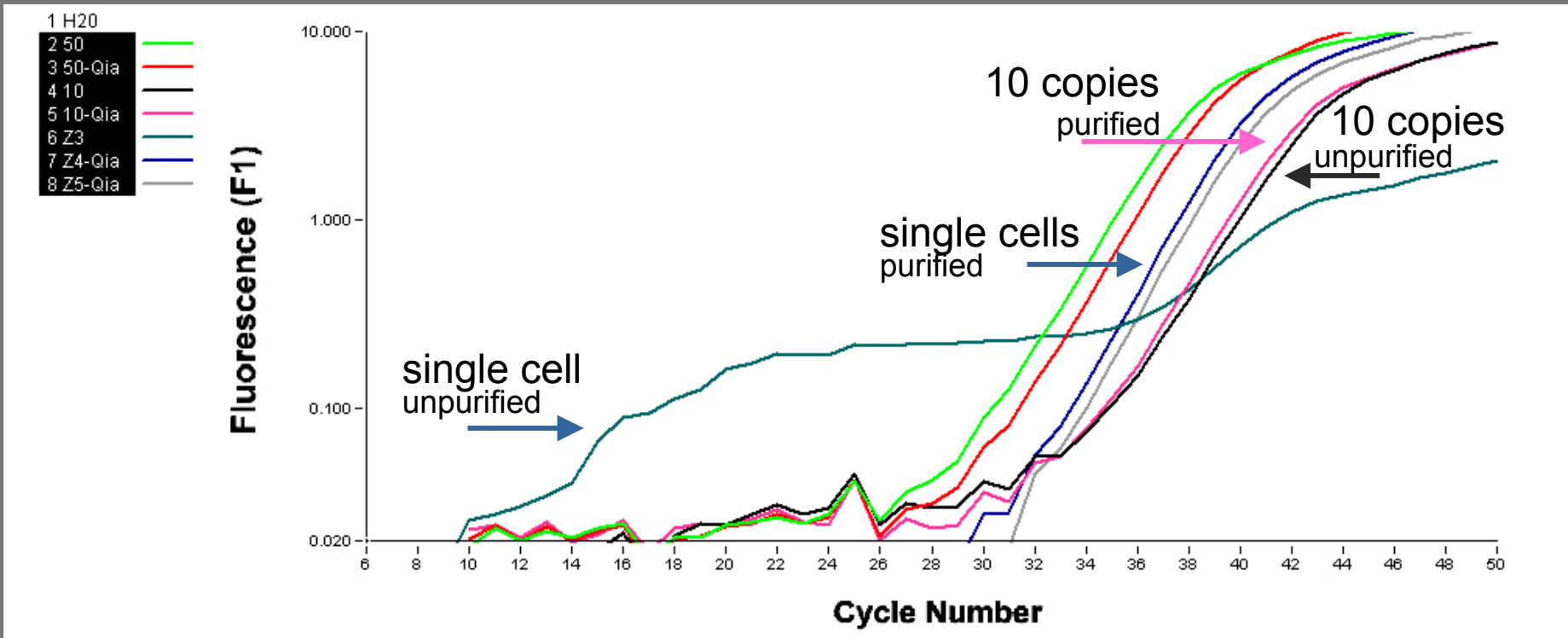


Rapid cell harvest from an acute brain slice



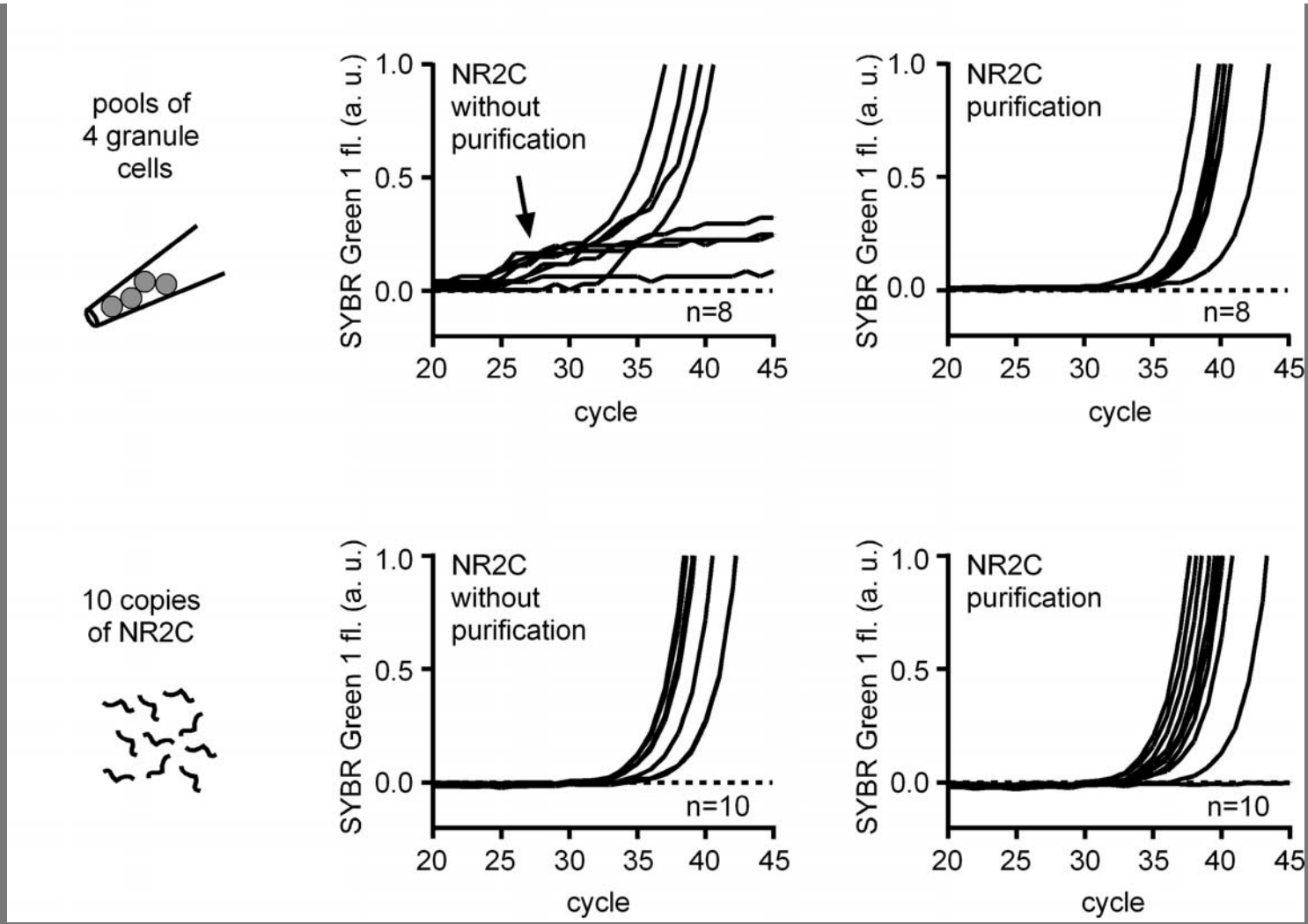
Rat cerebellum (P12); harvest of a granule cell, internal granule cell layer,
cell load: 10µm Fura PE3 AM, pipette: 100µm fluoresceine

Working with single cells: problem No. 1



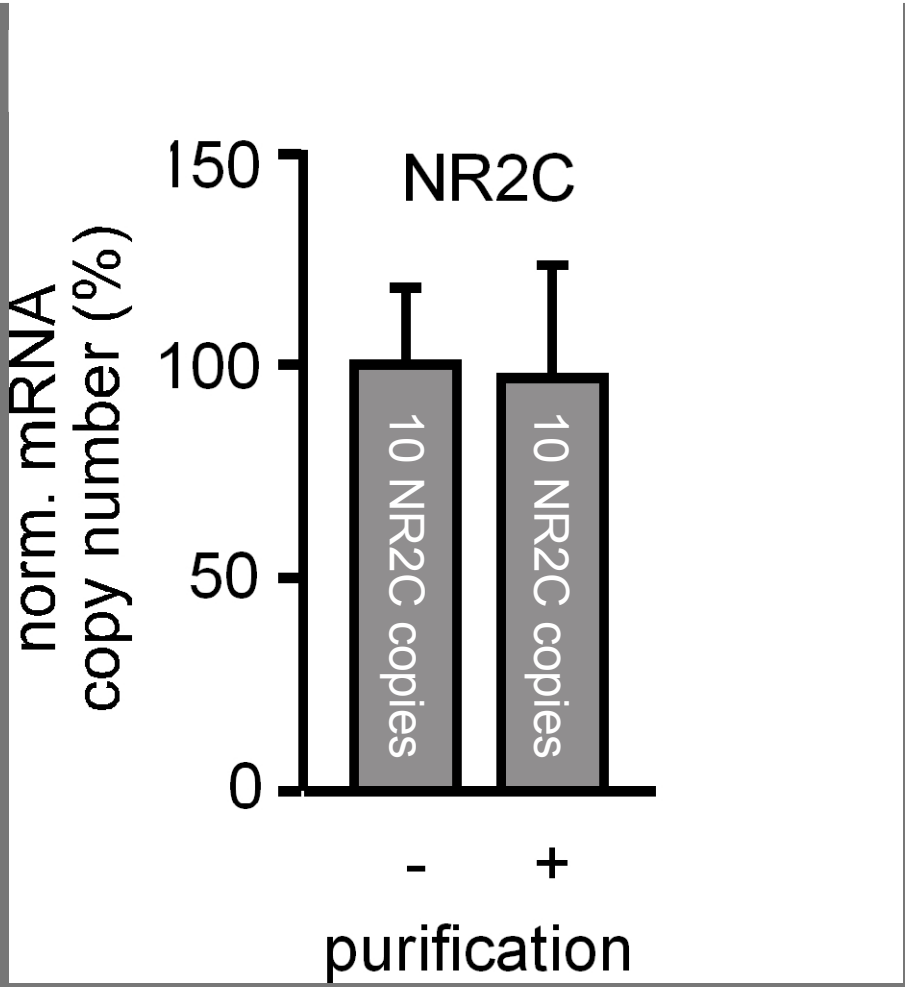
- single cell RT-material reduces the PCR effiency.
- Fast and easy: QiaExII DNA binding matrix-protocol.

Fast cDNA purification from single cell RT reactions



➲ cDNA purification using DNA binding matrix rescues the PCR kinetic.

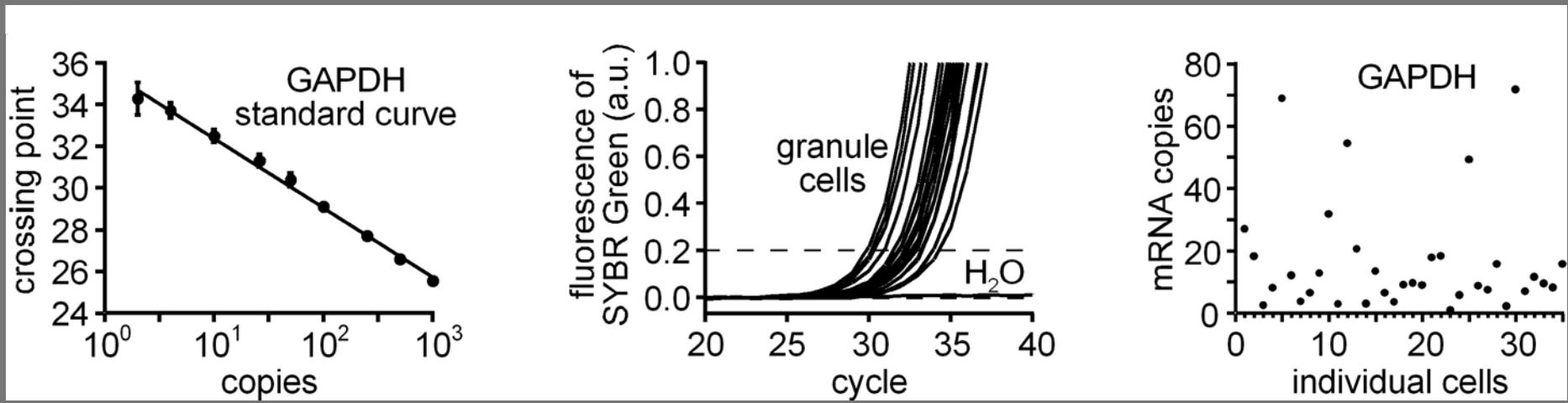
Fast and quantitative cDNA purification



➲ The cDNA purification procedure is `quantitative´.

Quantitative single cell RT-PCR of a housekeeping gene

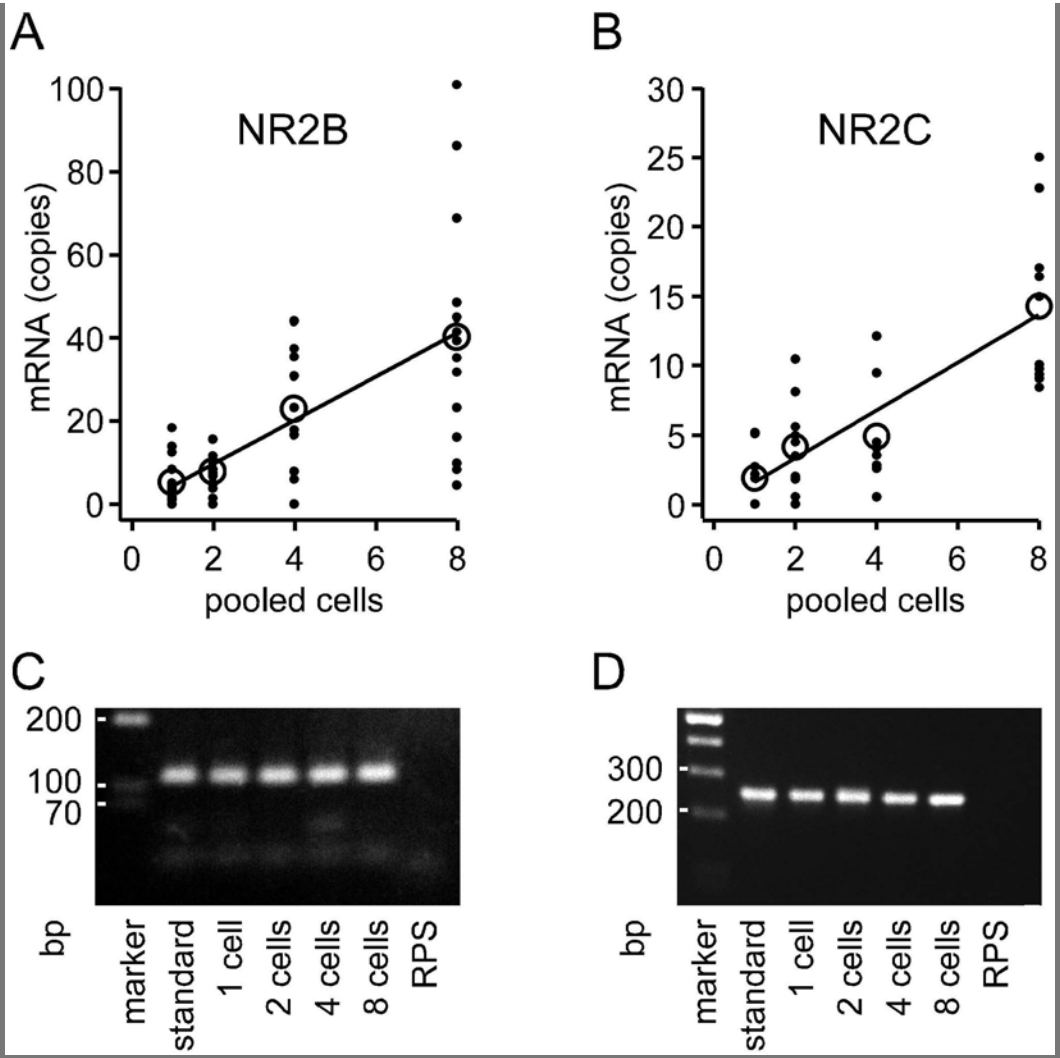
Y_i=35.99
ms=-3.44 (-3.32)
ql=4 ss copies
E=1.98



- GAPDH cannot be used as an internal standard (denominator problem).
- Copy numbers per cell (denominator) have to be determined.
- Success rate: 100%

Linearity of quantitative single-cell RT-PCR

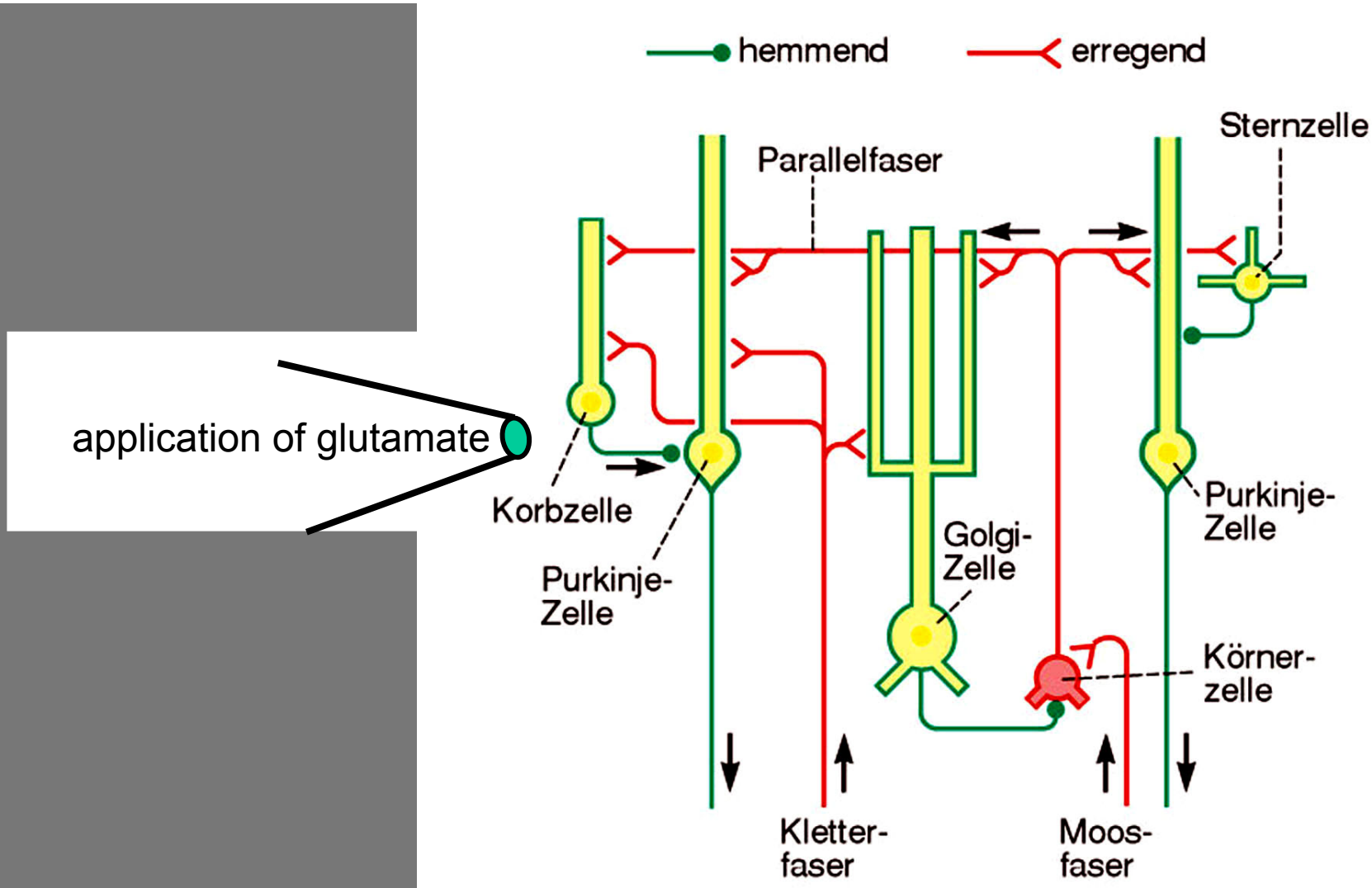
NR2B
r=0.672
P<0.0001
ms=5.27



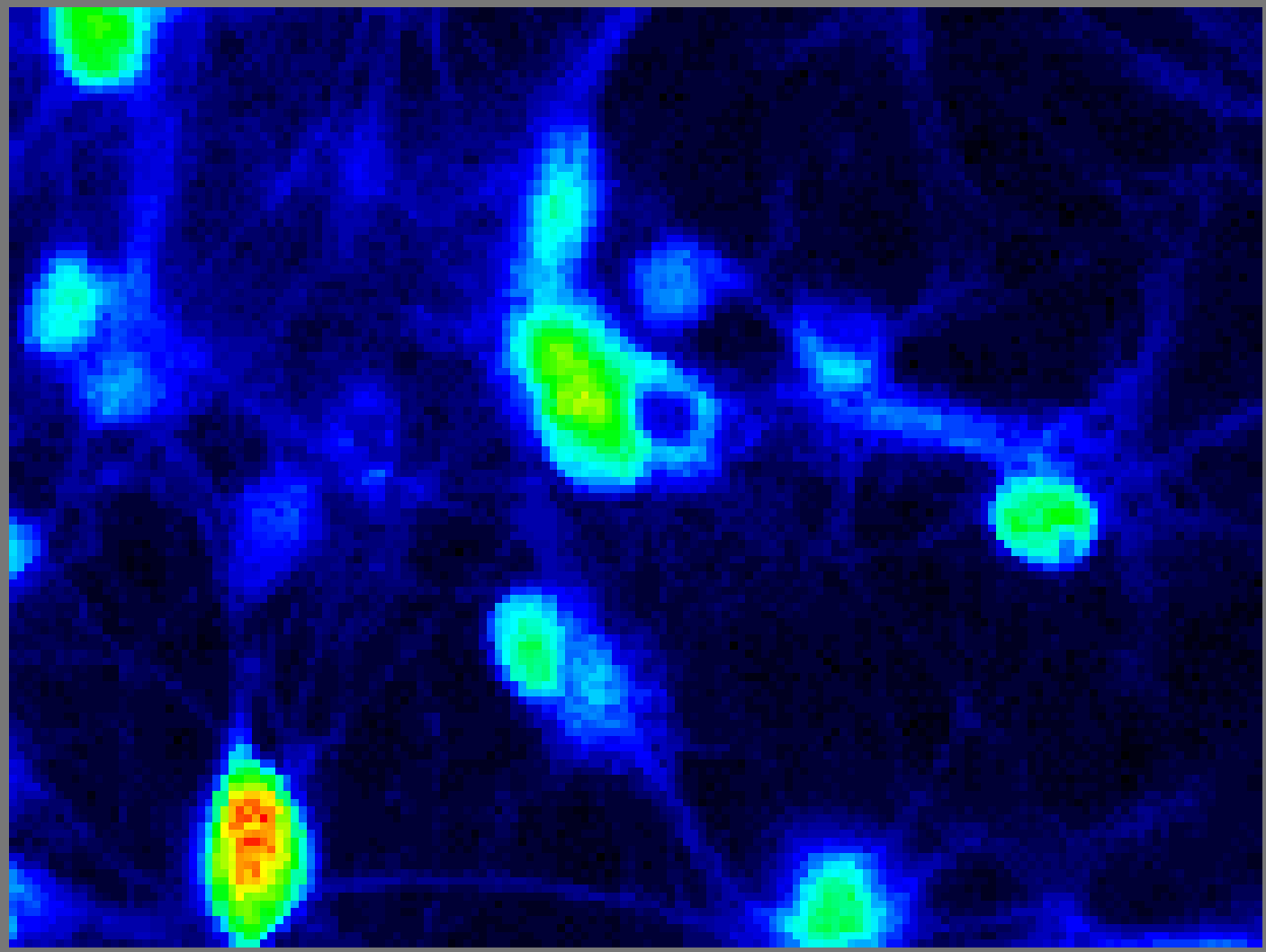
NR2C
r=0.761
P<0.0001
ms=1.72

NR2B: one cell=3.9±1.7 copies; eight cells 42.5±14.3 copies
NR2C: one cell=2.1±0.8 copies, eight cells=14.3±1.9 copies

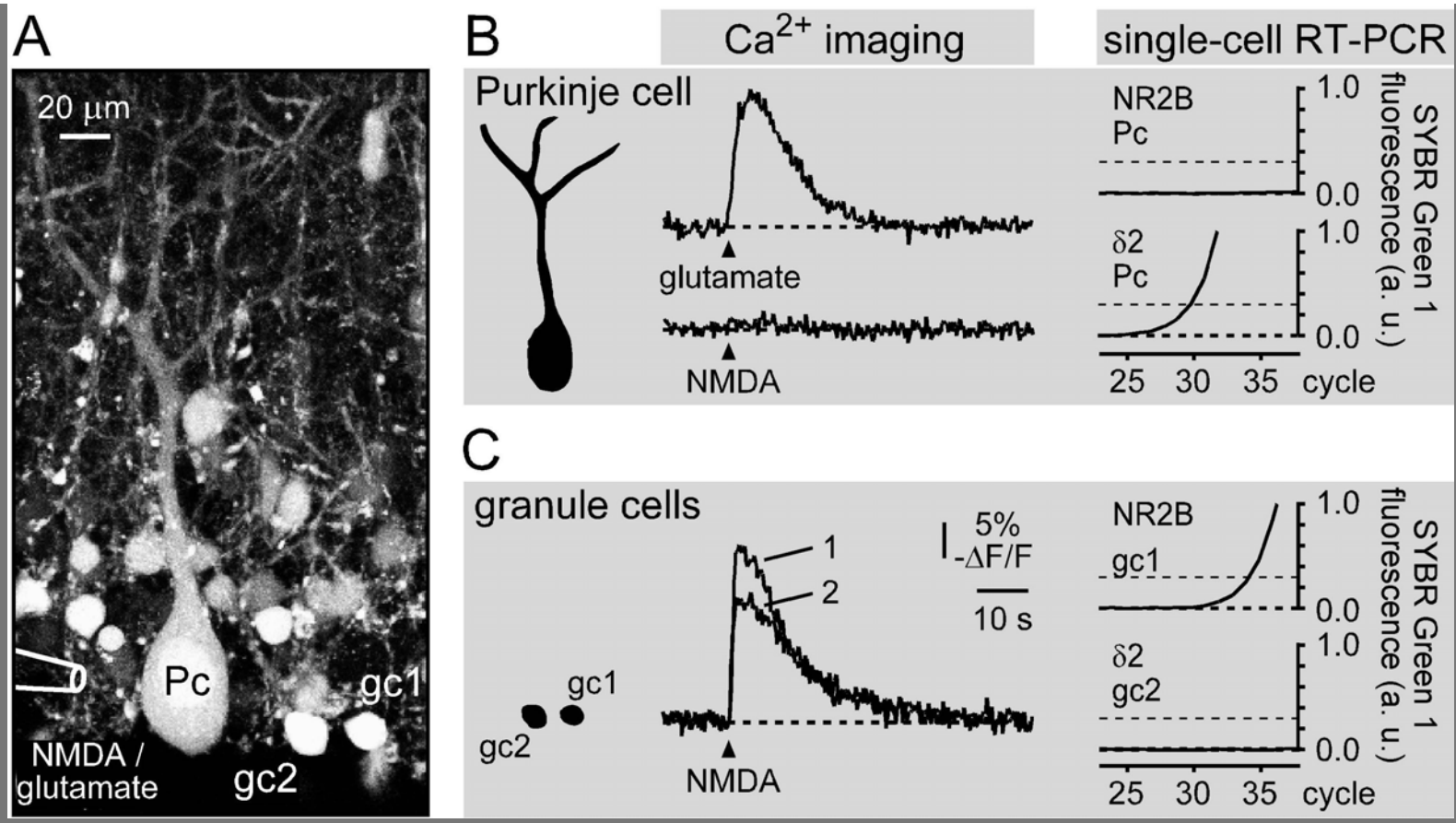
Calcium imaging in multicellular networks



Calcium imaging in multicellular networks

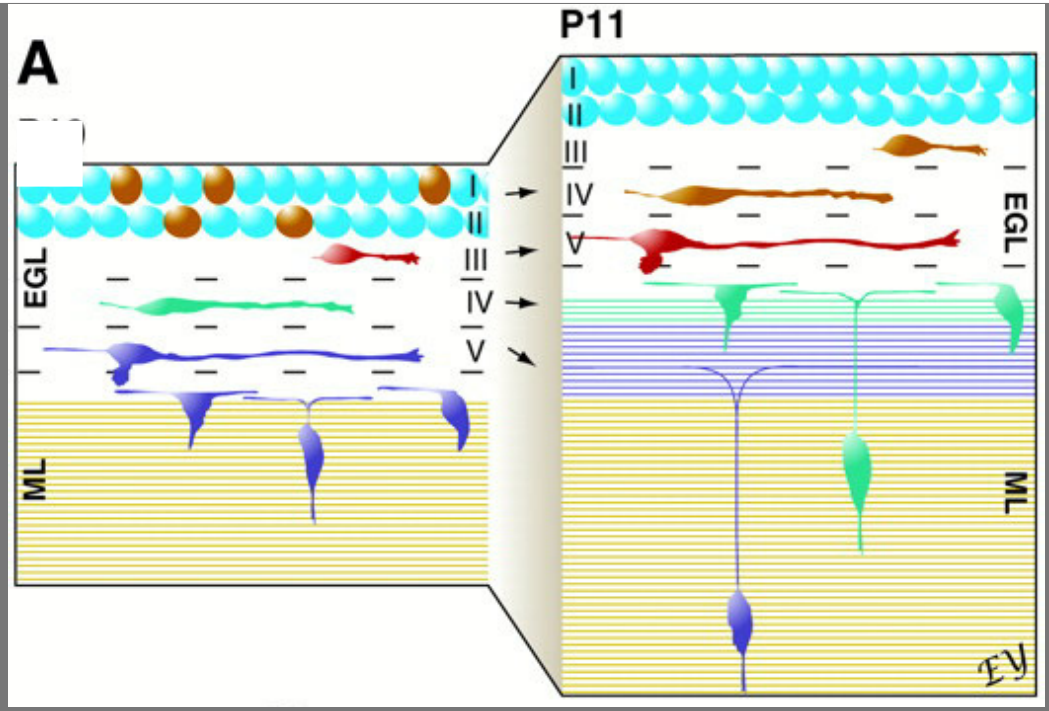


Quantitative single-cell RT-PCR and Ca^{2+} imaging in slices

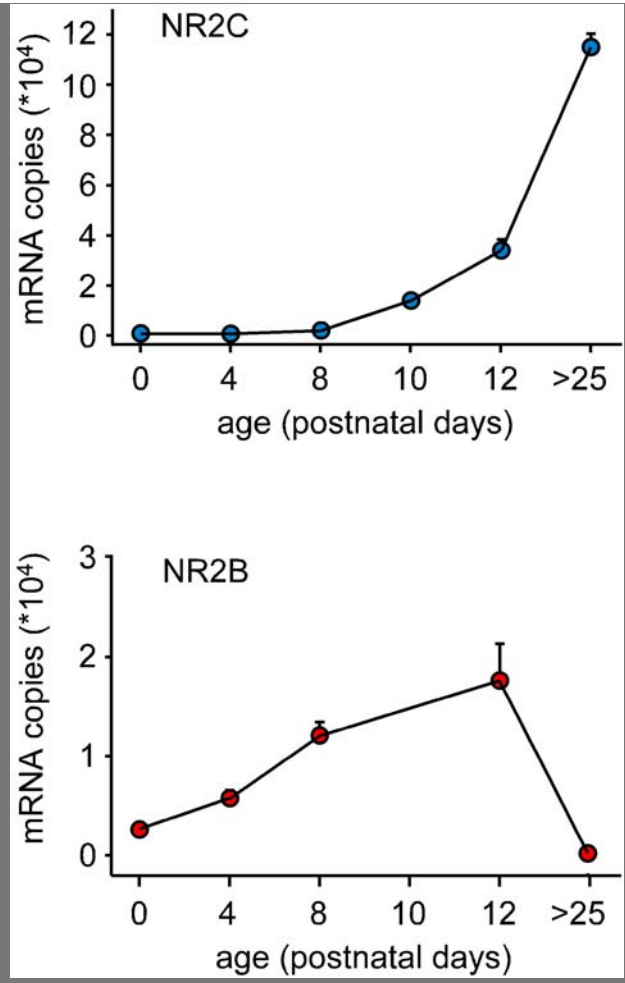


- Quantitative single cell RT-PCR can be combined with functional analysis.
- Functional imaging using fluorescent dyes does not interfere with real time PCR
- Molecule-function relation in multicellular networks

Developmental switch of NR2-subunit mRNA in granule cells

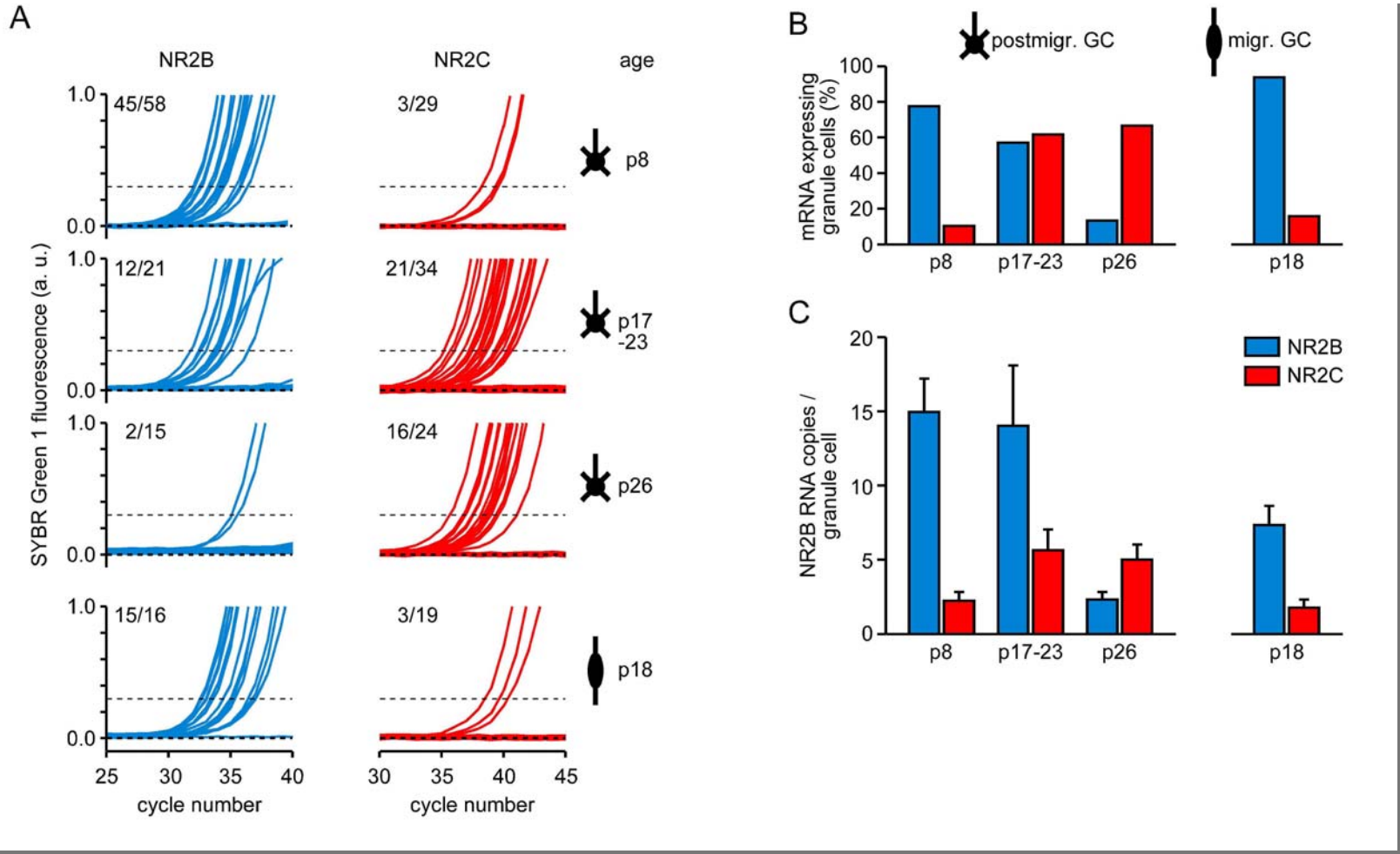


from: Komuro et al. (2001) J. Neurosci. 21, 527ff.



- NR2B subunit: early development
- NR2C subunit replaces NR2B in older animals (P12-P18)

Developmental switch of NR2-subunit mRNA in granule cells



- NR2 subunit switch: more cells express (a little bit more) NR2C.
- NR2B is down-regulated.
- NR2B is preferentially expressed in migrating granule cells.

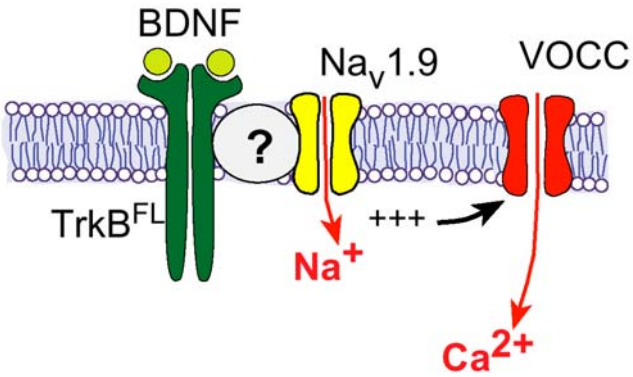
Finally, for example:

1) The advantage of a defined quantification limit:
   - bad primers can be used!

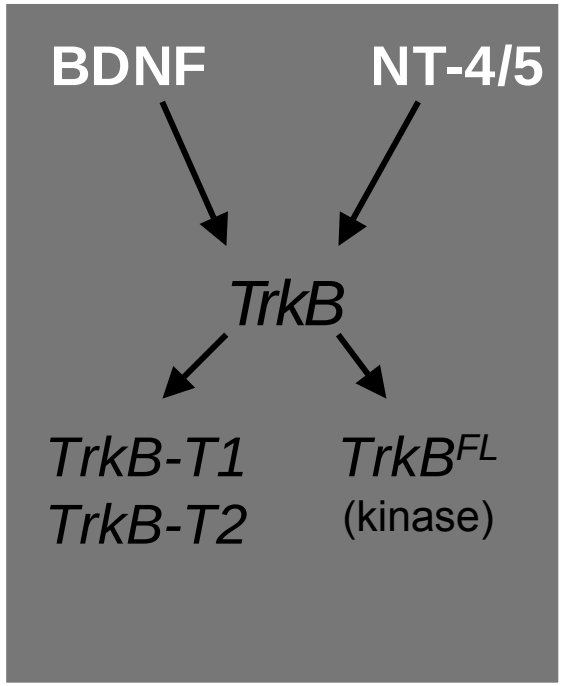
2) An expression level-dependent phenotype

Which TrkB receptor is expressed in glia cells

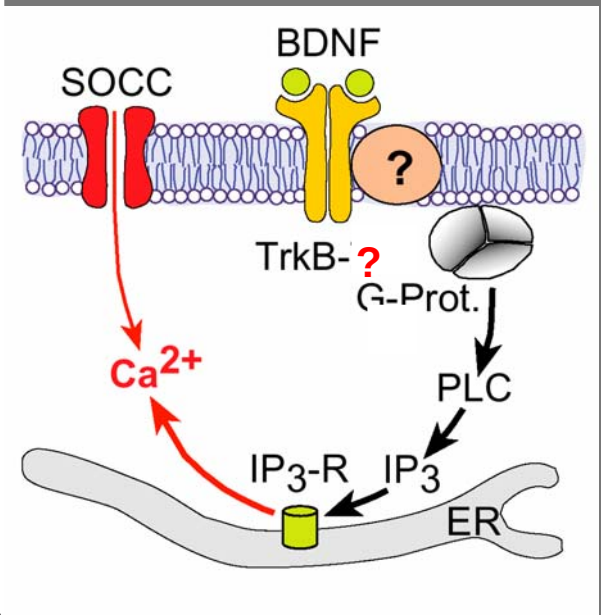
TrkB kinase !



Blum et al. (2002) Nature 419



TrkB ????

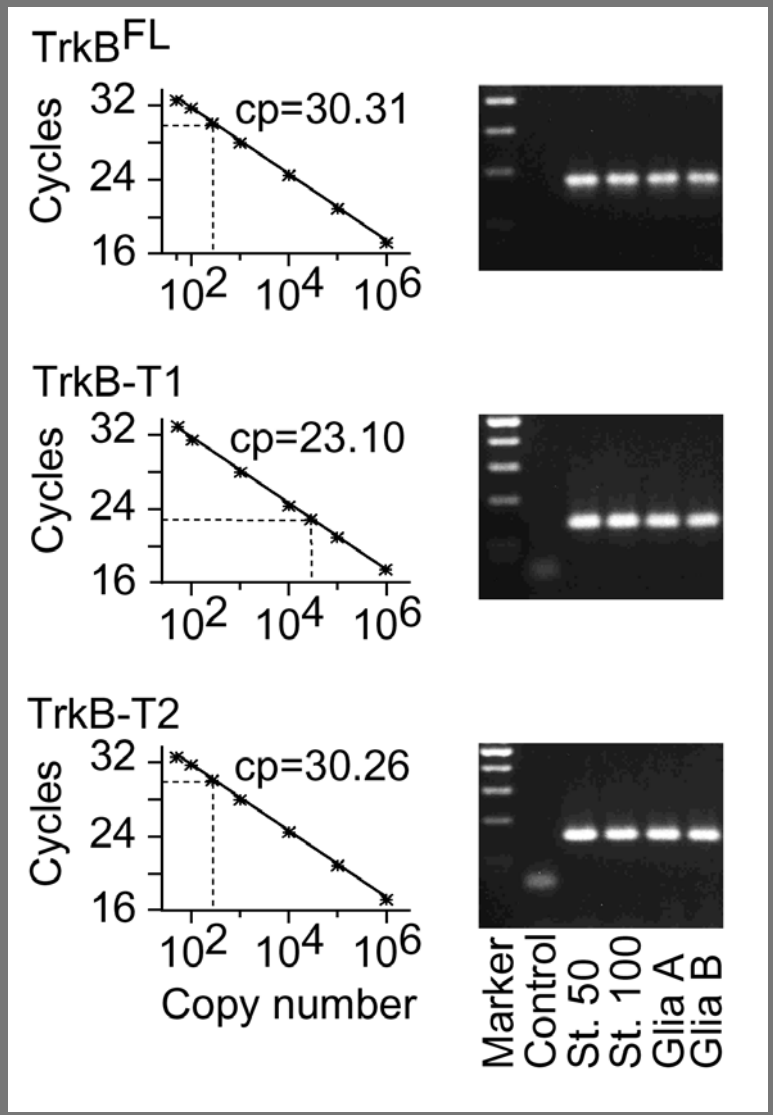


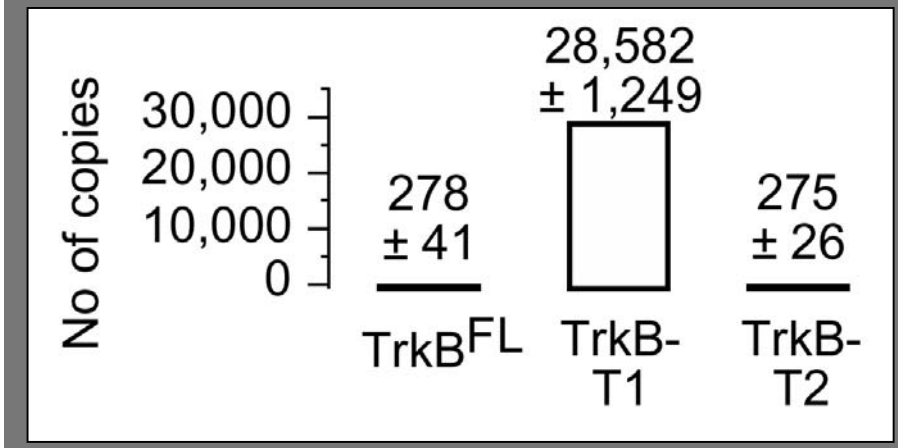
Rose et al. (2003) Nature 426

Problems:

- no TrkB-kinase or TrkB-T2 specific antibody available.
- TrkB-T2 protein has never been detected in natural tissues.
- TrkB-T1 and TrkB-T2 differ in only 11 amino acids at the C-terminus.

Quantitative RT-PCR of TrkB receptors



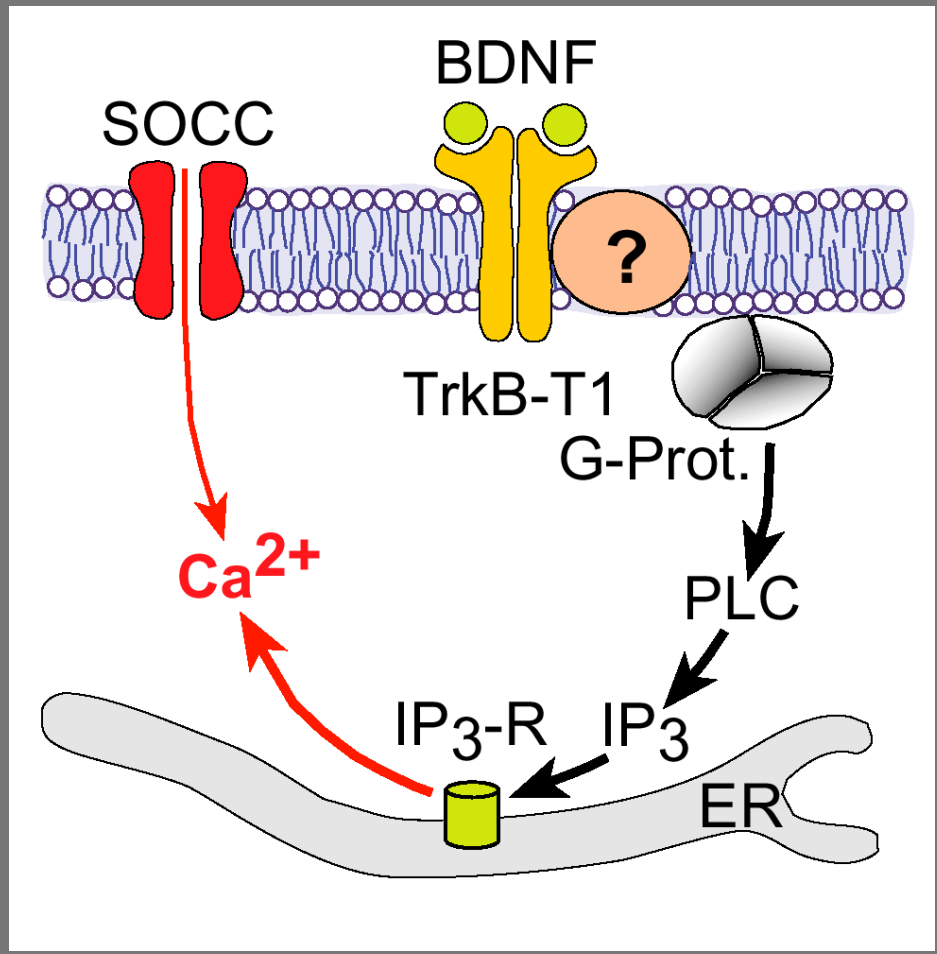


Quantification limit for TrkB-T2: 50 copies

minimal amount of RNA: >2.4 ng glia RNA

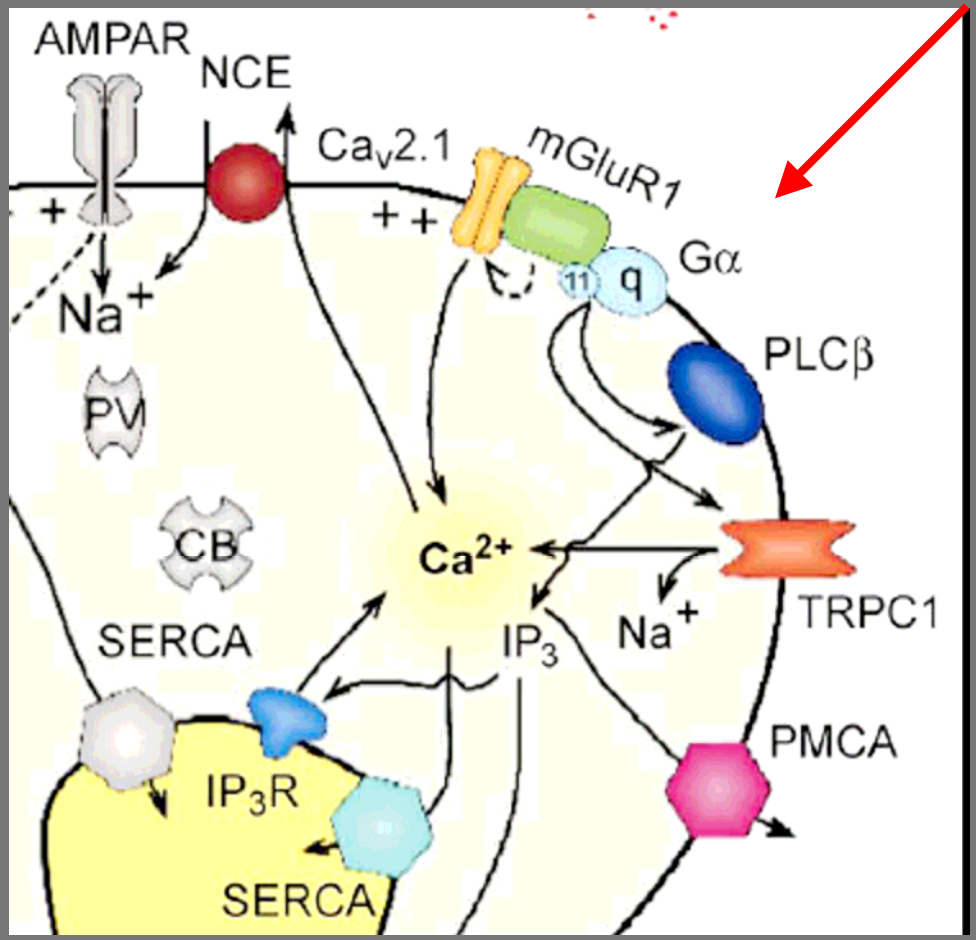
Used amount: 2.4 ng **x 5** = 13ng total RNA

Truncated TrkB-T1 mediates neurotrophin-evoked calcium signalling in glia cells



Rose, Blum, Pichler, Lepier, Kafitz & Konnerth (2003) Nature 426

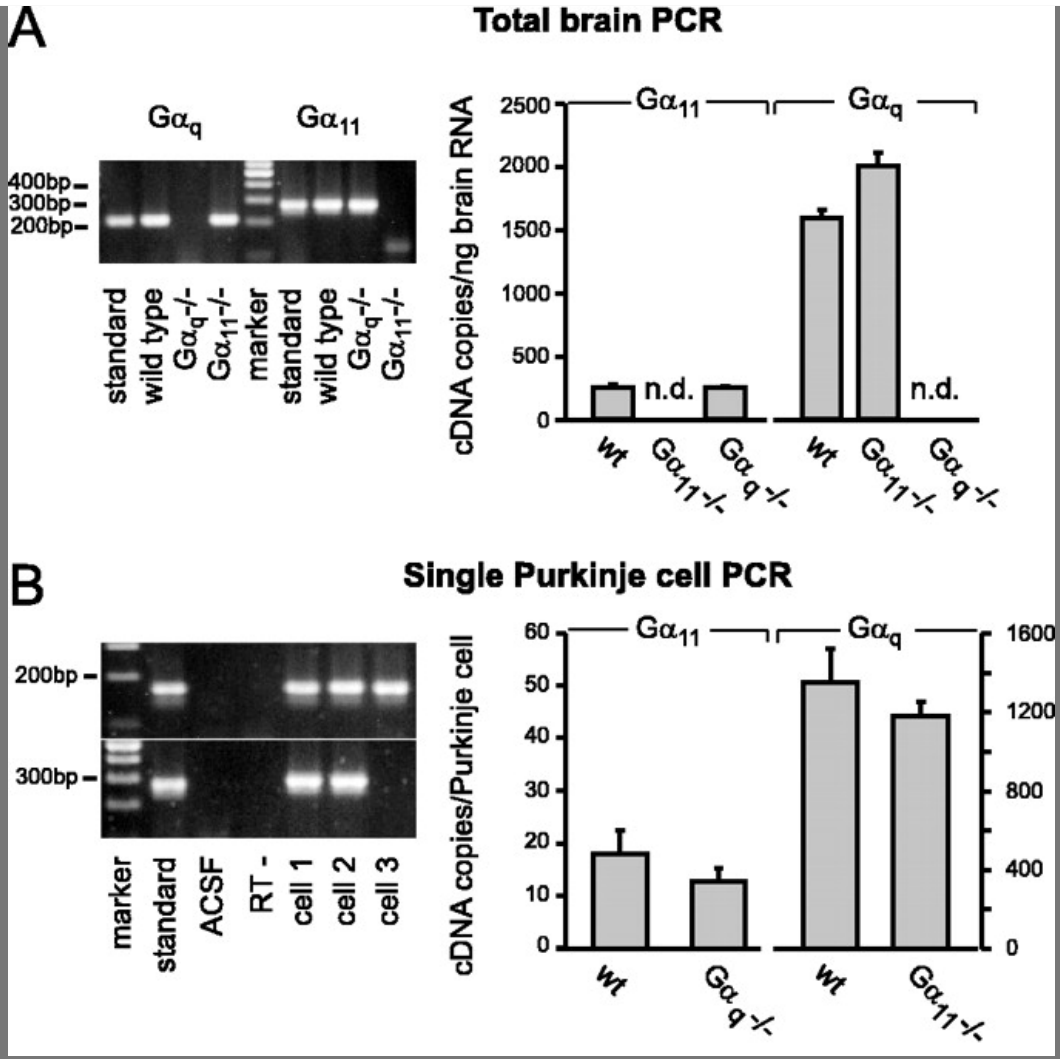
Distinct roles of $G\alpha_q$ and $G\alpha_{11}$ for Purkinje cell signalling ?



Hartmann & Konnerth (2005) *Cell calcium* 37

➲ Offermanns et al. (1997) Gαq ko-motor deficits, Gα11 ko: not

Single Purkinje cells express >100-fold more $G\alpha_q$ than $G\alpha_{11}$



but:
They express about
15 copies $G\alpha_{11}$

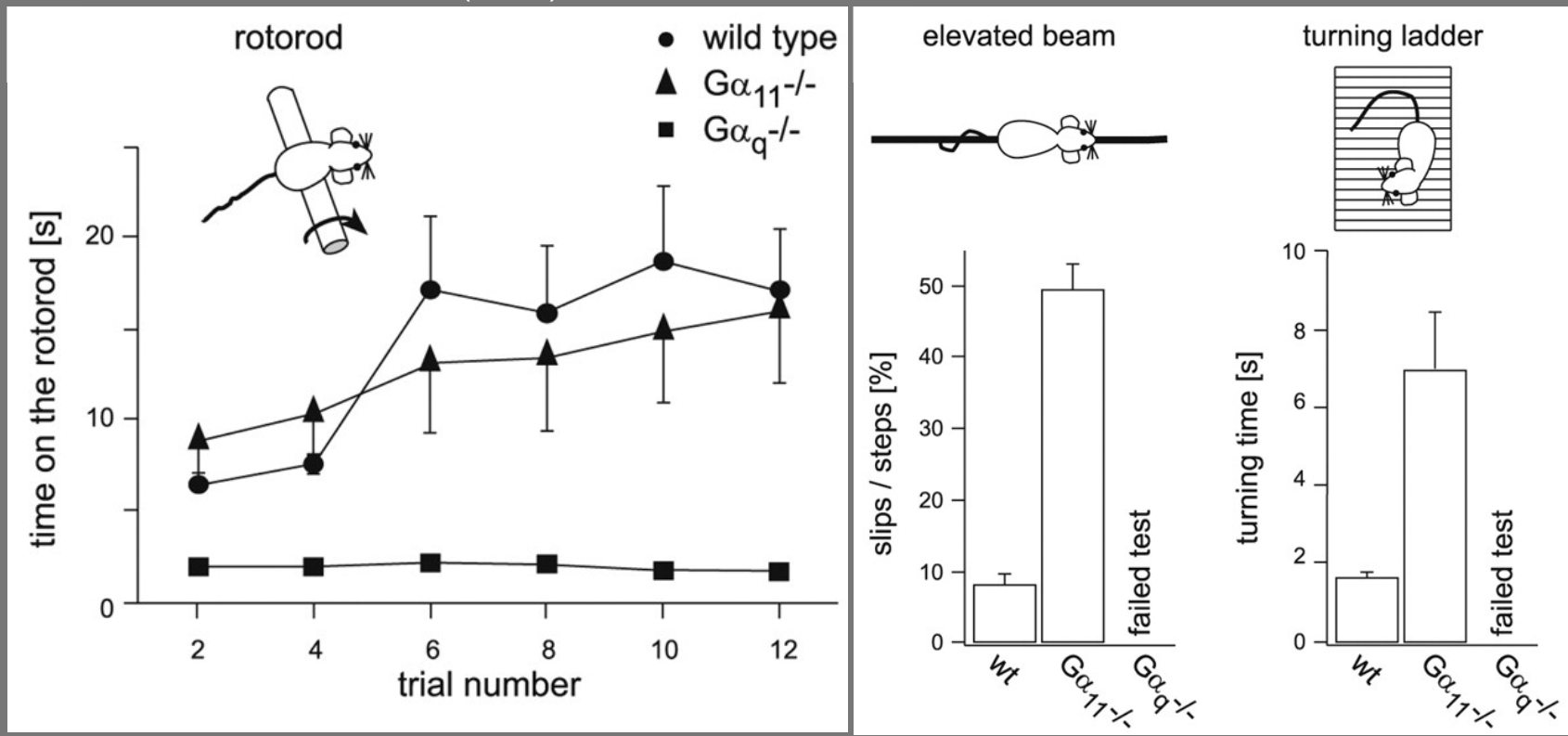
Question:

Is there a hidden phenotype in $G\alpha_{11}$ KO mice?

Hartmann, Blum, Kovalchuk, Adelsberger, Kuner, Durand, Miyata, Kano, Offermanns, Konnerth (2004) *J. Neurosci.* 24

Distinct roles of $G\alpha_q$ and $G\alpha_{11}$ for Purkinje cell signalling

Hartmann, Blum, Kovalchuk, Adelsberger, Kuner, Durand, Miyata, Kano, Offermanns, Konnerth (2004) *J. Neurosci.* 24



➲ Low expression rate, no phenotype, no function ?

No, here: expression-level dependent phenotype!

SUMMARY

Single-cell quantitative rapid cycle real-time PCR:

is possible ☺.

Institut für Physiologie

Robert Blum

Guylaine Durand

Nima Marandi

Simone Herberger

Arthur Konnerth

Quantitative single-cell RT-PCR and Ca^{2+} imaging in brain slices

Guylaine M. Durand[1], Nima Marandi[1], Simone D. Herberger,
Robert Blum[*] & Arthur Konnerth

European Journal of Physiology, Pflügers Archive (in press)

Optimization of real-time RT- PCR

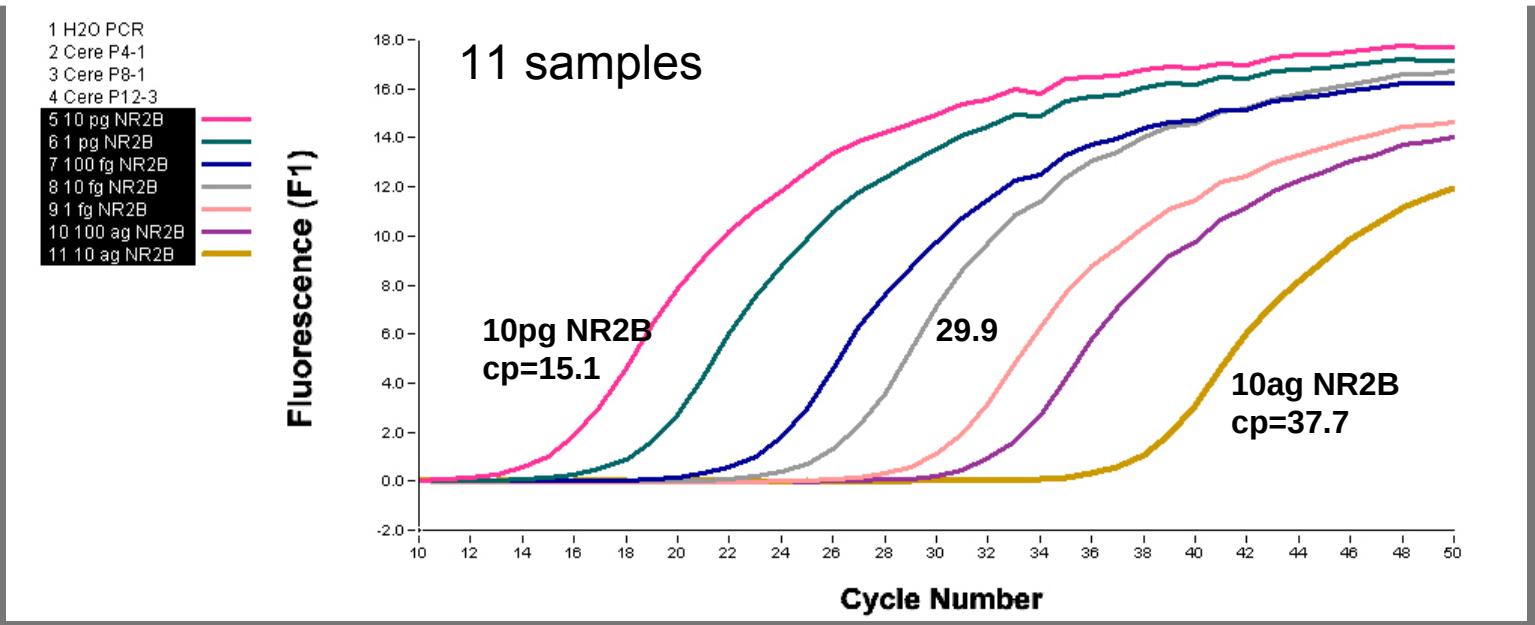
For very small amounts of cDNA

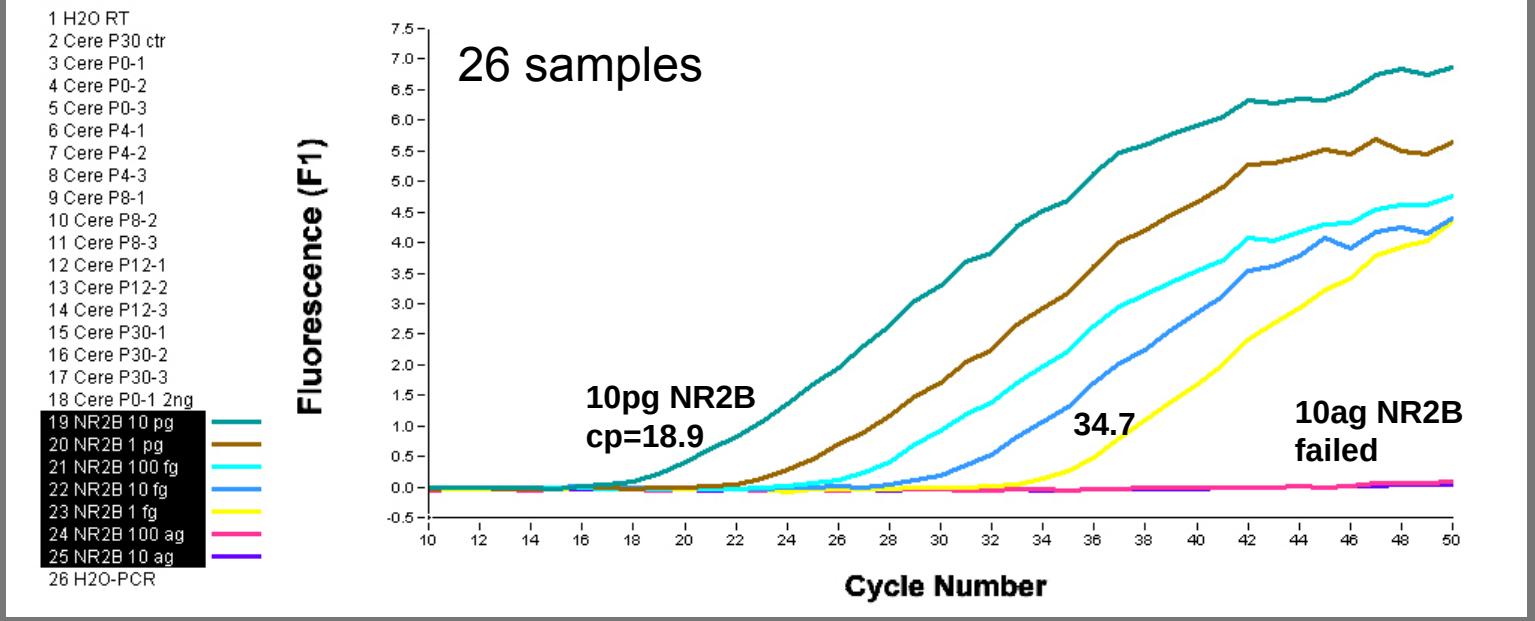
- not more than 12 samples per Lightcycler run.

Before the next PCR run

- cool the thermal chamber of the LC to 21–23°C.

Optimization of real-time RT- PCR



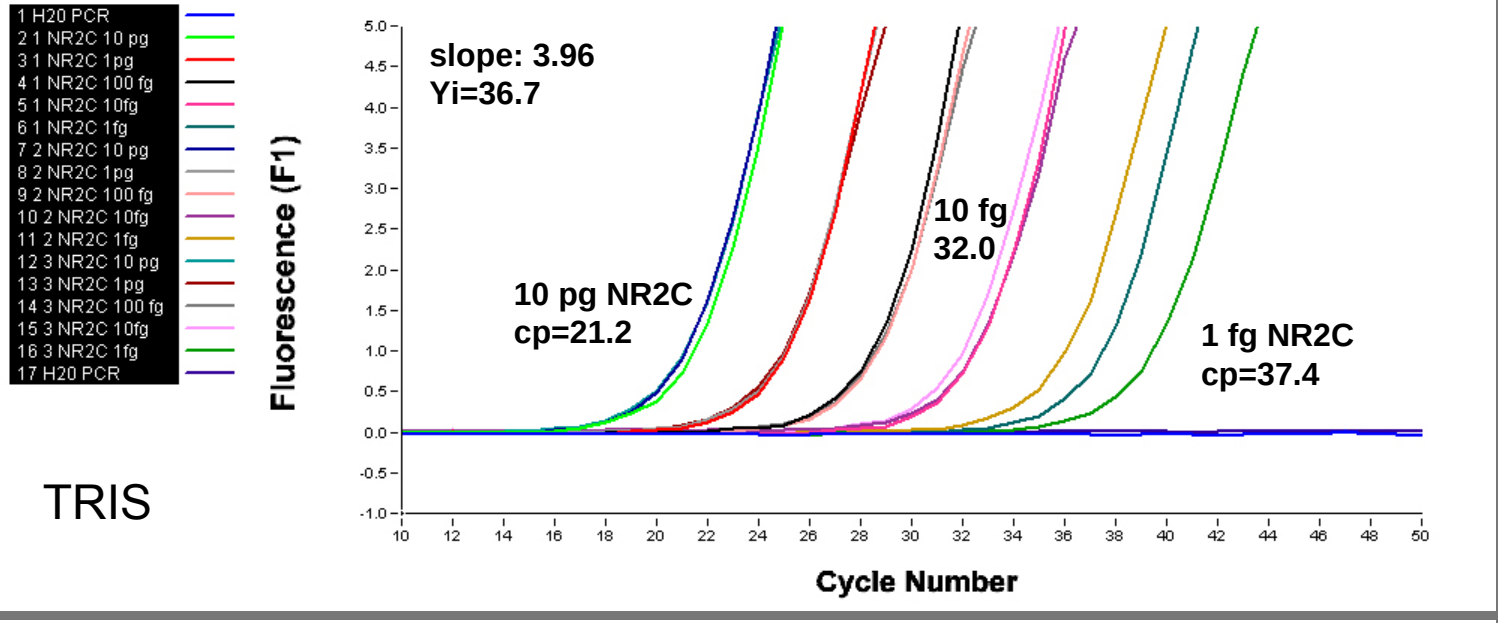


Optimization of real-time RT- PCR

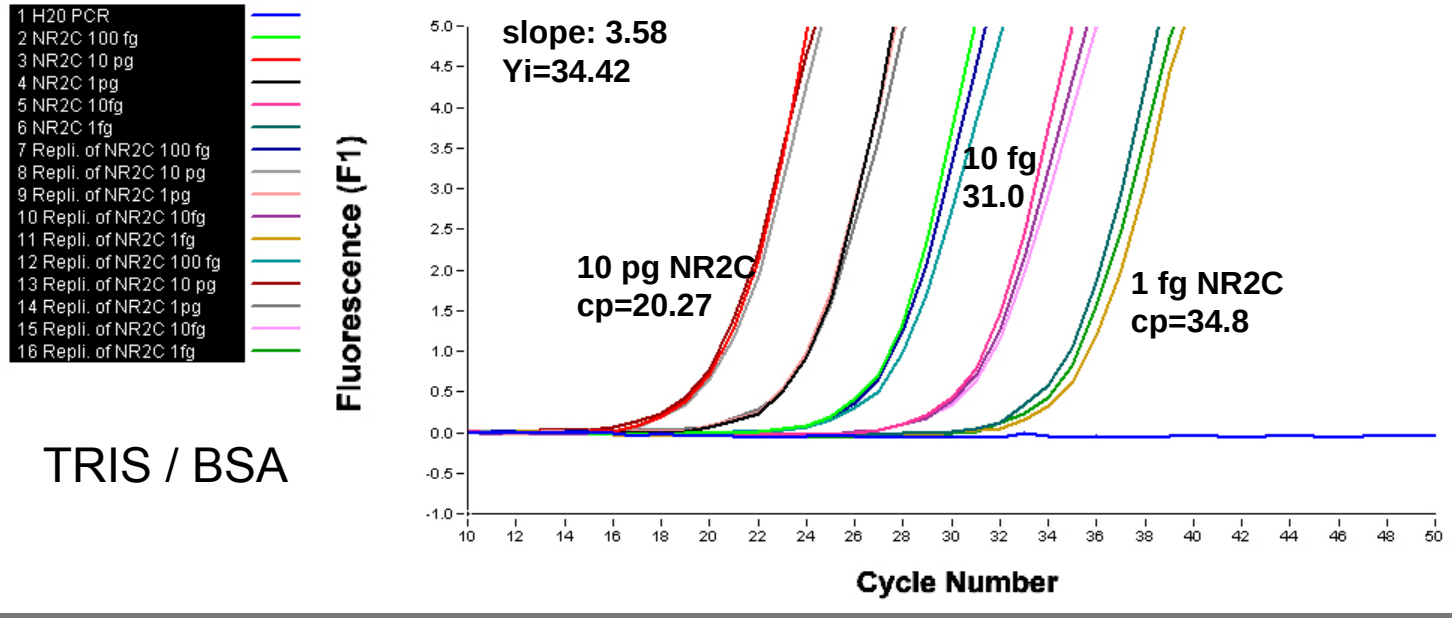
cDNA standard preparation

- volume: 200-400 µl
- buffer: 10mM Tris, pH 8.5, 1mg/ml purified BSA
- precoating of the tubes
- TRIS-BSA >>TRIS > H_2O

Optimization of real-time RT- PCR



TRIS



TRIS / BSA