Supplement S1

The following pages include preparation sheets for conducting GTPase assays with 1, 2, or 3 sample rows.

Assay number: Name: Date:
Incubation:

	5 µL 2xGTP solution	5 µL protein	10 µL Glo solution	20 µL Det. reagent
A				
B				
C				
D				
E				
F				
G				
H				
I				
J				
K				
L				
M				
N				
O				
P				

Assay preparation/ protein dilutions:

2x GTP solution:			Glo solution:				Det. Reag.:
500 µL	1000 µL		500 µL	750 µL	1000 µL		
494 µL	989 µL	protein buffer	496 µL	745 µL	993 µL	Glo buffer	
5 µL	10 µL	DTT	2.5 µL	3.75 µL	5 µL	**1mM** ADP (2µL **10mM** ADP + 18µL mQ)	
0.5 µL	1 µL	GTP	1 µL	1.5 µL	2 µL	Glo-reagent	

Assay number: Name: Date:
Incubation:

	5µL 2xGTP	5µL protein	10µL Glo	20µL Det reag		5µL 2xGTP	5µL protein	10µL Glo	20µL Det reag
A					A				
B					B				
C					C				
D					D				
E					E				
F					F				
G					G				
H					H				
I					I				
J					J				
K					K				
L					L				
M					M				
N					N				
O					O				
P					P				

Assay preparation/ protein dilutions:

2x GTP solution:			Glo solution:				Det. Reag.:
500 µL	1000 µL		500 µL	750 µL	1000 µL		
494 µL	989 µL	protein buffer	496 µL	745 µL	993 µL	Glo buffer	
5 µL	10 µL	DTT	2.5 µL	3.75 µL	5 µL	**1mM** ADP (2µL **10mM** ADP + 18µL mQ)	
0.5 µL	1 µL	GTP	1 µL	1.5 µL	2 µL	Glo-reagent	

Assay number: Name: Date:
Incubation:

	5	5	10	20		5	5	10	20		5	5	10	20
A					A					A				
B					B					B				
C					C					C				
D					D					D				
E					E					E				
F					F					F				
G					G					G				
H					H					H				
I					I					I				
J					J					J				
K					K					K				
L					L					L				
M					M					M				
N					N					N				
O					O					O				
P					P					P				

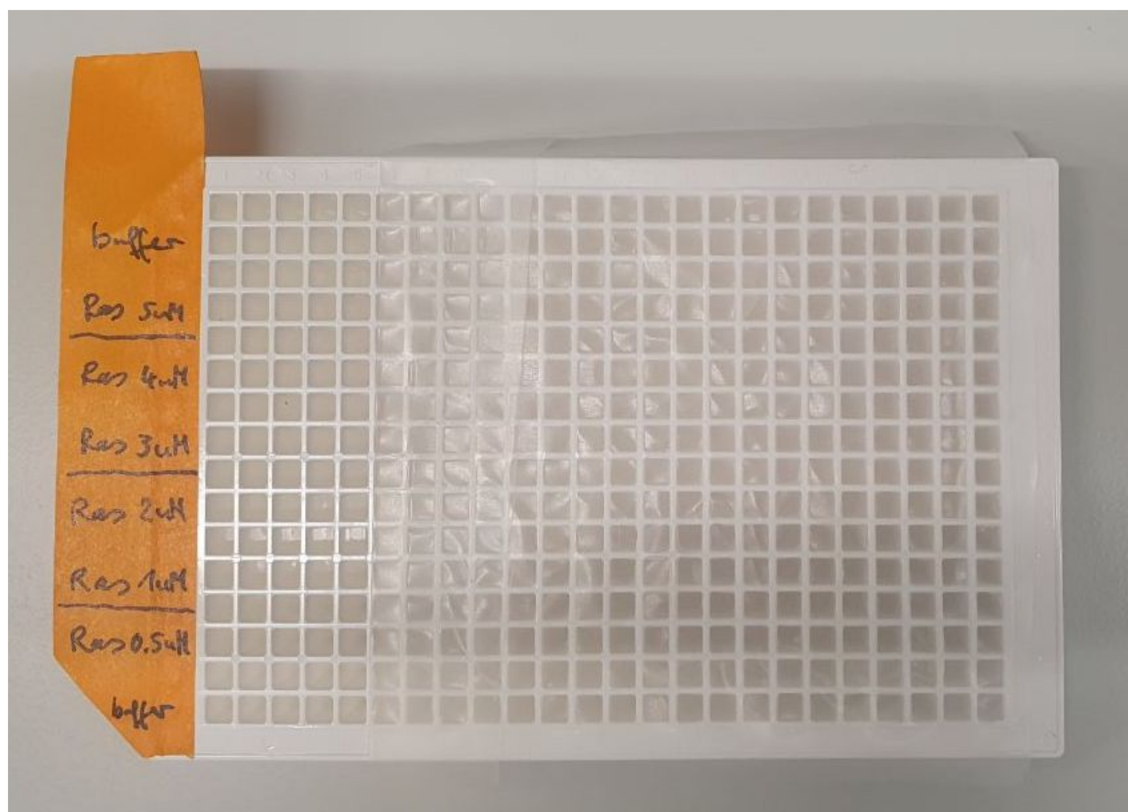
Assay preparation/ protein dilutions:

2x GTP solution:			Glo solution:				Det. Reag.:
500 µL	1000 µL		500 µL	750 µL	1000 µL		
494 µL	989 µL	protein buffer	496 µL	745 µL	993 µL	Glo buffer	
5 µL	10 µL	DTT	2.5 µL	3.75 µL	5 µL	**1mM** ADP (2µL **10mM** ADP + 18µL mQ)	
0.5 µL	1 µL	GTP	1 µL	1.5 µL	2 µL	Glo-reagent	

Supplement S2

This supplement shows a prepared plate and preparatory sheet for a GTPase assay of 6 Ras GTPase serial dilutions (example 1) (see basic protocol).

It leads to data that can be found in 'example1.xlsx', tab: 'E1', 'E2'.



S2 Figure 1. Preparation of a plate for a GTPase assay containing 6 Ras GTPase serial dilutions (example 1).

Assay number: 1 | Name: ST | Date: 2023-03-30

Incubation: 94min 30°C

Wells 1-5	5 µL 2xGTP solution	5 µL protein	10 µL Glo solution	20 µL Det. reagent
A				
B buffer				
C				
D Ras 5µM				
E				
F Ras 4µM				
G				
H Ras 3µM				
I				
J Ras 2µM				
K				
L Ras 1µM				
M				
N Ras 0.5µM				
O				
P buffer				

Assay preparation/ protein dilutions:

Ras stock ~160µM

Serial dilutions:

- per dilution: 5 x 5µL + 5µL = 30µL
- dilutions:

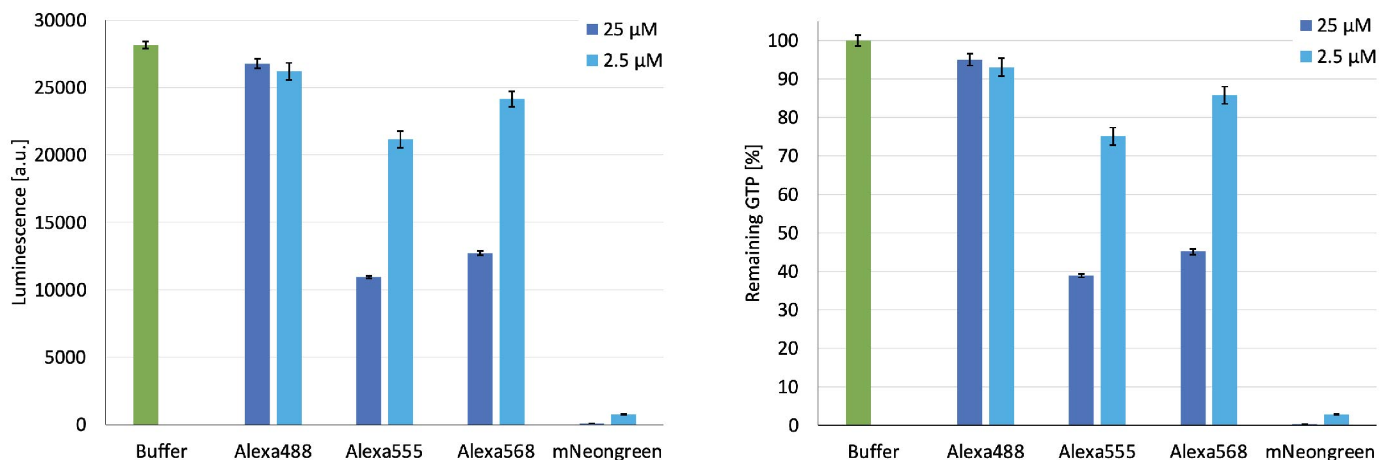
	2x5µM = 10µM	2x4µM = 8µM	2x3µM =6µM	2x2µM =4µM	2x1µM =2µM	2x0.5µM =1µM
Ras [µL]	6.5 (of 160µM)	68 (of 10µM)	51 (of 8µM)	36 (of 6µM)	23 (of 4µM)	15 (of 2µM)
Protein buffer [µL]	97.5	17	17	18	23	15
Total vol. [µL]	104-68 = 36	85-51 = 34	68-36 = 32	54-23 =31	46-15 = 31	30

2x GTP solution:	8x5x5µL=200µL		Glo solution:	400µL			Det. Reag.: 800µL Prepare 1mL
500 µL	1000 µL		500 µL	750 µL	1000 µL		
494 µL	989 µL	protein buffer	496 µL	745 µL	993 µL	Glo buffer	
5 µL	10 µL	DTT	2.5 µL	3.75 µL	5 µL	**1mM** ADP (2µL **10mM** ADP + 18µL mQ)	
0.5 µL	1 µL	GTP	1 µL	1.5 µL	2 µL	Glo-reagent	

S2 Figure 2. Preparation of a GTPase assay of 6 Ras GTPase serial dilutions (example 1) (made using templates provided in S1).

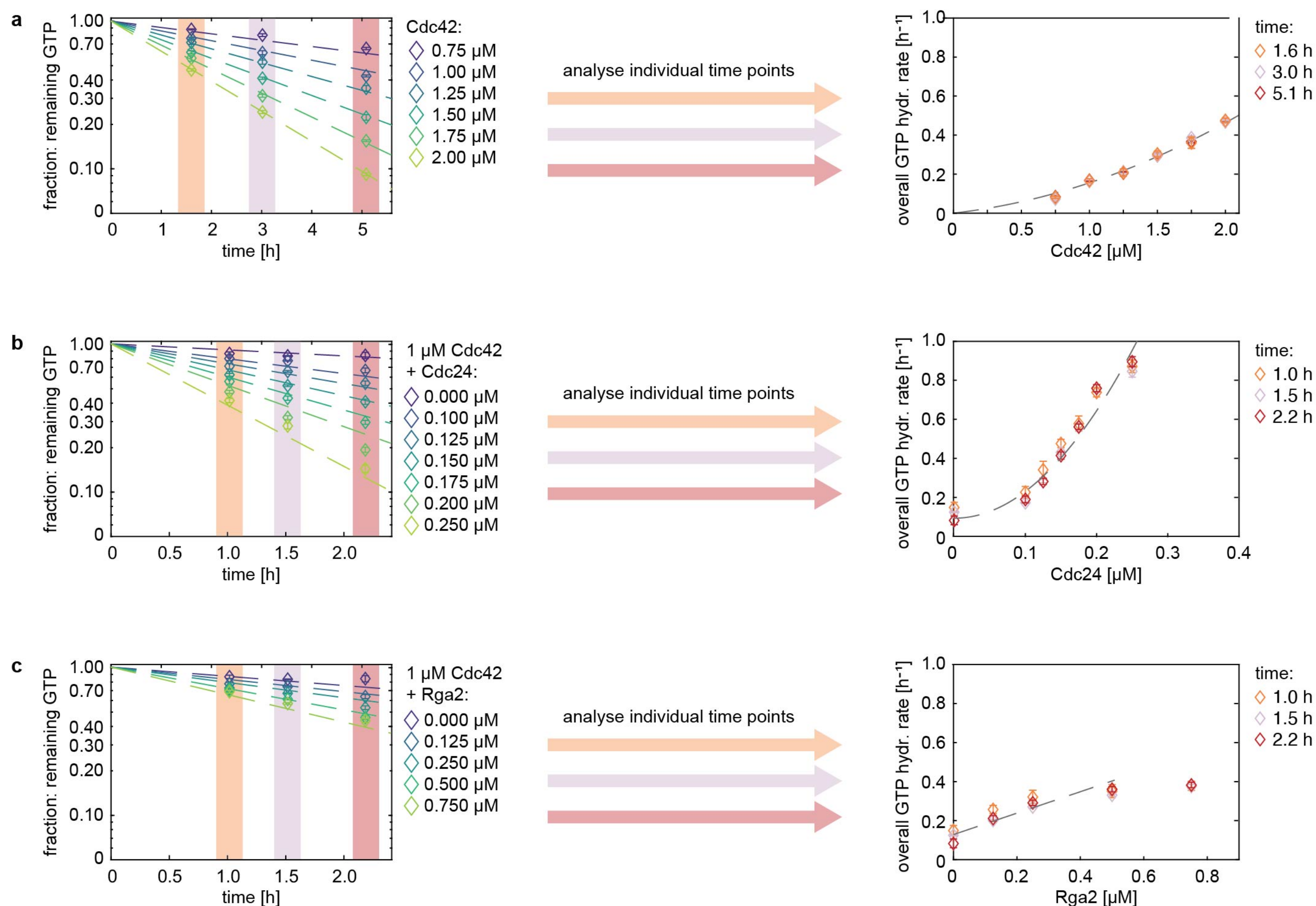
Supplement S3

Before including fluorescently labelled proteins into the GTPase assay, assess if the fluorescent tags interfere with the assay readout (= luminescence). Some fluorescent tags (e.g. Alexa488) reduce the luminescence almost completely (e.g. mNeon-green), while others only affect it mildly (e.g. Alexa488) (S3 Fig. 1).



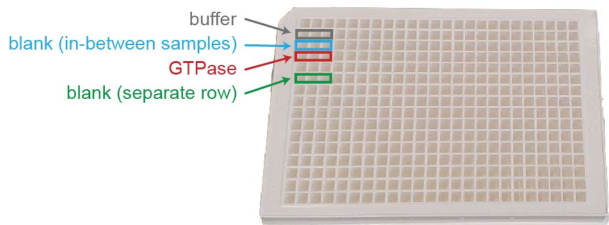
S3 Figure 1. Fluorescent tags can interfere with the GTPase assay readout: Luminescence (left) and perceived amount of remaining GTP (right) of four fluorophores (normalised to buffer). Fluorophores are not GTPase enzymes and are not expected to hydrolyse GTP. A drop in luminescence signal (and thus a decrease in the perceived amount of remaining GTP) is likely due to absorption of some of the luminescence signal by the fluorophores.

Supplement S4



S4 Figure 1. The GTP concentration declines exponentially with time in GTPase reactions. Amount of remaining GTP for (a) Cdc42 concentrations (b) Cdc42 Cdc24 mixtures, and (c) Cdc42 Rga2 mixtures, each for three time points (measured as one individual assay per time point). The remaining GTP content declines exponentially with time (left). Data of each individual time point shows the same overall GTP hydrolysis cycling rate for each GTPase - effector mixture. Thus, only one time point per assay condition is needed, to fit the data (right).

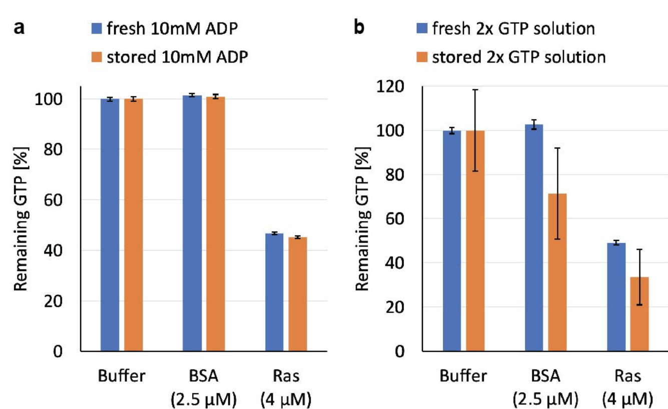
Supplement S5



Well	buffer	GTPase	blank in-between samples	blank separate row
1	30376	22725	373	30
2	31316	22891	451	31
3	30448	22553	425	31
Average (Luminescence)	30713	22723	416	30
Std. err. mean (Lum.)	302	98	23	0
Remaining GTP [%]	100,0	74,0	1,4	0,1
Error [%]	1,4	0,8	0,1	0,0

S5 Figure 1. Leave one empty row between all sample rows to avoid any spill-over of luminescence signal between samples. The row 'blank (in-between samples)' (light blue) does not contain any solution, it is empty. It is placed between a row that contains buffer and a row with a GTPase sample, both of which have a strong luminescence signal (as is expected). This leads to a small, but detectable luminescence signal in this row. In comparison, a similar blank/empty row that is not placed next to a sample row (green), exhibits a 10× reduced background luminescence. This spill-over of luminescence signal in in-between sample rows (light blue) translates to an 1% increase in remaining GTP - a small but unnecessary error to the assay's accuracy.

Supplement S6

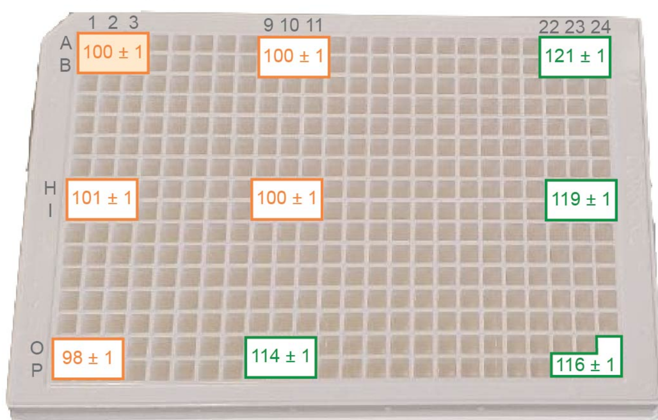


S6 Figure 1. In GTPase assays the by the kit provided 10 mM ADP solution can be re-used (here: 3 freeze/thaw cycles) (a). In contrast, the 2× GTP solution can not be stored (b)! The graphs show the amount of remaining GTP for buffer (used for normalisation), BSA, and Ras GTPase. BSA is not a GTPase and does not change the GTP content. Ras is a GTPase and hydrolyses GTP, decreasing the amount of remaining GTP. Re-using 2× GTP solution results in huge variations between replicas of the same sample, leading to large error bars.

Supplement S7

We advice to aliquote the detection reagent to reduce the number of freeze-thaw cycles and decrease the time required for thawing. Before using the detection reagent in GTPase assays, prepare a sufficient volume (e.g. through mixing of several aliquots) and vortex for proper mixing. We strongly advice against using separate detection reagent aliquots in one assay. *In some cases* this results in a large shift in luminescence, negating assay reliability (S7 Fig. 1).

Remaining GTP [%]:



detection reagent: aliquot 1

reference wells used for nomalisation

detection reagent: aliquot 2

Well	A1-A3 B1-B3	A9-A11 B9-B11	H1-H3 I1-I3	H9-H11 I9-I11	O1-O3 P1-P3	A22-A24 B22-B24	H22-H24 I22-I24	O9-O11 P9-P11	O24 P22-P24
1	34293	34202	34944	34710	34271	41728	41384	39597	
2	33683	33608	34298	33252	33075	41069	41524	38479	
3	34268	34596	34876	34442	33015	40874	40438	37845	39692
4	35463	33775	35026	35490	33531	42263	41625	40377	40512
5	34616	35616	35646	34335	34150	42724	40857	40479	39558
6	34704	35814	35265	35350	35035	42505	41042	40395	41698
Average (Luminescence)	34465	34359	34958	34446	33608	41731	41165	39355	39921
Std. err. mean (Lum.)	291	358	215	360	262	349	225	520	298
Remaining GTP [%]	100	100	101	100	98	121	119	114	116
Error [%]	1	1	1	1	1	1	1	2	1

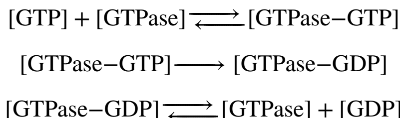
S7 Figure 1. Luminescence (and resulting remaining GTP) values of a GTPase assay where buffer was added to all wells. In wells marked in orange one detection reagent aliquot was used and in wells marked in green a separate detection reagent aliquot was used. The use of distinct aliquots resulted in this case in a large difference in luminescence between both groups, propagating to an up to 20% difference in the perceived amount of remaining GTP.

Supplement S8

We developed a GTPase activity model for determining the GTPase cycling rates *k*. It is briefly described in the following. (An extended version is given in S11.)

A GTPase cycling model

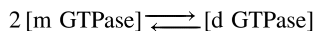
GTPase cycling involves three steps: (1) A GTP molecule from solution binds to the GTPase. (2) The GTPase hydrolyses GTP. (3) The GTPase releases GDP.



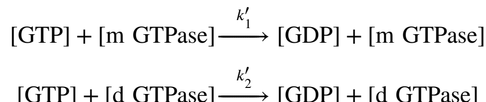
The activity of some GTPases can further be upregulated by effector proteins: GAPs have been shown to enhance GTP hydrolysis by the GTPase (step 2), GEFs enhance the release of GDP from the GTPase (step 3) [Bos et al., 2009, Vetter and Wittinghofer, 2001, Cherfils and Zeghouf, 2013].

To quantitatively describe the GTPase reaction cycle, we coarse-grained the GTPase reaction steps with

To account for possible GTPase dimerisation and cooperativity, we included the following reactions into the model:
(1) Some GTPase enzymes can dimerise [Zhang and Zheng, 1998, Zhang et al., 1999, Zhang et al., 2001, Kang et al., 2010]:



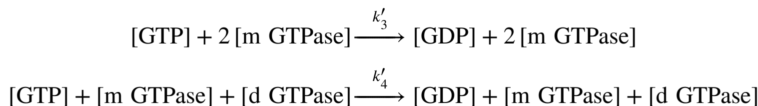
and both monomeric and dimeric forms of the GTPase can contribute to the overall GTP hydrolysis with different rates:



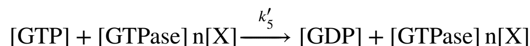
Assuming that the majority of the GTPase enzyme is in its monomeric form ([m GTPase] $< C_d$, with C_d as the concentration at which half of the total GTPase is dimeric), we can approximate

$$\begin{aligned} \text{[d GTPase]} &= \frac{\text{[m GTPase]}^2}{2C_d} \\ \text{[m GTPase]} &\approx \text{[GTPase]} - \frac{\text{[GTPase]}^2}{C_d} \end{aligned} \tag{6}$$

(2) Next to cooperativity from dimerisation, cooperativity can also emerge when GTPase proteins come in close contact with each other - they can affect each other's behaviour without forming a stable homodimer, effectively functioning as an effector protein for themselves:



(3) Effector proteins, such as GAPs and GEFs, affect the speed of the GTP hydrolysis cycle:



Here X is an effector protein and $n \in \mathbb{N}$.

Our data showed that the amount of remaining GTP follows an exponential decline over time (S4):

$$\text{[GTP]}_t = \text{[GTP]}_{t_0} \exp(-Kt)\text{, using } \text{[GTP]}_{t_0} = 1 \tag{7}$$

Considering reactions (1) - (3), we can thus define K in Eq. 7 as

$$K = k_1'[\text{m GTPase}] + k_2'[\text{d GTPase}] + k_3'[\text{m GTPase}]^2 + k_4'[\text{m GTPase}][\text{d GTPase}] + k_5'[\text{GTPase}][X]^n$$

Using Eq. 6, and considering only up to second-order terms, results in

$$\begin{aligned} K &= k_1'[\text{GTPase}] + \left(\frac{k_2'}{2C_d} + k_3' - \frac{k_1'}{C_d}\right)[\text{GTPase}]^2 + k_5'[\text{GTPase}][X]^n \\ &= k_1[\text{GTPase}] + k_2[\text{GTPase}]^2 + k_{3,X}[\text{GTPase}][X]^n \end{aligned} \tag{8}$$

where k_1 refers to GTP hydrolysis cycling rates of monomeric GTPase, k_2 includes effects of cooperativity and dimerisation and k_3 represents the rate of GTPase - effector interaction. We refer to K as 'overall GTP hydrolysis rate'.

Variability between assays

Eq. 8 with [X]=0 can be used to determine the rates of the GTPase alone. Then assays with the GTPase and an effector protein can be conducted to determine k_3. While doing so one needs to account for assay variability, i.e. for the observation that the rates for the GTPase can vary between assays. Possible reasons for this include small concentration differences introduced though pipetting of small volumes (as are required for this assay), temperature and shaker speed fluctuations during the incubation step, and/or intrinsic changes in the protein activities due to other external conditions. To account for this variance, we introduced the parameter c_{corr}. It maps all factors that lead to variations between assays onto the GTPase concentration.

The assay data, including samples containing only GTPase and GTPase - (effector X) mixtures, are fitted with

$$K = k_1 c_{corr}[\text{GTPase}] + k_2(c_{corr}([\text{GTPase}])^2 + k_{3,X} c_{corr}[\text{GTPase}][X]^n \tag{9}$$

to determine c_{corr} and $k_{3,X}$ (using k_1 and k_2 determined earlier) (with n either 1 or 2).

c_{corr} values are usually close to 1.0 (e.g. [Tschirpke et al., 2023b, Tschirpke et al., 2023a]), showing that the variation between assays is small. We advice to exclude assays with a big or very small c_{corr}, as these indicate that the GTPase behaviour/assay conditions are unusual.

GTPase - effector interactions

The accompanying matlab code allows to fit GTPase - effector mixtures that depend either linearly ($n = 1$) or quadratically ($n = 2$) on the effector concentration $[X]$ (Eq. 9). If the effectors show neither a linear nor a quadratic concentration-dependence (e.g. due to saturation), we advice to either only include the linear/quadratic regimes into the analysis or extend our fitting model to match the specific case.

The model allows to fit GTPase - effector mixtures with up to two effectors present:

$$K = k_1 c_{corr}[\text{GTPase}] + k_2(c_{corr}[\text{GTPase}])^2 + k_{3,X_1} c_{corr}[\text{GTPase}][X_1]^n + k_{3,X_2} c_{corr}[\text{GTPase}][X_2]^m + k_{3,X_1,X_2} c_{corr}[\text{GTPase}][X_1]^n[X_2]^m \tag{10}$$

with n and m either 1 or 2.

Pooling of cycling rates k and error propagation

The way rate values are weighted for pooling and how errors are propagated is explained in detail in S11.

Supplement S9

This supplement describes a simple python script that reads in GTPase data analysed in a spreadsheet editor (Support Protocol 1) and re-formats it into the input required for Support Protocol 2.
The script is illustrated using data of example 1 (Fig. 5).

Necessary resources

- Python script file: 'Ras_example.ipynb'
- Data file: 'example1.xlsx'
- a spreadsheet editor
- software to run a python script

Steps

1. Open 'Ras_example.ipynb', state the input data and relevant tab names:

```
datafilename = 'example1.xlsx'
tabnamelist = ['E1', 'E2']
```

2. Run the python script. It will generate two excel sheeets: 'E1.xlsx' and 'E2.xlsx'.
3. Copy data of both outputs into one excel sheet, but only include one header (S9 Fig. 1)! This will be the input for the matlab script used in Support Protocol 2.
4. Use the find/replace option of the spreadsheet editor to replace '.' (a point) with ',' (a comma).
   The python script generates numbers of the format '1.00' while the matlab script requires the format '1,00'.

General considerations on how the script operates

The spreadsheet data needs to conform to the following formatting to be processed by the python script (S9 Fig. 2):

- The script processes values in the spreadsheet area A80-Z89.
  This area can only contain relevant numbers. If comments are placed in this area, the script will given an error.
- The incubation time, stated in hours, must be stated in cell C82 (S9 Fig. 2 blue box).
- The error of the buffer must be stated in cell E81 (S9 Fig. 2 blue box).
- Remaining GTP values must be stated in cells F80-Z80. Cells not in use must remain empty (S9 Fig. 2 red box).
- Remaining GTP error values must be stated in cells F81-Z81. Cells not in use must remain empty (S9 Fig. 2 red box).
- Protein names must follow the formatting `'ProteinName_conc'` and be stated in A83-A89. Cells not in use must remain empty (S9 Fig. 2 orange box).
- Protein concentration values must be stated in the area B83-Z89. It is important to state the concentration of each protein listed in the protein name section here. (I.e. if a protein is not part of a sample, its concentration is 0.) Cells not in use must remain empty (Fig. 2 orange box).

Run	Time	GTP_remaining	Error	Buffer_error	Ras_conc
E1	1,566666667	0,453944399	0,005618298	0,016198535	5
E1	1,566666667	0,622129048	0,008046552	0,016198535	4
E1	1,566666667	0,764956994	0,010290555	0,016198535	3
E1	1,566666667	0,886149288	0,015009141	0,016198535	2
E1	1,566666667	0,984468547	0,01192282	0,016198535	1
E1	1,566666667	0,992636905	0,01654503	0,016198535	0,5
E2	1,75	0,378457167	0,002579438	0,008967404	5
E2	1,75	0,557180709	0,008588182	0,008967404	4
E2	1,75	0,739454841	0,006747585	0,008967404	3
E2	1,75	0,878599651	0,013720581	0,008967404	2
E2	1,75	0,95195911	0,008710646	0,008967404	1
E2	1,75	0,977303953	0,00689024	0,008967404	0,5

S9 Figure 1. Required input format for the matlab scirpt (Support Protocol 2).

	A	B	C	D	E	F	G	H	I	J	K
69						Ras					
70	Well			buffer	buffer	5uM	4uM	3uM	2uM	1uM	0.5uM
71	1			35330	34324	15662	21294	26750	31392	33846	35117
72	2			35668	33599	15829	21478	26466	30209	34289	34076
73	3			35151	33313	15579	21739	26105	30286	33948	33736
74	4										
75	5										
76											
77	Average				34564	15690	21503	26440	30629	34027	34310
78	Std. err. mean				396	74	129	187	382	134	415
79											
80	Remaining GTP [%]				100,0	45,4	62,2	76,5	88,6	98,4	99,3
81	Error [%]				1,6	0,6	0,8	1,0	1,5	1,2	1,7
82	time [min; h]	94	1,567								
83	Ras_conc					5	4	3	2	1	0,5
84											
85											
86											
87											
88											
89											
90											

E1 | E2 | matlab | +

S9 Figure 2. Required input format for the python scirpt.

Supplement S10

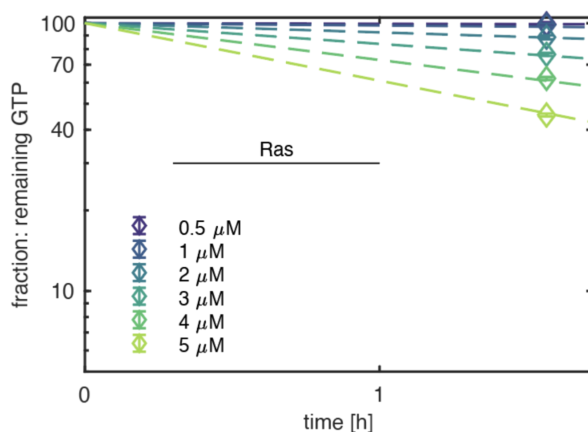
To use the plotting scripts, a 'Data_assays.mat' file (output of Support Protocol 2) is required. All scripts automatically save the plot as '.tif' and '.pdf'.

Plot_Semilog_GTP_time.m

This script produces plots showing of the amount of GTP over time fitted with an exponential, as shown in S4. It can be used for assays of GTPases and GTPase effector mixtures with one effector.

1. Run 'Plot_Semilog_GTP_time.m'.
2. Select a 'Data_assays.mat' file.
3. Choose an assay name, as stated in the previously used 'assaylist.xlsx' file. Then choose which assay (shown by assay number) should be plotted.
   An example plot is given in S10 Fig. 1.

   This script is especially useful to assess/ verify that the amount of GTP declines exponentially. To do so, several assays (e.g. 3) of different incubation times using the same protein concentrations need to be conducted. To plot all (3) assays in the same plot, give these assays ***the same*** *assay number before analysing them using 'Process_assays.m'.*

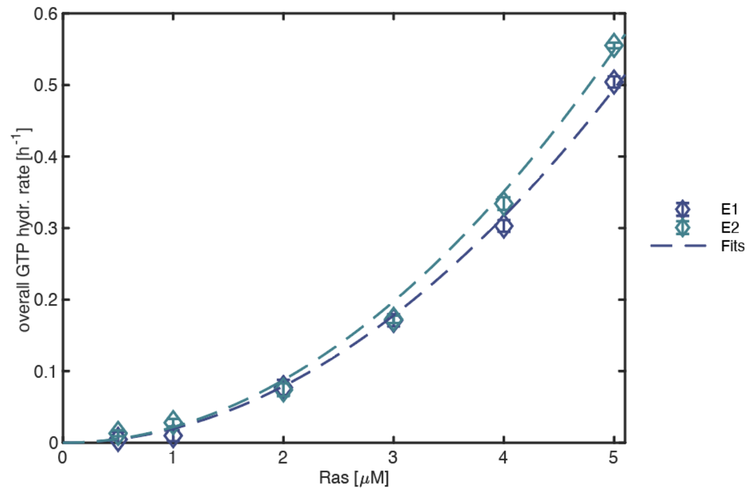


S10 Figure 1. Exponential fit for the amount of amount of GTP over time for Ras concentrations. Generated for assay name 'Ras' and experiment number 'E1' (using 'Plot_Semilog_GTP_time.m' and 'Data_assays.mat' in the folder 'example1and2 matlab output').

Plot_rate_concentration.m

This script produces allows to plot of the overall GTP hydrolysis rate for several assays in one figure. It can be used for assays of GTPases and GTPase effector mixtures with one effector.

1. Run 'Plot_rate_concentration.m'.
2. Select a 'Data_assays.mat' file.
3. Choose an assay name, as stated in the previously used 'assaylist.xlsx' file.
   An example plot is given in S10 Fig. 2.



S10 Figure 2. Overall GTP hydrolysis rate over Ras concentration for assays 'E1' and 'E2'. Generated for assay name 'Ras' (using 'Plot_rate_concentration.m' and 'Data_assays.mat' in the folder 'example1and2 matlab output').

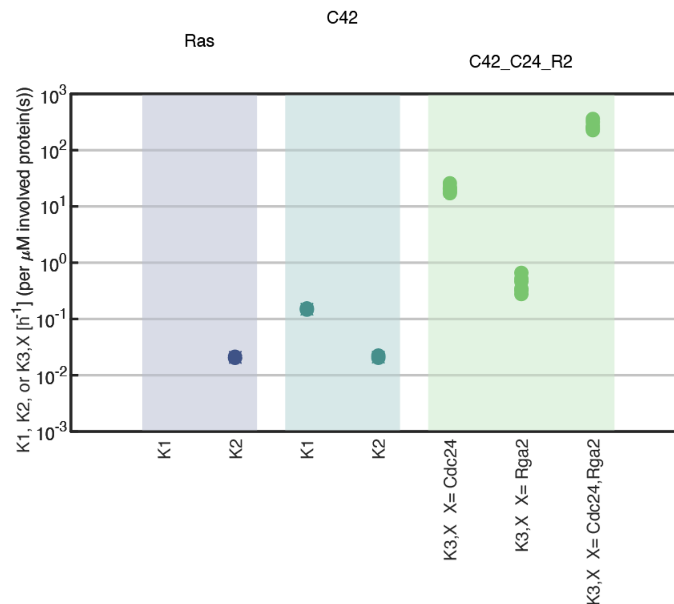
Plot_pooled_values_std_err.m

This script produces plots of the pooled rates k_1, k_2 k_3 (that are also shown in the 'Data_summary.xlsx' file).

1. Run 'Plot_pooled_values_std_err.m'.

   The parameter 'y_limits' in the code can be used to modify the limits of the y-axis. It is currently set to [1e-3 1e3].
2. Select a 'Data_assays.mat' file.
3. Choose an assay name, as stated in the previously used 'assaylist.xlsx' file.

   An example plot is given in S10 Fig. 3.



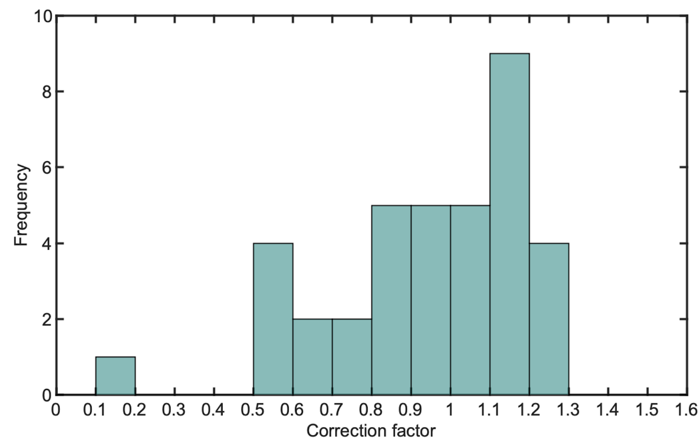
S10 Figure 3. Plot of rates K_1, K_2 for Ras (left), K_1, K_2 for Cdc42 (middle), and K_3, X for Cdc42-Cdc24-Rga2 assays. The assay name is stated on top. Rate values of individual experiments are shown as filled dots. The average is shown as a cross and error bars represent the standard error. Generated for assay names 'Ras', 'C42', and 'C42-C24-R2' (using 'Plot_pooled_values_std_err.m' and 'Data_assays.mat' in the folder 'example1and2 matlab output').

Plot_c_corr_histogram.m

This script produces a histogram plot of c_{corr} values. Before running the script, first copy/paste all c_{corr} values that should be plotted into a spreadsheet file.

1. Copy/paste all c_{corr} values that should be plotted from 'Data_summary.xlsx' into the first column of a spreadsheet file. All values should be in column A. Change the number formatting from ',' to '.' (i.e. change the number formatting from '0,863' to '0.863'.
2. Run 'Plot_c_corr_histogram.m'.
   An example plot is given in S10 Fig. 4.

   The parameter 'bin_size' , 'x_start' , and 'x_end' in the code can be used to modify the plot. 'bin_size' states the bin size for the histogram segmentation, and 'x_start' and 'x_end' define the x-axis limits (i.e. minimum and maximum c_{corr} values that will be plotted).

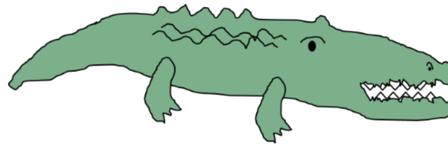


S10 Figure 4. Histogram plot of c_{corr} using a bin size of 0.1. Generated for all c_{corr} values of example 2 (using 'Plot_c_corr_histogram.m' and 'example2-histogram.xlsx' in the folder 'example2 matlab output').

Supplement S11

GTPase (CROCODILE) model

Mathematical appendix



General model outline II

Pooling III

Case 1: No effectors IV

Case 2: Adding a single effector VI

GTPase concentration correction factors VII

Linear and quadratic effectors VIII

Case 3: Adding two effectors IX

Fitting restrictions on parameters X

Error propagation XI

Optional crowding addition XI

Single effector case XI

Double effector case XII

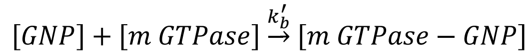
Bibliography XII

Crocodile stands for Crowding, cooperativity and dimerization in luminescence experiments. The purpose of this model is to describe, dissect and interpret the results of GTPase assays with, also in combination with other effectors, for example effector Cdc24 in the case Cdc42 is the GTPase. In these assays, GTP is hydrolyzed over time by a GTPase at a rate dependent on the concentration of proteins involved. The following section describe how we model the rate and what assumptions underlie this description.

General model outline

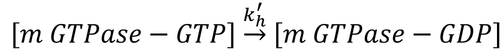
We consider a GTPase in solution with nucleotides that get hydrolyzed through GTPase cycles. Effectors may also be present to speed up (parts of) the GTPase cycle. Chemically, the GTPase cycle consists of three steps, which are nucleotide binding, hydrolysis and nucleotide release, and effectors influence the rates of one or more of these steps. First considering monomeric GTPases with concentration $[m\ GTPase]$, possible in complex with a nucleotide $[m\ GTPase - GNP]$, this cycle constitutes the following reaction schemes (with GNP representing a GTP or GDP nucleotide):

Nucleotide binding (reaction rate constant k_b')



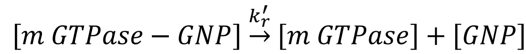
$$[GNP] + [m\ GTPase] \xrightarrow{k_b'} [m\ GTPase - GNP]$$

Hydrolysis (reaction rate constant k_h')



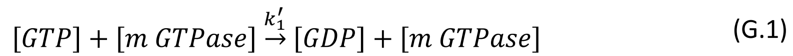
$$[m\ GTPase - GTP] \xrightarrow{k_h'} [m\ GTPase - GDP]$$

Nucleotide release (reaction rate constant k_r')



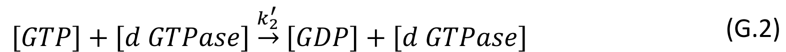
$$[m\ GTPase - GNP] \xrightarrow{k_r'} [m\ GTPase] + [GNP]$$

Effectors will influence the reaction rates and to avoid making concrete assumption on the molecular mechanism of each effector, and to reduce the number of fitting parameters later, we coarse-grain this GTPase cycle to a single step with rate constant k_1'. This will also help us to deal with the rate variability across replicate experiments as we will see further on.



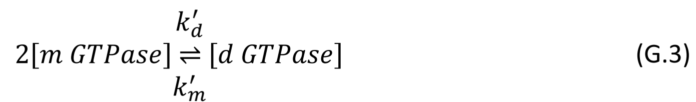
$$[GTP] + [m\ GTPase] \xrightarrow{k_1'} [GDP] + [m\ GTPase] \qquad \text{(G.1)}$$

GTPases may also dimerize. To take this into account, we also consider the possible reaction (with rate constant k_2'):



$$[GTP] + [d\ GTPase] \xrightarrow{k_2'} [GDP] + [d\ GTPase] \qquad \text{(G.2)}$$

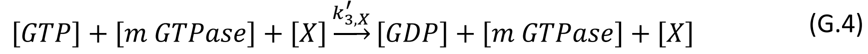
where GTPase dimers results from the monomers through the reaction:



$$2[m\ GTPase] \underset{k_m'}{\overset{k_d'}{\rightleftharpoons}} [d\ GTPase] \qquad \text{(G.3)}$$

with monomeric and dimeric rate constants k_m' and k_d' respectively.

When involving another protein X into this coarse-grained cycle such as an effector, we have (with rate constant $k_{3,X}'$:):



$$[GTP] + [m\ GTPase] + [X] \xrightarrow{k'_{3,X}} [GDP] + [m\ GTPase] + [X] \tag{G.4}$$

Generally, the overall uncorrected hydrolysis rate K^* will then take the form (correction explanation follows later):

$$\frac{d[GTP]}{dt} = -k'_1[m\ GTPase][GTP] - k'_2[d\ GTPase][GTP] \\ - \sum_X k'_{3,X}[X][m\ GTPase][GTP] = -K^*[GTP] \tag{G.5}$$

adding all contributions of each individual cycle reaction to the overall hydrolysis, potentially having multiple proteins X that contribute to the summation. This equation retains the same form if we instead assume complex formation between X and the GTPase, such that we have $[GTP] + [m\ GTPase - X] \xrightarrow{k'_{3,X}} [GDP] + [m\ GTPase - X]$. This alternative leads to:

$$\frac{d[GTP]}{dt} = -k'_1[m\ GTPase][GTP] - k'_2[d\ GTPase][GTP] - \sum_X k'_{3,X}[m\ GTPase - X][GTP]$$

but as we expect $[m\ GTPase - X] \propto [m\ GTPase][X]$ from the rate equation of $[m\ GTPase] + [X] \rightleftarrows [m\ GTPase - X]$ in equilibrium, this is still equivalent to G.5.

Assuming the GTP concentration contains the only time-dependence on the right-hand side (i.e., all proteins have had time to equilibrate their reaction with each other), we can write:

$$-\frac{d\log[GTP]}{dt} = K^* = k'_1[m\ GTPase] + k'_2[d\ GTPase] + \sum_X k'_{3,X}[X][m\ GTPase] \tag{G.6}$$

and this yields an exponential decay for the GTP nucleotides:

$$[GTP] = [GTP]_{t=0}\exp(-Kt) \tag{G.7}$$

Pooling

Generally, we have multiple runs for an experiment with a GTPase, each yielding estimates for e.g., k_1 and k_2 with standard errors. To get a single estimate, we must pool these. This is done by performing a weighted average to obtain the pooled estimates, as explained in the Supplements of (Tschirpke, Daalman & Laan, 2023).

In short, we model for example the individual run estimates $\hat{k}_{1,i}$ (with $i = 1, 2, \dots, n$ with n as the number of runs) to deviate from the pooled $k_{1,p}$ through normally distributed errors, but with heteroscedasticity:

$$\hat{k}_{1,i} = k_{1,p} + \varepsilon_i$$

with errors $\varepsilon_i \sim \mathcal{N}(0, \sigma_i)$ and σ_i as the standard errors of each run estimate. By multiplying both sides by the weights $w_i = 1/\sigma_i$, the resulting weighted estimates per run now follow a standard normal distribution. As a consequence, estimates that have large uncertainty are awarded a lower weight for the weighted estimated of $k_{1,p}$ that follows. This weighted estimate of $k_{1,p}$ is constructed by minimizing the (sum of) weighted errors, realized by squaring these first to treat positive and negative errors equally. This minimization amounts to a weighted least squares regression to yield the estimate $\hat{k}_{1,p}$ of $k_{1,p}$, see e.g., (Heij, de Boer, Franses, Kloek, & van Dijk, 2004), which is a weighted average of the run estimates:

$$\hat{k}_{1,p} = \frac{\sum_{i=1}^{n} \hat{k}_{1,i}/\sigma_i^2}{\sum_{i=1}^{n} 1/\sigma_i^2}$$

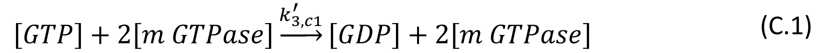
with standard error:

$$\sigma_{\hat{k}_{1,p}} = \frac{\frac{1}{n-1}\sum_{i=1}^{n}\left(\hat{k}_{1,i}/\sigma_i - \hat{k}_{1,p}/\sigma_i\right)^2}{\left(\sum_{i=1}^{n} 1/\sigma_i^2\right)^2}$$

By the same token, we can construct other pooled estimates such as $\hat{k}_{2,p}$.

Case 1: No effectors

Even in absence of effectors, reactions G.1 and G.2 is not necessarily the only reaction taking place. GTPase can exhibit cooperativity and/or dimerization, Thus, we also consider reaction G.4 with X as another GTPase molecule, and this reaction reduces to (with rate constant $k'_{3,c1}$):



$$[GTP] + 2[m\ GTPase] \xrightarrow{k'_{3,c1}} [GDP] + 2[m\ GTPase] \quad \text{(C.1)}$$

which implies cooperativity. Presence of dimers can also be taken into account with X = d GTPase, so we also have (with rate constant $k'_{3,c2}$):

$$[GTP] + [m\ GTPase] + [d\ GTPase] \xrightarrow{k'_{3,c2}} [GDP] + [m\ GTPase] + [d\ GTPase] \quad \text{(C.2)}$$

Theoretically, even higher order encounters (e.g., dimer + dimer) of GTPase molecules may take place, but we assume these get progressively unlikelier.

Given the previous reactions, the overall uncorrected cycling rate G.6 for the change in GTP concentration over time becomes (with $X = \{[m\ GTPase], [d\ GTPase]\}$:

$$K^* = -\frac{d\log[GTP]}{dt} = \overbrace{k'_1[m\ GTPase]}^{monomers} + \overbrace{k'_2[d\ GTPase]}^{dimers} + \overbrace{k'_{3,c1}[m\ GTPase]^2}^{cooperativity\ 1} + \overbrace{k'_{3,c2}[m\ GTPase][d\ GTPase]}^{cooperativity\ 2} \quad \text{(C.3)}$$

The overall cycling rate is hence a function of the monomeric and dimeric GTPase concentration. However, we supply a specific total GTPase concentration, so we want to rewrite all terms to this known $[GTPase]$ concentration. Assuming the monomeric and dimeric pool are in equilibrium with each other, the rate equation resulting from the reaction in G.3 reads

$$\frac{d[m\ GTPase]}{dt} = k_m'[d\ GTPase] - k_d'[m\ GTPase]^2 = 0$$

$$\Longrightarrow [d\ GTPase] = \frac{k_d'}{k_m'}[m\ GTPase]^2 \qquad \text{(C.4)}$$

We can thus write the Hill equation:

$$\frac{[m\ GTPase]}{[GTPase]} = \frac{[m\ GTPase]}{[m\ GTPase] + 2[d\ GTPase]} = \frac{[m\ GTPase]}{[m\ GTPase] + \frac{2k_d'}{k_m'}[m\ GTPase]^2}$$

$$= \frac{1}{1 + \frac{[m\ GTPase]}{C_d}}$$

Consequently, C.4 then reads as:

$$2C_d[d\ GTPase] = [m\ GTPase]^2 \qquad \text{(C.5)}$$

with $C_d = k_m'/(2k_d')$. So, there is a critical concentration above which the monomeric fraction is low compared to the dimeric fraction. This critical concentration is higher if monomerization reaction rate k_m' is high relative to dimerization reaction rate k_d'.

Therefore, we can write the monomeric concentration as:

$$[GTPase] = [m\ GTPase] + 2[d\ GTPase] = [m\ GTPase] + \frac{1}{C_d}[m\ GTPase]^2$$

$$\Longrightarrow [m\ GTPase] = -\frac{C_d}{2} + \sqrt{C_d[GTPase] + \frac{C_d^2}{4}} = -\frac{C_d}{2} + \frac{C_d}{2}\sqrt{1 + 4\frac{[GTPase]}{C_d}}$$

We can then use Taylor expansion to approximate this square root. The order up to which we need to expand depends on the GTPase concentration relative to the critical concentration, i.e. the size of the monomeric fraction relative to the dimeric fraction. If $[GTPase] \ll C_d$, then most GTPase molecules are monomeric and expansion up to second order (around the point $4[GTPase]/C_d = 0$) suffices. As our data shows these terms suffices for fitting the GTPase dilution data well, we continue the derivation with the second order expansion. If other GTPase dilution data shows signs of higher order $[GTPase]$ dependencies, this derivation must be adapted to include higher order expansion terms of the square root.

$$[m\ GTPase] \approx -\frac{C_d}{2} + \frac{C_d}{2}\left(1 + \frac{2[GTPase]}{C_d} - 2\frac{[GTPase]^2}{C_d^2}\right) = [GTPase] - \frac{[GTPase]^2}{C_d} \qquad \text{(C.6)}$$

Similarly, using $[GTPase] = [m\ GTPase] + 2[d\ GTPase]$ and substituting E.4, we get

$$[d\ GTPase] = \frac{[GTPase] - [m\ GTPase]}{2} \approx \frac{[GTPase]^2}{2C_d} \quad \text{(C.7)}$$

and also, combining E.3 and E.5, we get:

$$[m\ GTPase]^2 = 2C_d[d\ GTPase] \approx [GTPase]^2 \quad \text{(C.8)}$$

This means for the hydrolysis rate C.3, using the previous three equations:

$$-\frac{d\log[GTP]}{dt} = k_1'\left([GTPase] - \frac{[GTPase]^2}{C_d}\right) + \frac{k_2'}{2C_d}[GTPase]^2 + k_{3,c1}'[GTPase]^2 + \frac{k_{3,c2}'}{2C_d}\left([GTPase] - \frac{[GTPase]^2}{C_d}\right)[GTPase]^2$$

$$-\frac{d\log[GTP]}{dt} = k_1'[GTPase] + \left(-\frac{k_1'}{C_d} + \frac{k_2'}{2C_d} + k_{3,c1}'\right)[GTPase]^2 + \mathcal{O}([GTPase]^3) \quad \text{(C.9)}$$

Ignoring the higher order terms $\mathcal{O}([GTPase]^3)$ and defining $k_1 = k_1'$ and $k_2 = -\frac{k_1'}{C_d} + \frac{k_2'}{2C_d} + k_{3,c1}'$, we obtain:

$$\frac{d\log[GTP]}{dt} = \underbrace{k_1[GTPase]}_{K_1^*} + \underbrace{k_2[GTPase]^2}_{K_2^*} \quad \text{(C.10)}$$

where k_1 and k_2 are cycling rates and K_1^* and K_2^* are uncorrected overall rate contributions to K^*. The former involves monomeric GTPase contributions, and the latter also <u>co</u>operativity and <u>di</u>meric contributions. In the section on optionally adding crowding effects, we see that <u>cro</u>wding would also present itself in the second term, explaining the origin for the 'crocodile' model term.

So, using G.7 we obtain for the GTP concentration:

$$[GTP] = \exp(-k_1[GTPase]t - k_2[GTPase]^2 t) \quad \text{(C.11)}$$

Case 2: Adding a single effector

When an effector is added to the GTPase, more terms result from G.6 than those in C.11. Assuming the number of monomers and dimers is not significantly affected by the presence of GTPase-effector complexes, the same derivation as without effectors applies from G.6 up to C.9, only with an added term:

$$-\frac{d\log[GTP]}{dt}=k_1'[m\ GTPase]+k_2'[d\ GTPase]+\sum_X k_{3,X}'[X][m\ GTPase] \tag{C.12}$$

$$=k_1'[m\ GTPase]+k_2'[d\ GTPase]+k_{3,c1}'[m\ GTPase]^2+k_{3,c2}'[m\ GTPase][d\ GTPase]+k_{3,X}'[X][GTPase]\Rightarrow$$

$$-\frac{d\log[GTP]}{dt}=k_1'[GTPase]+\left(-\frac{k_1'}{C_d}+\frac{k_2'}{2C_d}+k_{3,c1}'\right)[GTPase]^2$$
$$+k_{3,X}'[X][GTPase]+\mathcal{O}([GTPase]^3) \tag{C.13}$$

Defining $k_1=k_1'$, $k_2=-\frac{k_1'}{C_d}+\frac{k_2'}{2C_d}+k_{3,c1}'$ and $k_3=k_{3,X}'$, and ignoring the higher order terms $\mathcal{O}([GTPase]^3)$ leads to:

$$-\frac{d\log[GTP]}{dt}=\overbrace{k_1[GTPase]}^{K_1^*}+\overbrace{k_2[GTPase]^2}^{K_2^*}+\overbrace{k_3[GTPase][X]}^{K_3^*} \tag{C.14}$$

The new cycling rate k_3 and uncorrected overall rate contribution K_3^* reflect the interaction between effector and GTPase. Note that a low value of k_3 may be interpreted as absence of interaction, but can also be due to low functionality of the effector, or that the effector accelerates a step in the GTPase cycle which was already fast relative to the others. As we coarse-grain the full GTPase cycle into one step, the overall effectivity of the protein them appears low.

GTPase concentration correction factors

In principle, k_1 and k_2 are known from an GTPase serial dilution assay (without the effector). However, in practice a complication arises. Expected activity of the GTPase may vary slightly across experiments, for many reasons. This can be due to concentration variability introduced by pipetting, environmental variability from the ideal protocol situation or protein integrity variability. Moreover, the amount of GTPases molecules sequestered by complexes with the effectors may be non-negligible as previously assumed, which would lead to a lower effective monomer and dimer concentration and thus to a lower contribution of terms involving k_1 and k_2 than expected from the assay without effectors.

A modelling solution is to introduce a run-specific GTPase concentration correction factor for assays with effectors, to account for these run-specific effects. This factor is a single constant applying to all instances of $[GTPase]$. The correction factor also provides us with a diagnostic for run-specific issues, as ideally the value is ideally close to 1. Large deviations from 1 are indicative of incidental (e.g., a pipetting error) or systematic (e.g., strong sequestration of GTPases in complexes) problems in the assay (modelling). The code implementation of the model therefore allows the user to define what range of correction factors is still deemed acceptable, and those runs with correction factors that do not fall in this range are excluded for pooling single-run parameter estimates to the pooled estimates.

Including this correction factor c_{corr} into C.14, the corrected overall rate contribution K then becomes:

$$K = -\frac{d\log[GTP]}{dt} = \overbrace{k_1 c_{corr}[GTPase]}^{K_1} + \overbrace{k_2 c_{corr}^2[GTPase]^2}^{K_2} + \overbrace{k_3 c_{corr}[GTPase][X]}^{K_3} \quad \text{(C.15)}$$

with K_1, K_2 and K_3 as overall rate contributions. To fit this function, we still see it is of the form $a + b[X]$, where k_3 is contained in b. But before we can retrieve k_3, we must determine c_{corr}. This can be done by comparing the overall cycling rate K of the effector assay when $[X] = 0$, to the GTPase dilution assay cycling rate (which has no effector). Concretely, we compare:

$$k_1 c_{corr}[GTPase] + k_2 c_{corr}^2[GTPase]^2 = K_{effector\ assay}([X] = 0)$$

The $[GTPase]$ is set to the typical value in the effector assay. The left-hand side uses the k_1 and k_2 estimates that have been previously established from the GTPase serial dilution assay, while the right-hand side is the at $[X] = 0$.

$$c_{corr} = \frac{-k_1[GTPase] + \sqrt{k_1^2[GTPase]^2 + 4k_2[GTPase]^2 K([X] = 0)}}{2k_2[GTPase]^2}$$

$$\Rightarrow c_{corr} = \frac{-k_1 + \sqrt{k_1^2 + 4k_2 K([X] = 0)}}{2k_2'[GTPase]} \quad \text{(C.16)}$$

The rate of the effector assay can be measured directly or more reliably, inferred from evaluation at $[X] = 0$ of the fit based on all effector assay points, as in the computational implementation of the model. More specifically, this implementation generates random draws based on the fitting errors, while the fit also takes errors on the data points into account. Consequently, we can generate not only the point estimate of the correction factor, but also with the standard error. However, the code only uses the point estimate to excessively avoid inflating the errors on subsequent rate parameter estimates. For example, if we fit in practice $K = a + b[X]$, then $k_3 = \frac{b}{c_{corr}[GTPase]}$.

Linear and quadratic effectors

Some effectors form complexes with a GTPase, but can also dimerize themselves. If it can be assumed that this dimer is the most relevant form of the protein (due to its abundance or activity), this means that C.14 becomes (with X as dimer Y:$d\,Y$):

$$K = k_1 c_{corr}[GTPase] + k_2 c_{corr}^2[GTPase]^2 + k_3 c_{corr}[GTPase][d\,Y] \quad \text{(C.17)}$$

As dimer Y originates from two monomers, we have $[m\,Y] + [m\,Y] \underset{k_{mY}}{\overset{k_{dY}}{\rightleftharpoons}} [d\,Y]$, with the rate equation:

$$\frac{d[m\,Y]}{dt} = 2k_{mY}[d\,Y] - k_{dY}\,[m\,Y][m\,Y]$$

In equilibrium, this means $[d\,Y] \propto [m\,Y]^2$, and analogously to the GTPase monomer-dimer equilibrium, $[d\,Y] \propto [Y]^2$ (see (C.7)), such that K gets a quadratic dependence on effector concentration. The code implementation of the model accommodates both a linear dependency of the

overall rate on effector concentration and a quadratic dependency. Which case applies per effector can be defined by the user.

Case 3: Adding two effectors

With two effectors instead of one, the summation on the right hand side of C.12 will contain multiple terms for every effector combination. If we consider two effectors X_1 and X_2, the set whose rate contributions must be considered contains these two and a potential cross-term $X = \{X_1, X_2, X_1 - X_2\}$. As a result, C.12 will become:

$$-\frac{d\log[GTP]}{dt} = k_1'[m\ GTPase] + k_2'[d\ GTPase] + k_{3,X_1}'[X_1][m\ GTPase] \\ +k_{3,X_2}'[X_2][m\ GTPase] + k_{3,X_1-X_2}'[X_1 - X_2][m\ GTPase] \quad \text{(C.18)}$$

where we can replace $[X_1 - X_2]$ with $[X_1][X_2]$ as $[X_1] + [X_2] \underset{k_{m12}}{\overset{k_{d12}}{\rightleftharpoons}} [X_1 - X_2]$ leads to rate equation $\frac{d[X_1]}{dt} = k_{m12}[X_1 - X_2] - k_{d12}[X_!][X_2] = 0$ in equilibrium.

Proceeding analogously to the one effector case, ignoring the higher order terms $\mathcal{O}([GTPase]^3)$ again, we obtain:

$$-\frac{d\log[GTP]}{dt} = \overbrace{k_1'}^{k_1}[GTPase] + \left(\overbrace{\frac{k_2'}{2C_d} + k_{3,c1}' - \frac{k_1'}{C_d}}^{k_2}\right)[GTPase]^2$$

$$+\overbrace{k_{3,X_1}'}^{k_{3,X_1}}[X_1][GTPase] + \overbrace{k_{3,X_2}'}^{k_{3,X_2}}[X_2][GTPase] + \overbrace{k_{3,X_1-X_2}'}^{k_{3,X_1-X_2}}[X_1][X_2][GTPase] \Longrightarrow$$

$$-\frac{d\log[GTP]}{dt} = \overbrace{k_1[GTPase]}^{K_1^*} + \overbrace{k_2[GTPase]^2}^{K_2^*} + \overbrace{k_{3,X_1}[GTPase][X_1]}^{K_{3,X_1}^*} + \overbrace{k_{3,X_2}[GTPase][X_2]}^{K_{3,X_2}^*} \\ + \overbrace{k_{3,X_1-X_2}[GTPase][X_1][X_2]}^{K_{3,X_1-X_2}^*}$$

Taking into account the $[GTPase]$ correction factors, this becomes:

$$-\frac{d\log[GTP]}{dt} = \overbrace{k_1 c_{corr}[GTPase]}^{K_1} + \overbrace{k_2 c_{corr}^2[GTPase]^2}^{K_2} + \overbrace{k_{3,X_1} c_{corr}[GTPase][X_1]}^{K_{3,X_1}} \\ + \overbrace{k_{3,X_2} c_{corr}[GTPase][X_2]}^{K_{3,X_2}} + \overbrace{k_{3,X_1-X_2} c_{corr}[GTPase][X_1][X_2]}^{K_{3,X_1-X_2}}$$

The last term represents an interaction term between effectors, indicating a possible synergy between the effects of the proteins on the GTPase cycle. As with the single effector case, the effectors can be linear or quadratic, such that e.g., for a quadratic effector X_1 every instance of $[X_1]$ can be replaced by $[X_1]^2$ if X_1 is known to dimerize into a more active form and/or to be abundant in dimers.

Fitting restrictions on parameters

Summarizing the three cases of 0, 1 and 2 effectors, we have seen that the rate equations read respectively:

$$-\frac{d\log[GTP]}{dt} = k_1 c_{corr}[GTPase] + k_2 c_{corr}^2 [GTPase]^2 + \sum_{X=\{X_1,X_2,X_1-X_2\}} k_{3,X} c_{corr}[GTPase][X]$$

where $c_{corr} = 1$ for the zero-effector case. This means we have 2, 4 or 6 parameters in the 0, 1 and 2 effector cases respectively.

However, when fitting this function, we must also take into account restrictions on the parameters we know must exist. A GTPase by default must contribute positively to hydrolysis, So the main term with k_1 must be positive, i.e. $k_1 > 0$. As the 'crocodile term' k_2 might include multiple effects such as crowding, we do not impose this restriction on k_2. Similarly, for assay with effectors where only the effector concentration is varied, the equation takes the form of $a + b[X_1] + c[X_2] + d[X_1][X_2]$, where we assume the contribution a of the GTPases without effector must be positive.

For this purpose, the code implementation of the model transforms all k's to an alternative parameter space. Concretely, we define:

$$a = \log(k_1)$$

$$b = k_2$$

when fitting the no effector case:

$$-\frac{d\log[GTP]}{dt} = a[GTPase] + b[GTPase]^2$$

When fitting with effectors, we define:

$$a = \log(k_1 c_{corr}[GTPase] + k_2 c_{corr}^2 [GTPase]^2)$$

$$b = k_{3,X_1} c_{corr}[GTPase]$$

$$c = k_{3,X_2} c_{corr}[GTPase]$$

$$d = k_{3,X_1-X_2} c_{corr}[GTPase]$$

when fitting the effector case:

$$-\frac{d\log[GTP]}{dt} = a + b[X_1] + c[X_2] + d[X_1][X_2]$$

The individual rate constant estimate of k_3's can then be retrieved by dividing out the c_{corr} obtained as explained in the GTPase concentration correction factor section in Case 2: Adding a single effector.

Moreover, we restrict the concentration correction factor to the range 0.1 to 10 to avoid an apparent e.g., negative activity or extreme overactivity of a GTPase in a particular assay. Runs hitting these bounds for c_{corr} are indicative of a possible issue.

Error propagation

Ultimately, we obtain pooled estimates of cycling rates k_1 and k_2 and if applicable, k_3's which also has standard errors as explained in the pooling section in the General model outline. Originally, we have errors on the measurements of the GTP concentration. The squared reciprocals of these errors provide the weights for the non-linear regression in Matlab's fitnlm. We then generate random draws of the fitting parameter values in the alternative parameter space (see section Fitting restrictions on parameters) through multivariate normal random variables with the parameter covariance matrix of fitnlm. These draws are then transformed back and if needed divided by the concentration correction factor and the $[GTPase]$ to obtain the draws of the original parameters. Uncertainty in concentration correction factors are not propagated as mentioned in the section on these factors to not excessively inflate the rate parameter estimate errors. Finally, we use the standard deviations of these draws for the standard errors to use in the pooling regression as explained in the pooling section.

Optional crowding addition

Sometimes, crowding effects between proteins may be expected to play a role in the reaction rates. These effects can be accommodated in the Crocodile model. From literature, it is not evident whether we should expect a positive or negative contribution of crowding to the reaction rate (Kim and Yethiraj, 2009). Therefore, we approximate this as the same linear dependency of all reaction rate constants k', related to the GTPase cycle reactions, on the total protein concentration. This dependency can hence be positive or negative. Concretely, the rate constants then change through:

$$k_i' = k_{i,0}'(1 + k_s'[P_{tot}]) \qquad (O.1)$$

with $[P_{tot}]$ as the total concentration of proteins present and k_s' as a constant that can be positive, 0, or negative.

As we show below, we retain the same functional forms for the GTP hydrolysis rate. The only change is the way to interpret K_2 and the K_3's (and concordantly, k_2, the k_3's, K_2^* and the K_3^*'s), which now includes a crowding term. Incidentally, as K_2 now encompasses crowding, cooperativity and dimerization (of luminescence experiments), this can be dubbed the crocodile-term.

Single effector case

We can insert crowding in C.13 through O.1 such that e.g., $k_1' = k_{1,0}(1 + k_{s,0}')[P_{tot}]$ with $[P_{tot}] = [GTPase] + [X]$ and similarly for k_2' and k_3'. Ignoring as before the higher order terms $O([GTPase]^3)$, we get:

$$-\frac{d\log[GTP]}{dt} = \overbrace{k_{1,0}'}^{k_1}[GTPase] + \overbrace{\left(\frac{k_{2,0}'}{2C_d} + k_{3,c1,0}' - \frac{k_{1,0}'}{C_d} + k_{1,0}'\right)}^{k_2}[GTPase]^2$$

$$+ \overbrace{\left(k_{3,X,0}' + k_{1,0}'k_{s,0}'\right)}^{k_3}[X][GTPase]$$

This is the same functional form as before in C.14, only the interpretation of k_2 and k_3 contains crowding.

Double effector case

We follow the same logic starting from C.18, only this time incorporating crowding through O.1 with $[P_{tot}] = [GTPase] + [X_1] + [X_2]$. Ignoring the higher order terms $\mathcal{O}([GTPase]^3)$ yields:

$$-\frac{d\log[GTP]}{dt} = k'_{1,0}[GTPase] + \left(\frac{k'_{2,0}}{2C_d} + k'_{3,c1,0} - \frac{k'_{1,0}}{C_d} + k'_{1,0}k'_{s,0}\right)[GTPase]^2$$

$$+\left(k'_{3,X_1,0} + k'_{1,0}k'_{s,0}\right)[X_1][GTPase] + \left(k'_{3,X_2,0} + k'_{1,0}k'_{s,0}\right)[X_2][GTPase]$$

$$+\left(k'_{3,X_1-X_2,0} + k'_{1,0}k'_{s,0}\right)[X_1][X_2][GTPase] \Longrightarrow$$

$$-\frac{d\log[GTP]}{dt} = \overbrace{k_1[GTPase]}^{K_1^*} + \overbrace{k_2[GTPase]^2}^{K_2^*} + \overbrace{k_{3,X_1}[GTPase][X_1]}^{K_{3,X_1}^*} + \overbrace{k_{3,X_2}[GTPase][X_2]}^{K_{3,X_2}^*}$$
$$+ \overbrace{k_{3,X_1-X_2}[GTPase][X_1][X_2]}^{K_{3,X_1-X_2}^*}$$

Once more, we obtain the same functional form as without crowding, only the interpretation of k_2 and the k_3's is broadened.

Bibliography

Heij, C., de Boer, P., Franses, P. H., Kloek, T., & van Dijk, H. K. (2004). Econometric methods with applications in business and economics. Oxford University Press.

Kim, J.S., and Yethiraj, A. (2009). Effect of macromolecular crowding on reaction rates: a computational and theoretical study. Biophys. J. 96, 1333–1340.

Tschirpke, S., Daalman, W., and Laan, L. (2023). The GEF Cdc24 and GAP Rga2 synergistically regulate Cdc42 GTPase cycling. BioRxiv.