Parameter measurements for synthetic biology

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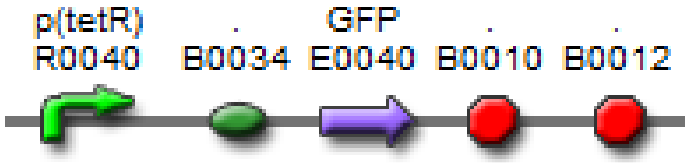
The problems

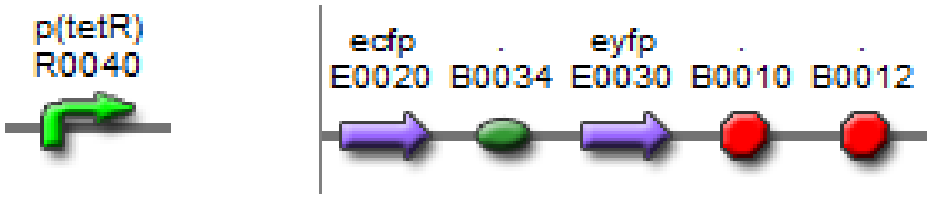
- Cells are inherently noisy environments.
- Measuring parameters *in vivo* is technically challenging.
- Many biological parameters may affect the performance of a part or device.
- A part may interact with the host cell in unexpected ways, altering its performance.
- Most parameters are measured from populations of cells. Even genetically identical cells show great heterogeneity in their proteome and physiology.

Current parts are mainly based on transcriptional activators/repressors



- Promoter – RNA polymerase binding
- RBS – Ribosome binding
- Gene – encodes protein to be expressed
- Terminator – prevents further transcription

What outputs would we like to measure?



- *Transcription* - **Po**lymerase **P**er **S**econd (PoPS):
  The number of times that an RNA polymerase molecule passes a specific point on DNA per unit time.
- *Translation* - **R**ibosomal **i**nitiations **P**er **S**econd (RiPS): The number of ribosome molecules that pass a point on mRNA each second, on a per mRNA copy basis (RiPSmc = RiPS per mRNA copy).

- PoPS and RiPS are calculated indirectly by introducing a test gene with the same regulatory region but with a GFP protein coding region and measuring the fluorescence. Use a simple ordinary differential equation model to estimate the steady state PoPS and RiPS levels (for details see Canton et al. (2008) Nature Biotech. 26:787-93).

What could affect these parameters?

- Host cell type
- Growth medium
  - Oxygen
  - Carbon
  - Nitrogen
  - Light
- Growth phase of cells
- Metabolic state of cells
- Total “load” on cells

- How do we control growth to minimise these variations? Use of fermentors.

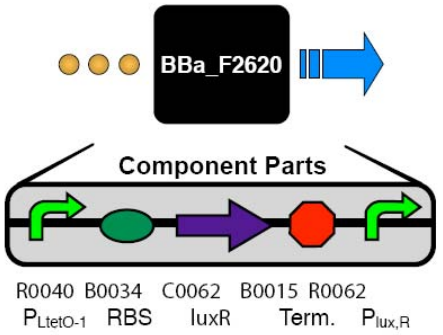
Input/output functions of a device

BBa_F2620

$3OC_6HSL \rightarrow$ PoPS Receiver

Mechanism & Function

A transcription factor (LuxR) that is active in the presence of a cell-cell signaling molecule ($3OC_6HSL$) is controlled by a regulated operator ($P_{LtetO-1}$). Device input is $3OC_6HSL$. Device output is PoPS from a LuxR-regulated operator. If used in a cell containing TetR then a second input such as aTc can be used to produce a Boolean AND function.



- Input – AHL
- Need to connect PluxR to reporter (eg GFP) to provide measurable output of protein production
- Convert from GFP protein molecules produced per cell to PoPS
- Output – PoPS gives mRNA production

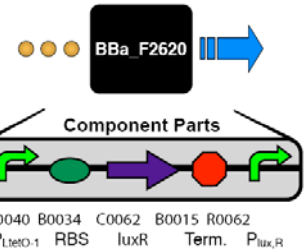
BioBricks Datasheet

- **Static performance:** Input/output relationship at steady state. Linear relationship between GFP production and PoPS.
- **Dynamic response:** Response kinetics to step change in AHL.
- **Input compatibility:** Specificity of output to correct AHL input.
- **Reliability:** Number of culture doublings over which performance is maintained.

- **Caution** – changing host cell, growth conditions and adding extra components may alter actual performance of a part!

BBa_F2620

3OC$_6$HSL → PoPS Receiver



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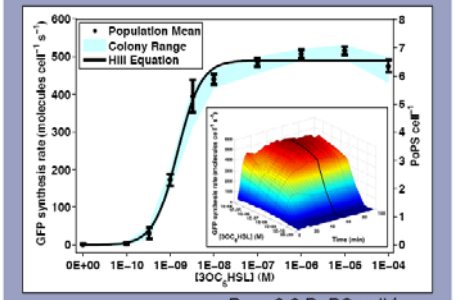
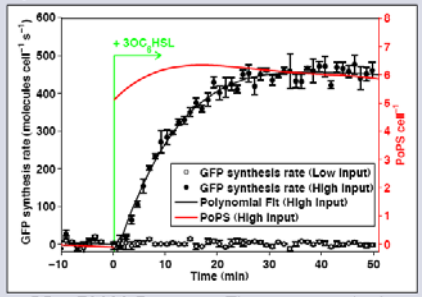
Static Performance*



$$P_{out} = \frac{P_{max}[3OC_6HSL]^n}{K^n + [3OC_6HSL]^n}$$

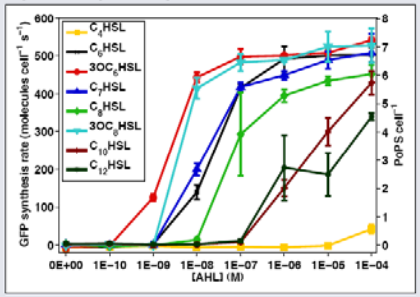
P_{max}: 6.6 PoPS cell^{-1}
K: 1.5E-09 M 3OC$_6$HSL
n: 1.6

Dynamic Performance*



BBa_F2620 Response Time: <1 min
BBa_T9002 Response Time: 6±1 min
Inputs: 0 M (Low), 1E-07 M (High) 3OC$_6$HSL

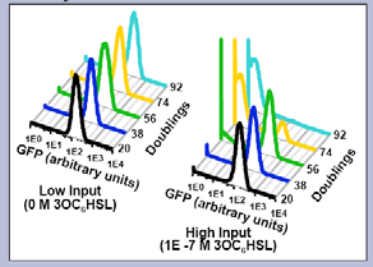
Input Compatibility*



Part Compatibility (qualitative)

Chassis: *MC4100*, *MG1655*, and *DH5α*
Plasmids: *pSB3K3* and *pSB1A2*
Devices: *E0240*, *E0430* and *E0434*

Reliability**



Genetic: >92/>56 culture doublings
Performance: >92/>56 culture doublings
(low/high input during propagation)

Transcriptional Output Demand (low/high input)
Nucleotides: **0 / 6xNt** nucleotides cell^{-1} s^{-1}
Polymerases: **0 / 1.5E-1xNt** RNAP cell^{-1}
(**Nt** = downstream transcript length)

Conditions (abridged)
Output: PoPS measured via *BBa_E0240*
Culture: Supplemented M9, 37°C
Plasmid: pSB3K3
Chassis: MG1655
**Equipment:* PE Victor3 multi-well fluorimeter
***Equipment:* BD FACScan cytometer

Authors: Barry Canton, Ania Labno
Updated: March 2008

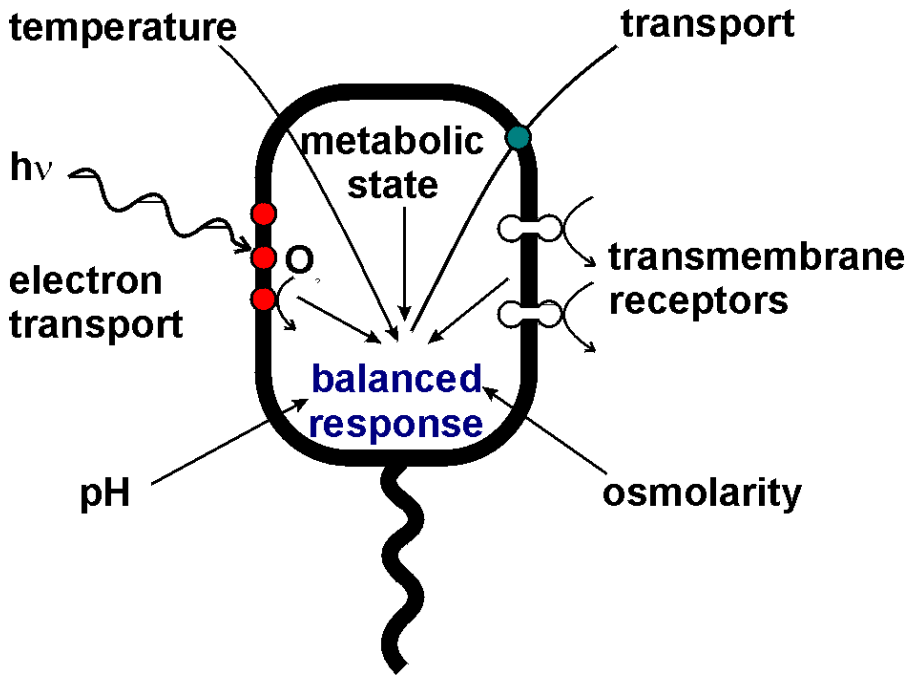
Registry of Standard Biological Parts
making life better, one part at a time

Signaling Devices

http://parts.mit.edu/registry/index.php/Part:BBa_F2620

Measuring parameters in natural bacterial signalling pathways

- We need to understand new biological modules before we can begin to exploit them for synthetic biology.
- We want to understand how sensitivity and robustness are achieved in natural biological systems.

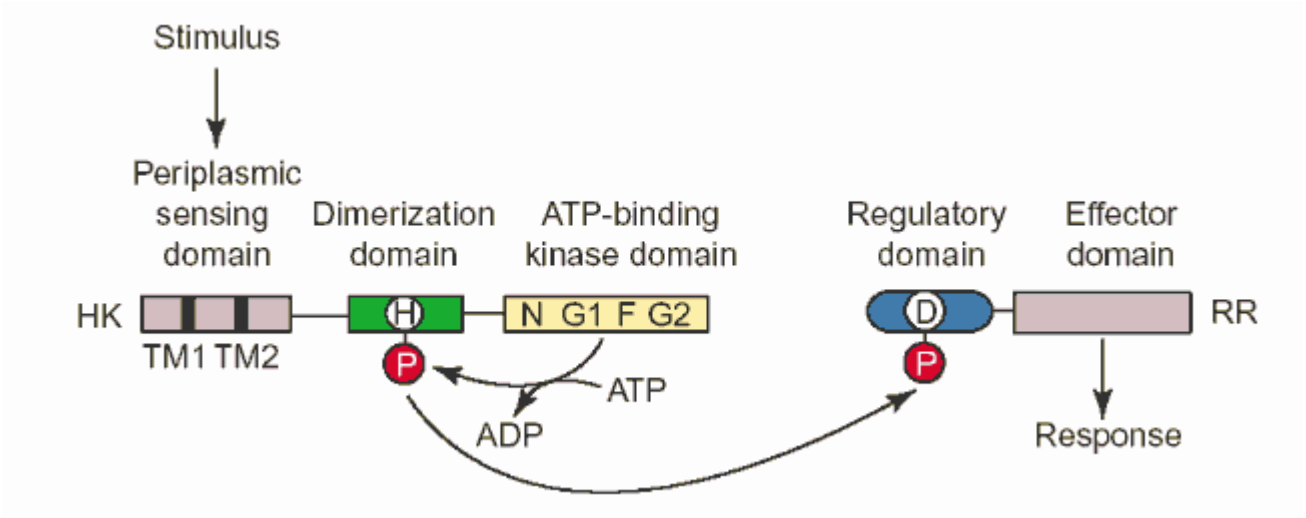


To do this we would like to know:

- the number of molecules of each protein within the pathway
- the localisation of each protein
- their binding kinetics
- their dynamics and turnover

Alternative pathways – phosphorylation cascades

- Two component signalling pathways

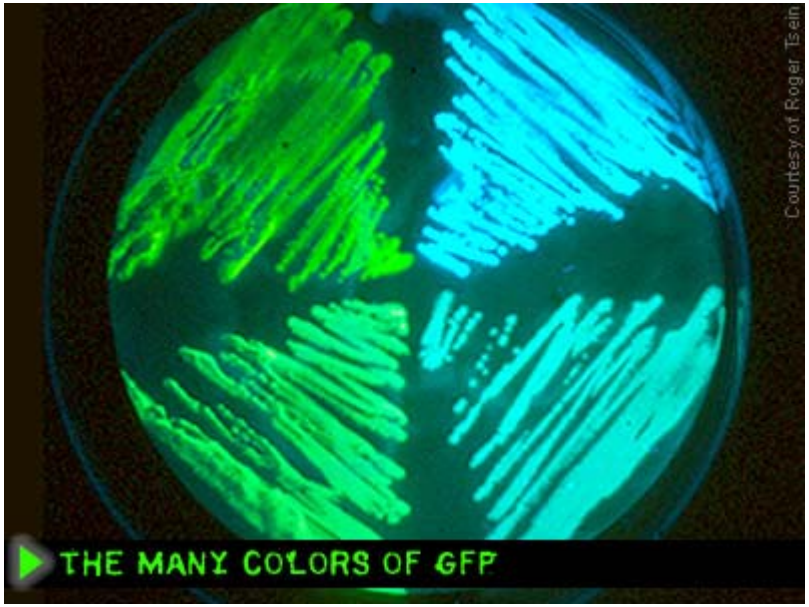


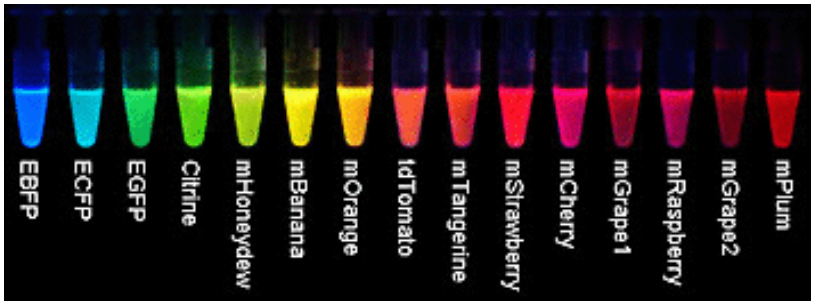
Bacterial cells can have as many as 250 of these pathways operating in a single cell.

Response is often a change in gene expression

Green Fluorescent Protein and its derivatives

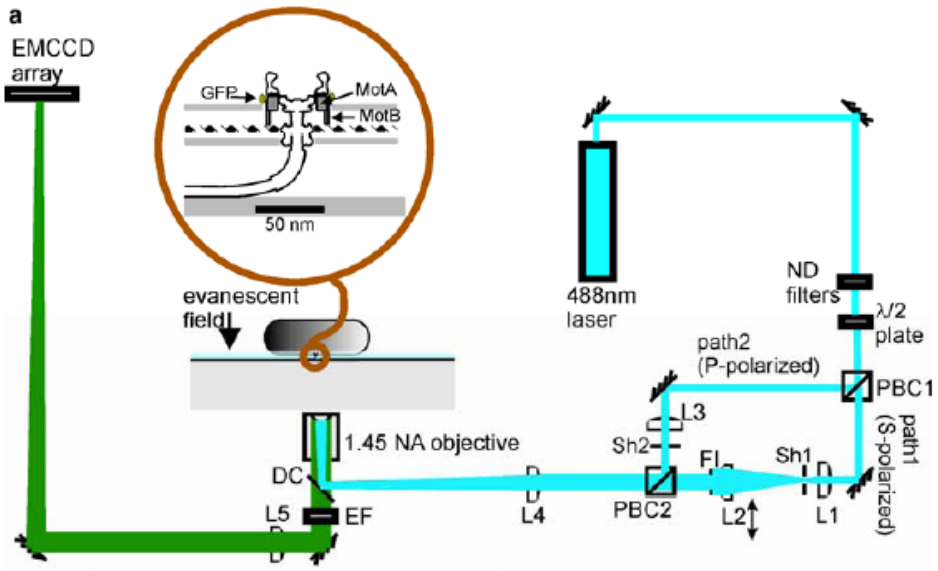
- Mw c. 28kDa
- Radius c. 4nm
- Monomeric in solution
- GFP fusion proteins usually retain biological activity (N- and C-terminal) but must check!
- Multiple GFP derivatives allow multiple tags to be detected in a single cell
- Easily measured by UV light, fluorescence microscopy, or FACS
- Applicable to many systems
- Chromophore maturation requires molecular oxygen





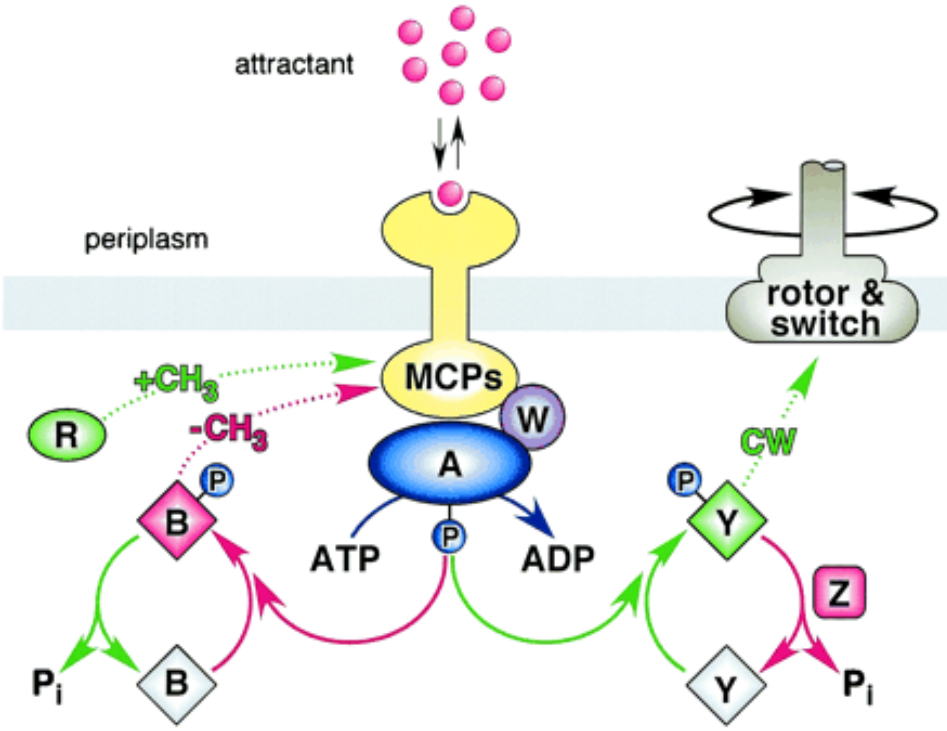
Protein visualisation and quantification

- **Fluorescence microscopy** (epi and TIRF)
  - protein localisation, concentration and dynamics
- **FACS**
  - protein concentration and cell separation
- **Fluorescence Correlation Spectroscopy**
  - protein concentration, dynamics and interactions

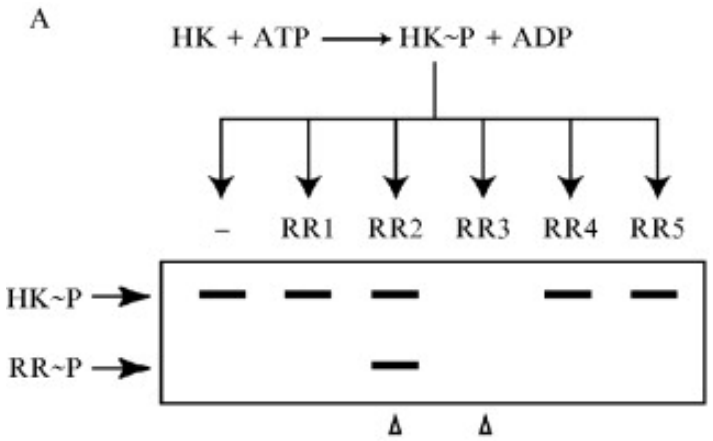


A “well characterised” signalling pathway in bacteria

- Decrease in [attractant] or increase in [repellent] causes the MCP to activate CheA autophosphorylation.
- CheA-P phosphotransfers to CheY and CheB
- CheY-P diffuses in ~100ms to the motor and causes switching
- CheZ causes signal termination
- Increased CheB-P causes receptor adaptation



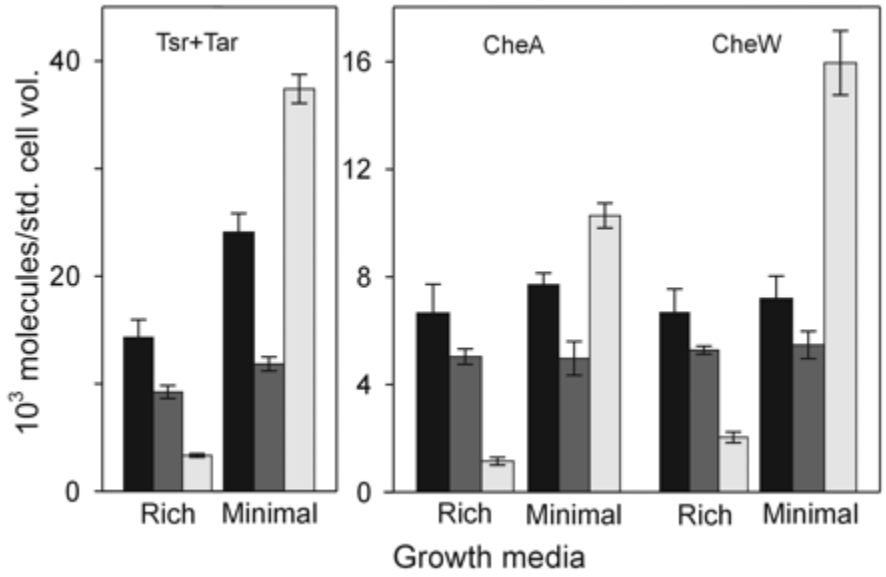
Different values for the same parameter (*in vitro*)!



- CheA —> CheAp

0.11 s^{-1}	With 5 mM Mg^{2+} and 2 mM ATP (Wolanin et al., 2006)
0.039 ± 0.004 s^{-1}	With 5 mM Mg^{2+} and 1 mM ATP (Zhao & Parkinson, 2006)
0.048 ± 0.005 s^{-1}	With 10 mM Mg^{2+}; K_M for ATP is 0.38 ± 0.02 mM (Stewart, 2005)
0.024 ± 0.005 s^{-1}	With 5 mM Mg^{2+} and 1 mM ATP (Jahreis et al., 2004)
0.12 ± 0.01 s^{-1}	With 5 mM Mg^{2+} and 2 mM ATP (Kott et al., 2004)
0.074 s^{-1}	With 5 mM Mg^{2+} and 4 mM ATP (Shrout et al., 2003)
0.4 s^{-1}	With 5 mM Mg^{2+} and 2 mM ATP (Francis et al., 2002)
0.106 ± 0.008 s^{-1}	With 5 mM Mg^{2+} and 2 mM ATP (Levit et al., 2002)
0.050 ± 0.005 s^{-1}	With 10 mM Mg^{2+} and 1 mM ATP; K_M for ATP is 0.3 ± 0.1 mM (Hirschman et al., 2001)

Differences in protein copy number with *E. coli* strain and growth medium

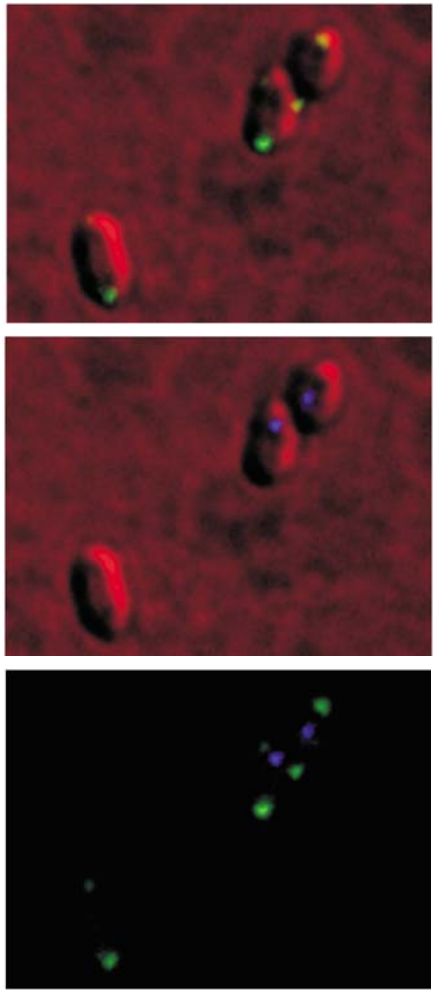


- Cellular content of chemotaxis proteins in three strains grown in two media. Despite wide variations in protein levels, the signalling pathway still works in all cases.

Li and Hazelbauer (2004). J. Bact. 186:3687-94

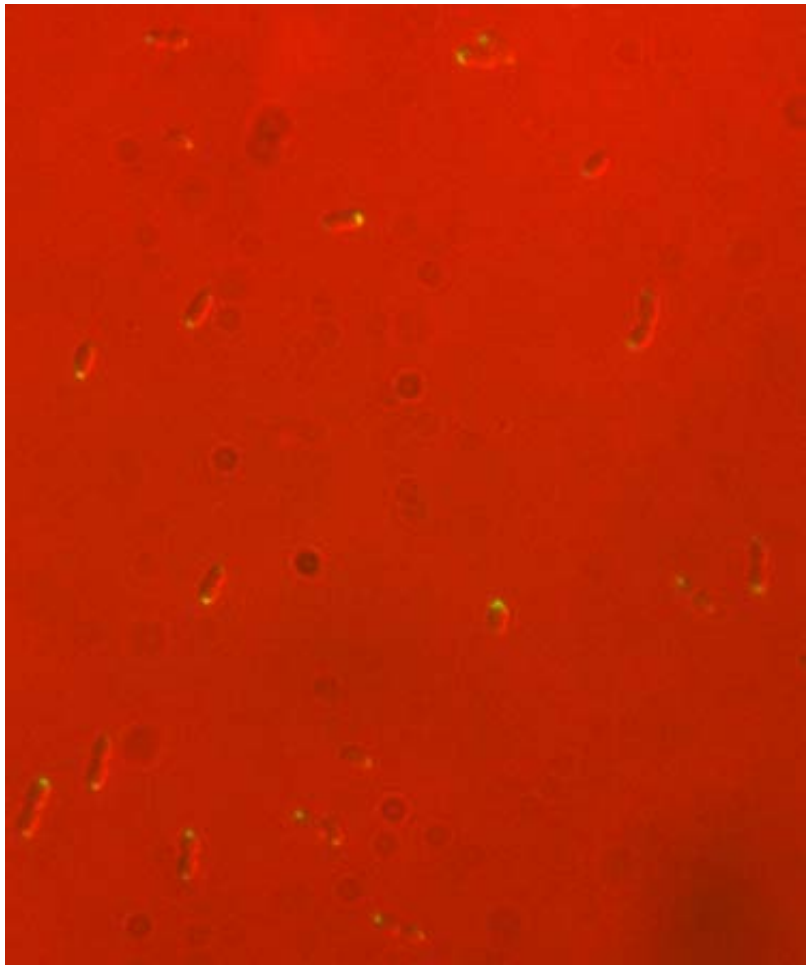
Localisation of chemotaxis proteins

- Protein localisation can effect local concentrations
- Reactions which happen *in vitro* may be prevented *in vivo* by spatial segregation of the proteins.
- This may effect their use in synthetic pathways
- These data are highlighting the importance of protein co-localisation in the kinetics of signalling pathways.



Concentration of chemotaxis proteins

- Calculate the exact number of molecules of a protein in an individual cell by fluorescence.
- Is the absolute concentration of proteins important or is the ratio of proteins in a particular system more important for function?
- How do protein concentrations and their fluctuations effect robustness and sensitivity of the system?

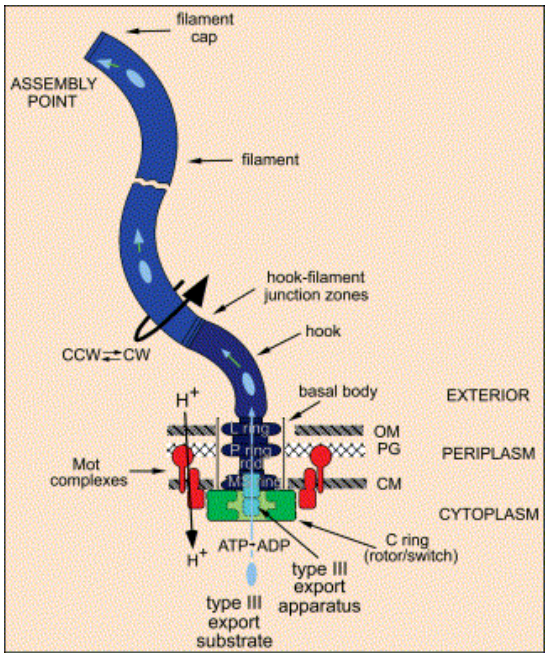


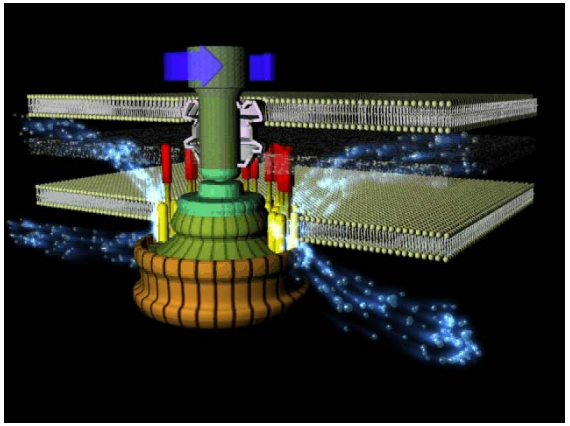
Lessons from studying chemotaxis proteins

- Host strains and growth conditions can have a huge effect on protein levels in cells
- Even genetically identical cells show significant heterogeneity in protein levels
- Protein localisation can effect local concentrations and reaction kinetics
- Even *in vitro* assays with purified proteins can give differing parameters
- It is essential to understand how parameters have been obtained if they are to be used successfully in synthetic biology

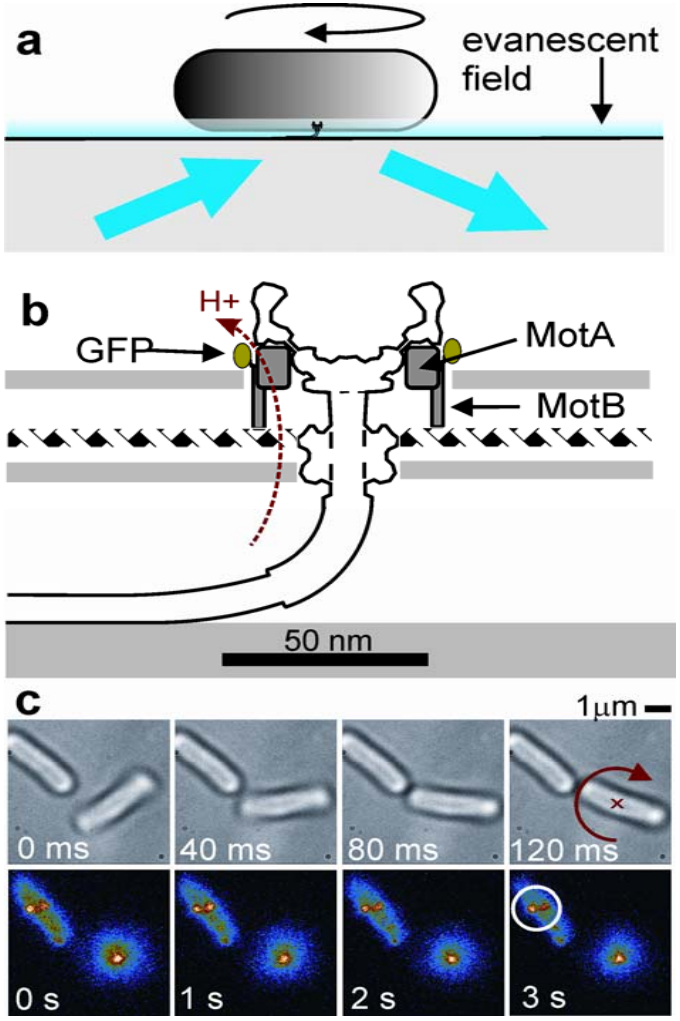
The flagellar motor

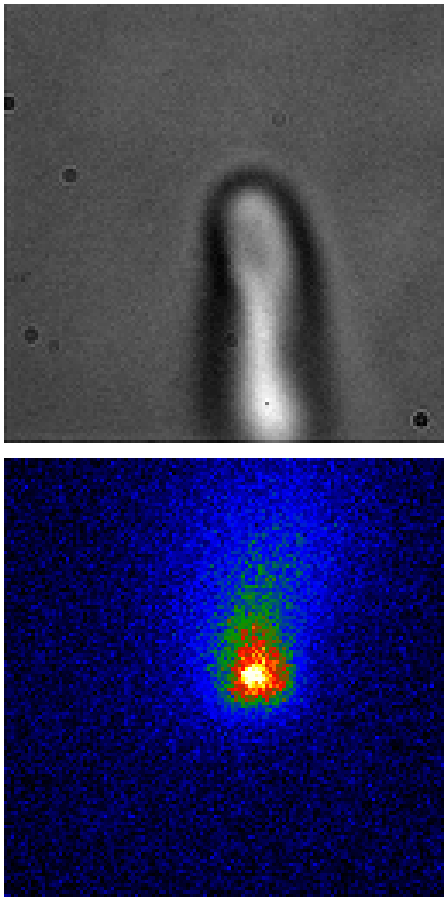
- Most complex biological rotary motor
- 45 nm diameter
- Rotates ~300Hz
- Bi-directional
- >30 different proteins, multiple copies of each
- Can not be isolated



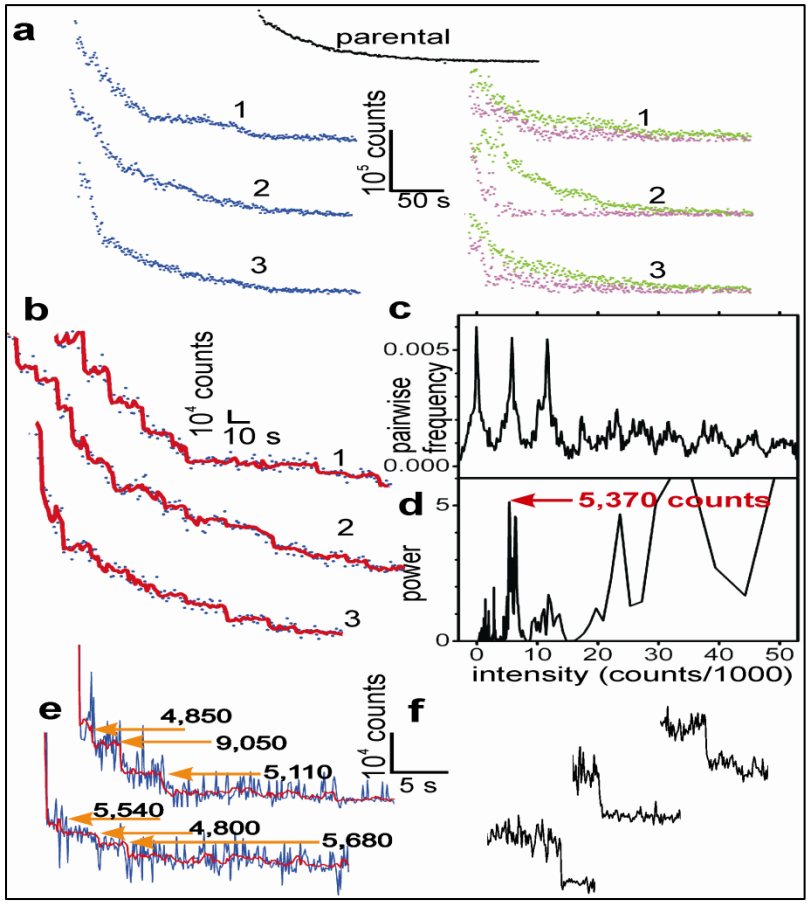


GFP-MotB: genomic replacement

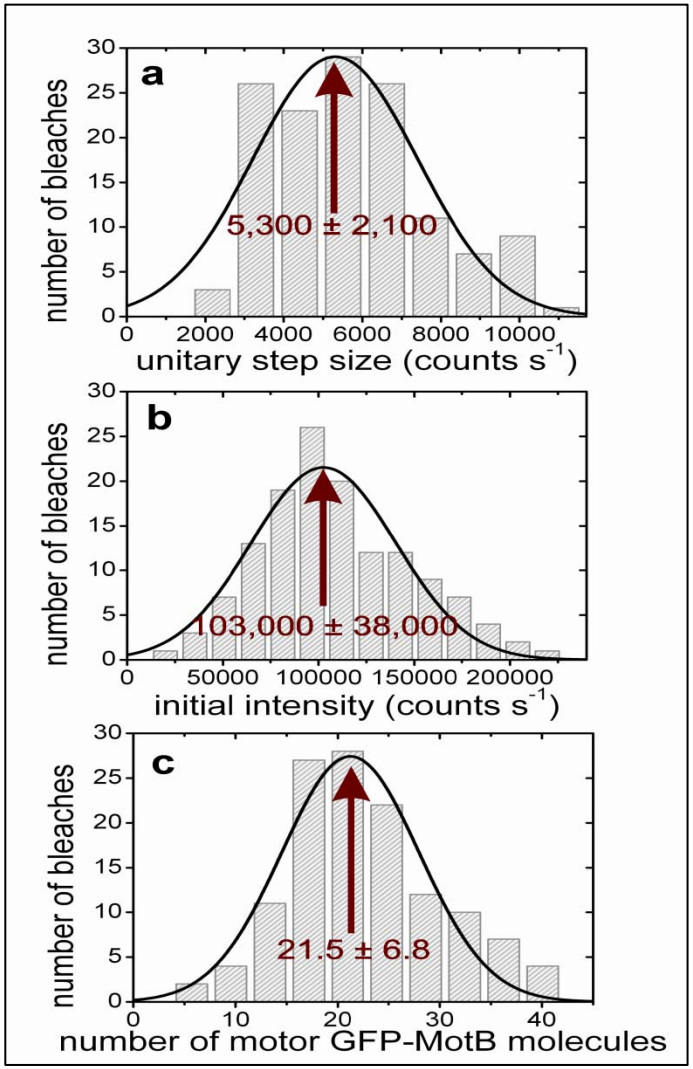




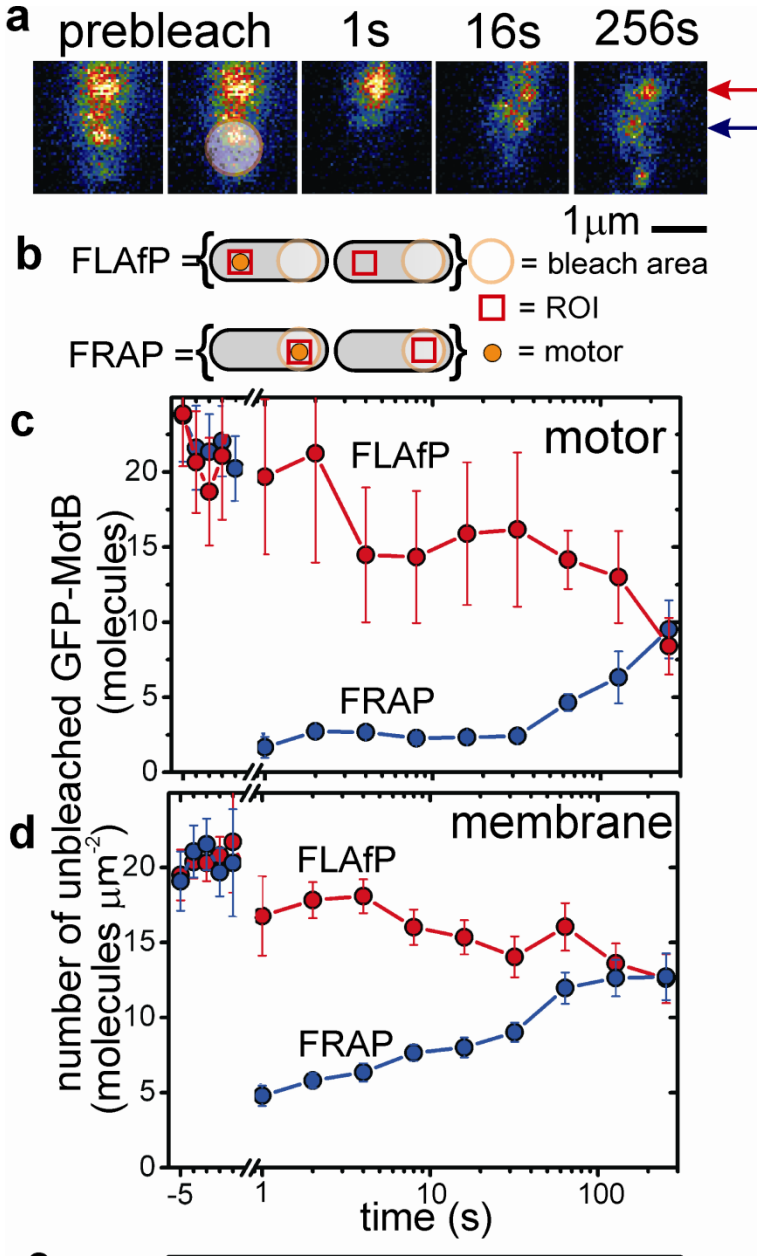
Bleaching rate

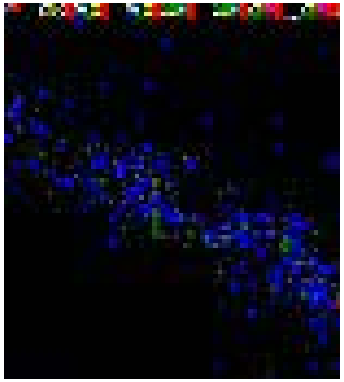


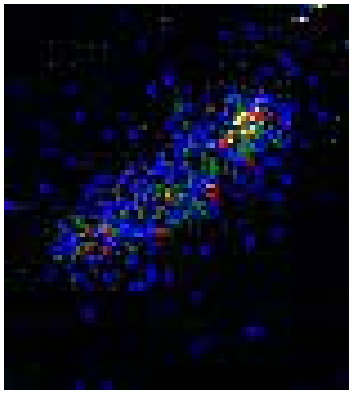
22 MotB's per motor (=11 stators)



Calculation of diffusion and turnover rate

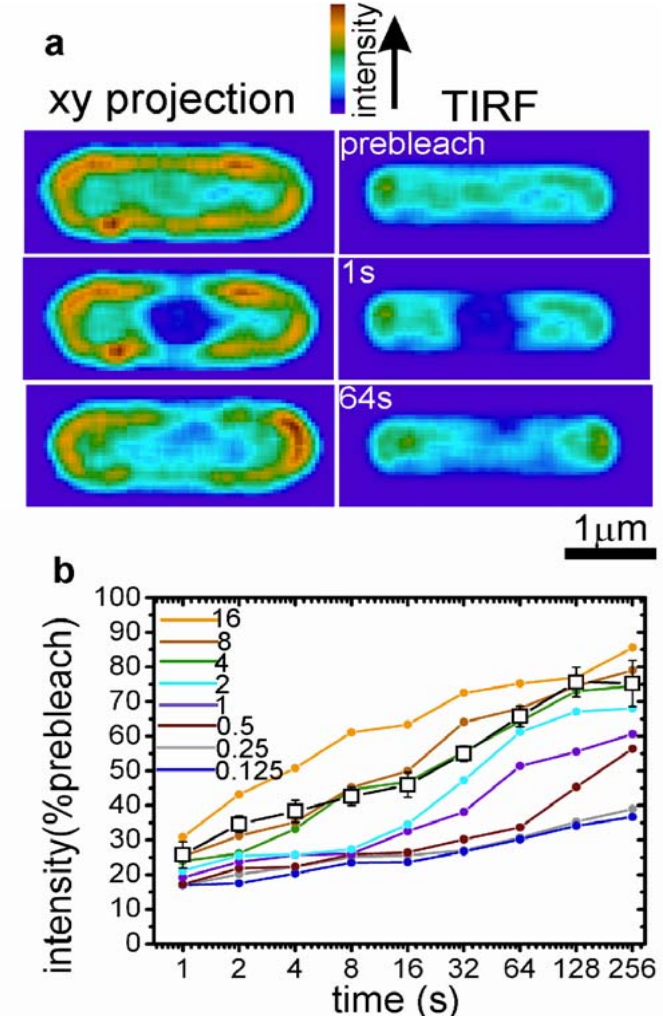






Examining dynamics of complexes, single proteins and interaction kinetics

Modelling of diffusion rate



- Model cell with 100 GFP-MotB dimers free to diffuse in 2D within the membrane
- Simulate photobleach
- Model recovery (FRAP and FLIP) with different *D* for the GFP-MotB dimers
- See which value of *D* best reflects observed data.

$D = 0.0075 \pm 0.0013\ \mu m^2\ s^{-1}$ (mean ± s.d.)

Stator turnover- first measurement of protein dynamics in functioning protein complex under native conditions

- ~11 MotB dimers per motor (it probably does vary), with membrane pool of ~200 MotB
- Dwell time of MotB in motor about 0.5s
- Dissociation rate ~0.04s^{-1} = exchange of ~0.44 stators per sec (complete stator ring per minute)

- Not all protein in a cell may be in a functional complex!
- Method for replacing faulty components?

Leake *et al.* Nature (2006) 443(7109):355-8

Current research

- Measure parameters of existing two component systems in bacteria
- Quantify effects of making targeted mutations that alter rates and specificities of phosphotransfer reactions
- Use modified versions of these components to generate novel and useful signalling pathways

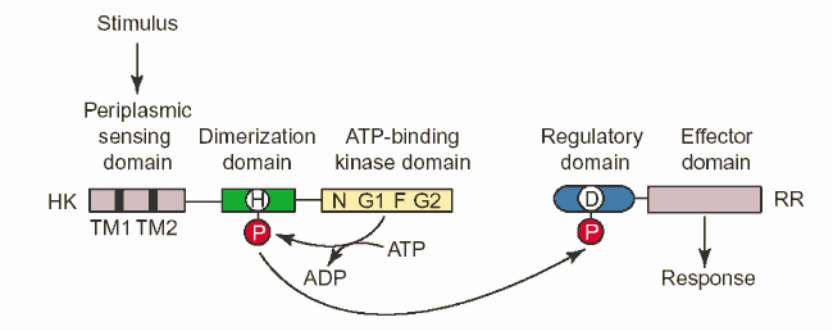
Applications to synthetic biology

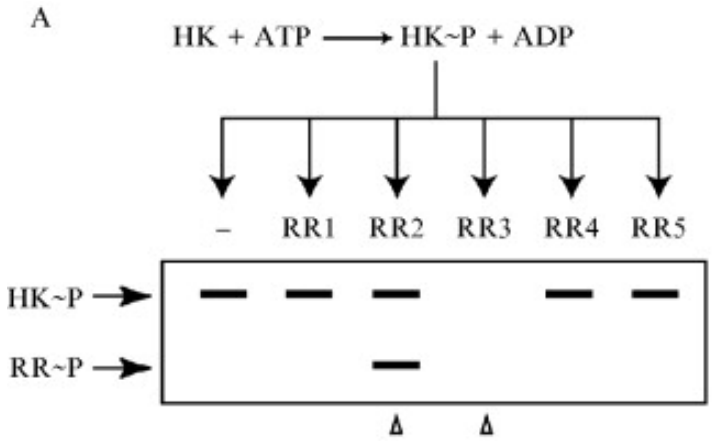
- Two component systems provide a wide range of useful modules for synthetic biologists.
- These modules must be characterised in their native hosts before we can adapt them for use in synthetic biology
- Measuring parameters in phosphorylation signalling pathways is technically challenging

- We are currently characterising and manipulating bacterial two component signalling systems for use as modules in synthetic signalling pathways

Changing phosphotransfer reactions

- Alter input or output domains
- Alter interacting partners
- Alter kinetics of phosphorylation and dephosphorylation
- Produce novel signalling pathways with defined input/output characteristics





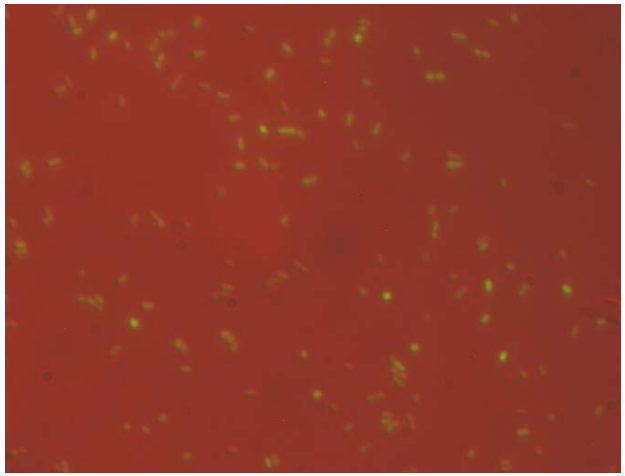
The choice of host cell type and cross-talk

- Introduce *E. coli* promoter into different species (*R. sphaeroides*)
- Introduce transcriptional activator OmpR
- Proves that *R. sphaeroides* can recognise *E. coli* promoters and induce transcription
- However, OmpR is constitutively phosphorylated in *R. sphaeroides*, presumably by a native kinase.

- Shows promise that systems can be transferred between species but highlights problems of cross-talk.

P*ompC-yfp*

P*ompC-yfp*, OmpR



Measurements in synthetic biology

- Host cell type and growth conditions greatly affect the levels of protein production.
- Not all proteins are evenly distributed throughout the cell and there is significant variation in protein copy number between genetically identical cells.
- Understanding the dynamics and interactions of proteins *in vivo* is essential for our understanding of the kinetics of biological pathways.
- Turnover of proteins in complexes may provide a mechanism for replacing faulty components.
- Two component systems may provide a vast resource which can be exploited in systems biology, but specifics such as parameters and cross-talk need to be carefully determined.

Acknowledgements

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