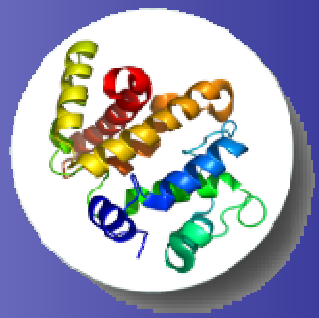
frank.niesen@sgc.ox.ac.uk http://www.thesgconline.org ftp://ftp.sgc.ox.ac.uk/pub/biophysics



SGC

Differential Scanning Fluorimetry: Detection of ligands and conditions that promote protein stability and crystallization

Frank Niesen

PX school 2008, Como, Italy

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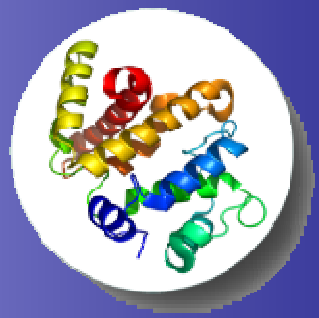
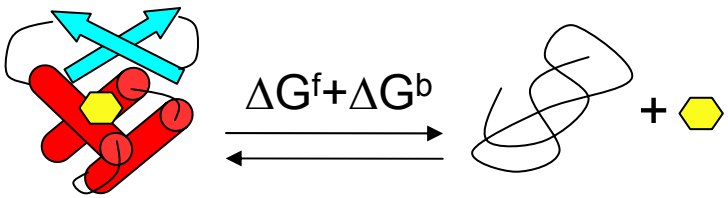


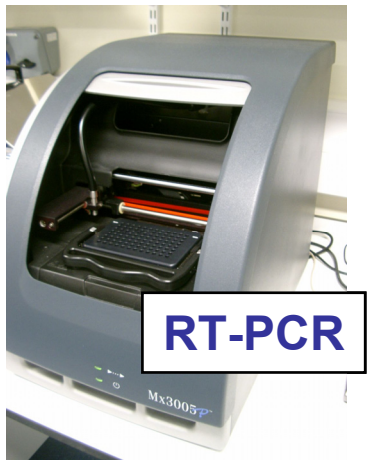
SGC

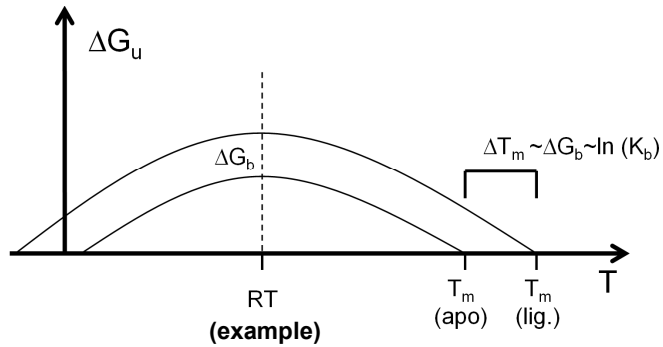
Topics

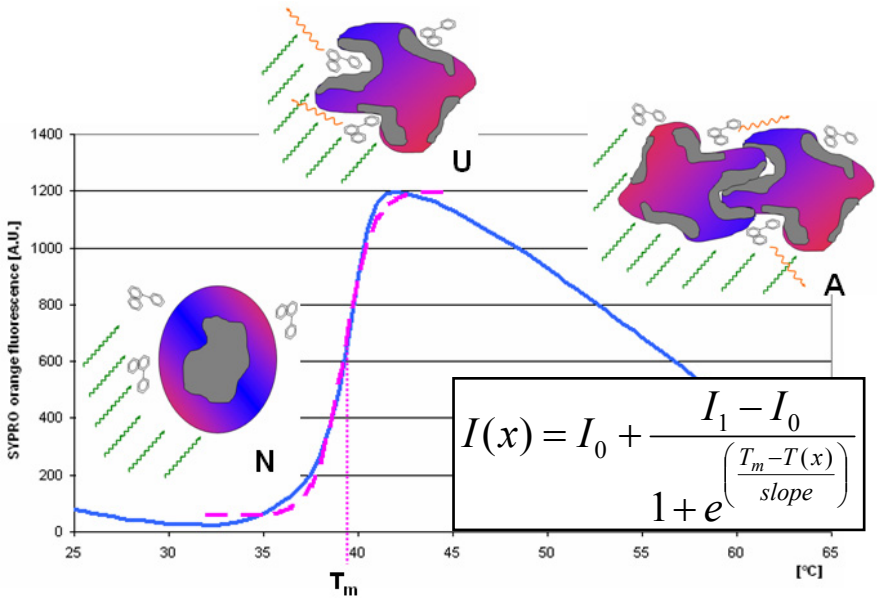
- Method introduction
- Applications and Limitations
- Examples for the application of DSF to the work of the SGC
- Analysis tools
- Outlook

Method introduction









- Stability of protein is related to ΔG_u (Gibbs free energy of unfolding); inverse proportional to the temperature
- Equilibrium: $\Delta G_u = 0$, $n_n = n_u$; T_m (mid-point temperature, "melting temperature")
- Free energy contribution of ligand, ΔG_b, most likely increases ΔG_u and T_m
- DSF monitors thermal unfolding in presence of a fluorescent dye (fluorescence quenched in aqueous solution, but not in non-polar environment, e.g. hydrophobic sites on unfolded proteins)

Niesen *et al*. (2007) *Nature Protoc.* **2(9)**, 2212-2221

Applications and Limitations

Applications

- Thermal stability?
- Increase in thermal stability by generic additives (salts, nucleotides, etc.) or change of pH?
- Detection of a stabilizing ligand?
- Extent of stabilization by a known ligand?

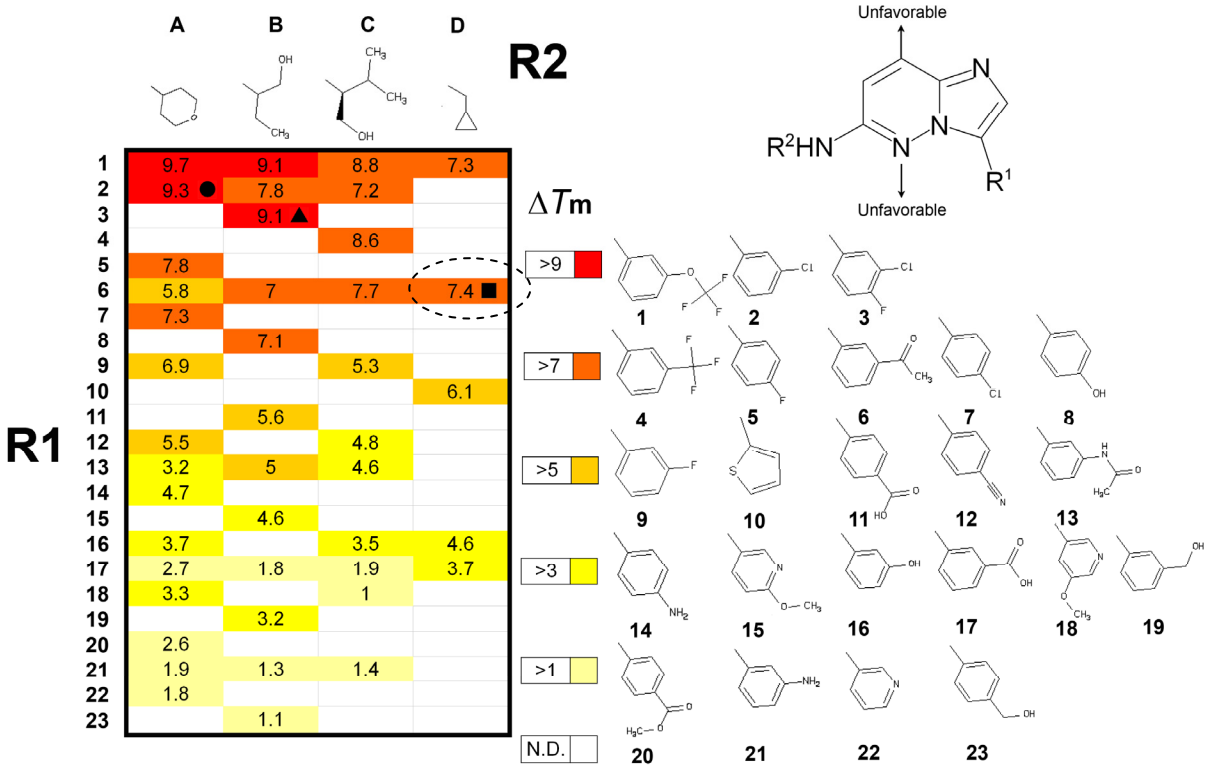
- Multi-domain protein – unfolding cooperatively?
- Influence of a known ligand on mode-of-unfolding (e.g. of multi-domain proteins)?
- High or low flexibility?
  'flexibility' ≡ number of differently stable conformational states:
  - steep transitions = highly cooperative unfolding
  - shallow transitions = high flexibility

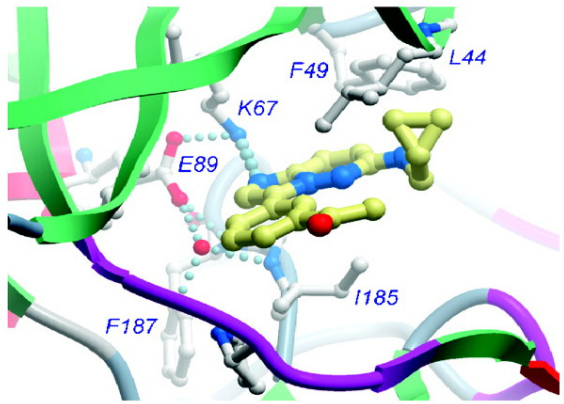
Limitations

- Interactions between compounds and dye may mask stabilization or give rise to artifacts.
- Coloured compounds may interfere with optical detection.
- Difficult interpretation of effects on multi-domain or oligomeric proteins that show non-two state (i.e. multi-phasic) unfolding
- Dyes currently available for high-throughput (RT-PCR) not applicable to conditions comprising hydrophobic additives such as detergents (most often necessary for membrane proteins).

Application of DSF to the work of the SGC

Identification of a novel family of PIM kinase inhibitors / compound SAR:





Pogacic *et al.* (2007): *Cancer Res.* **67(14)**, 6916-6924.
(collaboration of SGC [group S. Knapp] with J. Schwaller, University of Basel)

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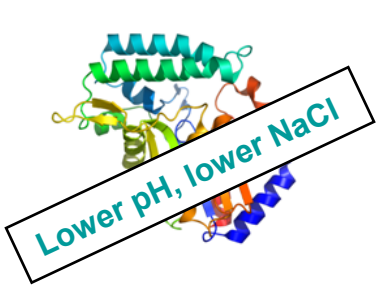
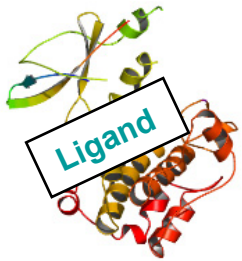
Application of DSF to the work of the SGC

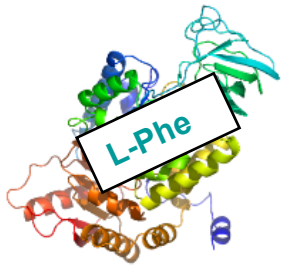
Aid in structure determination for human and Apicomplexan protein targets:



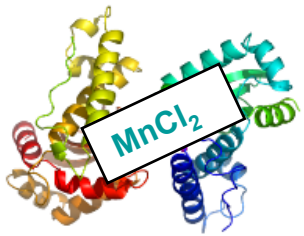
234. NT5C3
Human Cytosolic 5'-Nucleotidase III (cN-III)
PDB: 2cn1 (2006/05/17)



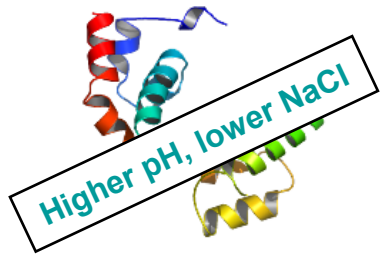
74. PAK4
Human p21(CDKN1A) activated kinase 4
PDB: 2BVA (2005/06/23)



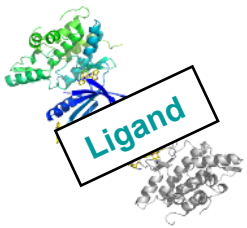
49. PKM2
Human Pyruvate Kinase, Muscle
PDB: 1ZJH (2005/04/28)



Pk-SOD1
Plasmodium knowlesi Superoxide Dismutase 1 with manganese bound
PDB: 2AWP (2005/09/01)



63. RGS17
Human regulator of G-protein signalling 17
PDB: 1ZV4 (2005/06/01)



68. STK16
Human Serine/Threonine kinase 16
PDB: 2BUJ (2005/06/13)

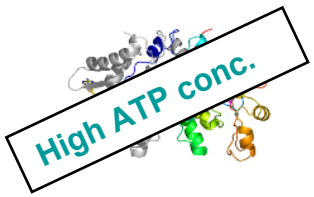
200. CSNK1G3
Human Casein Kinase I gamma 3
PDB: 2CHL (2006/03/16)

28. CLK1
Human Cdc-like Kinase
PDB: 1Z57 (2005/03/17)

134. RGS6
Human regulator of G-protein signalling 6
PDB: 2ES0 (2005/10/25)

140. MAP3K3
Human MAP/ ERK kinase 3, Phox/ PB1 domain
PDB: 2C60 (2005/11/04)

127. CSNK1G2
Human Casein Kinase 1 Gamma 2 in complex with 5-Iodotubercidin
PDB: 2C47 (2005/10/16)



110. PAPSS2
Human ATP kinase domain of 3'-phosphoadenosine-5'-phosphosulfate
PDB: 2AX4 (2005/09/02)

96. Py-ADA
Plasmodium yoelii Adenosine Deaminase
PDB: 2AMX (2005/08/11)

61. RGS16
Human regulator of G-protein signalling 16
PDB: 2BT2 (2005/05/25)

218. NEK2
Human never in mitosis gene A-related kinase 2
PDB: 2JAV (2006/04/25)

Vedadi *et al.* (2006) *Proc. Natl. Acad. Sci. USA* **103(43)**,15835-15840.

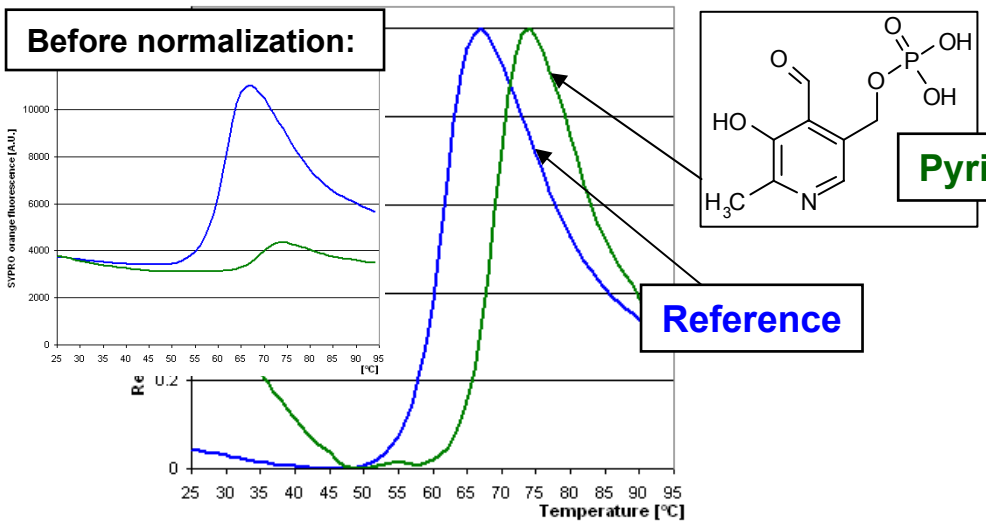
Application of DSF to the work of the SGC

Ligands of the C2 domain of Protein Kinase C gamma

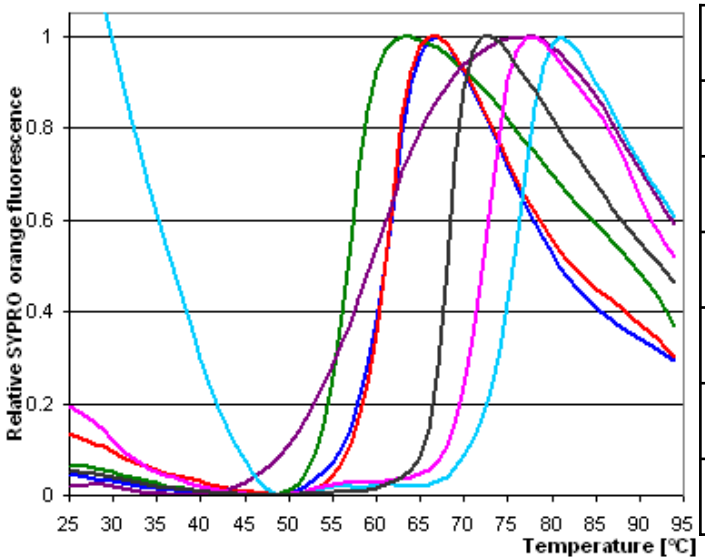
Because of a technical error, the following slide was missing in the presentation that was actually held at the PX school.

Application of DSF to the work of the SGC

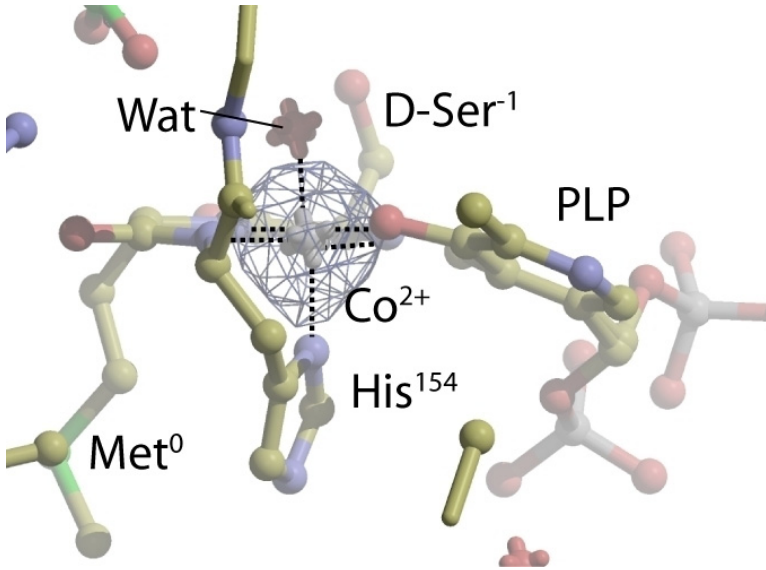
Ligands of the C2 domain of Protein Kinase C gamma



Pyridoxal-phosphate (PLP), ΔT_m = 7.5 ˚C



Divalent	ΔT_m (˚C)
Ca^{2+}	14.7
Co^{2+}	11.3
Mn^{2+}	7.2
Mg^{2+}	0.0
Zn^{2+}	-1.6*
Ni^{2+}	-4.1



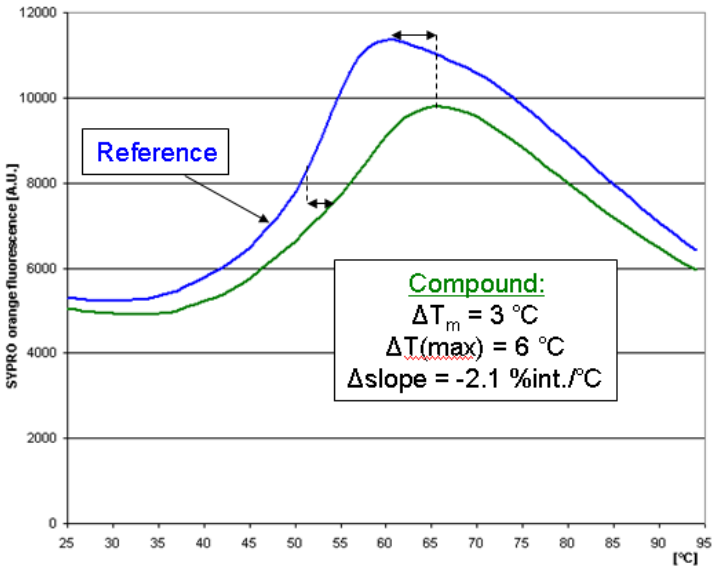
- C2 domain senses calcium levels – locates kinase to membrane.
- PLP forms Schiff's base with amino terminus, resulting in racemization of N-terminal serine to D-amino acid.
- PLP, Ser-1, Met0 and His154 coordinate cobalt ion very tightly (retained in denaturing mass spec - covalent?)

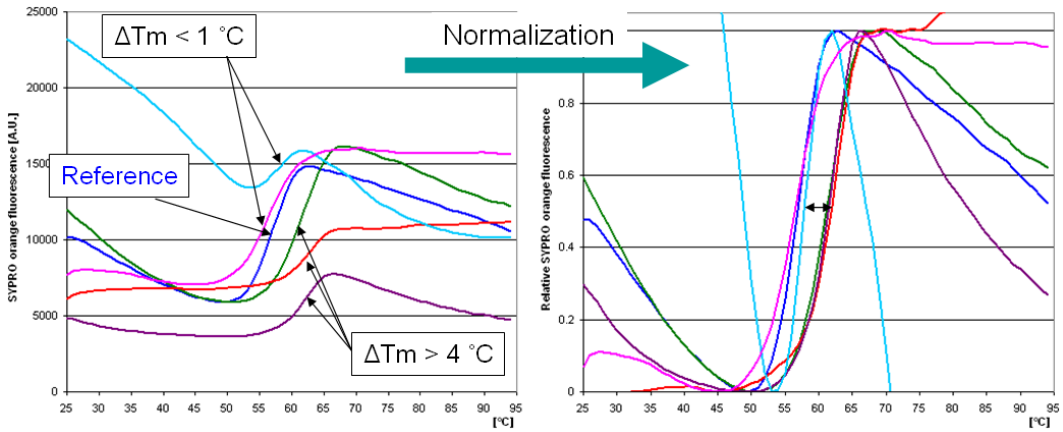
PDB: 2UZP; Purification, A. Amos; Screen, O. Fedorov; Crystallization, A. Amos; Crystallography, A. Pike; PI, S. Knapp

frank.niesen@sgc.ox.ac.uk http://www.thesgconline.org ftp://ftp.sgc.ox.ac.uk/pub/biophysics

DSF Analysis

- Development of analysis tools directed to protein-unfolding applications by RT-PCR manufacturers is strongly restricted by existing industrial patent (ThermoFluor)
- Our aim: build a highly automated analysis tool that enables fast and easy analysis of experiments in parallel, without need for specialized software
- Now available: *DSF Analysis 2.5*, requires only: Microsoft Excel + fitting software, e.g. GraphPad
- - customized transition range
  - data normalization
  - customized referencing
  - customized annotations
  - facilitated database entries
- Typical analysis: ~ 5 min





Free download of tools, templates and examples:
ftp://ftp.sgc.ox.ac.uk/pub/biophysics

Outlook

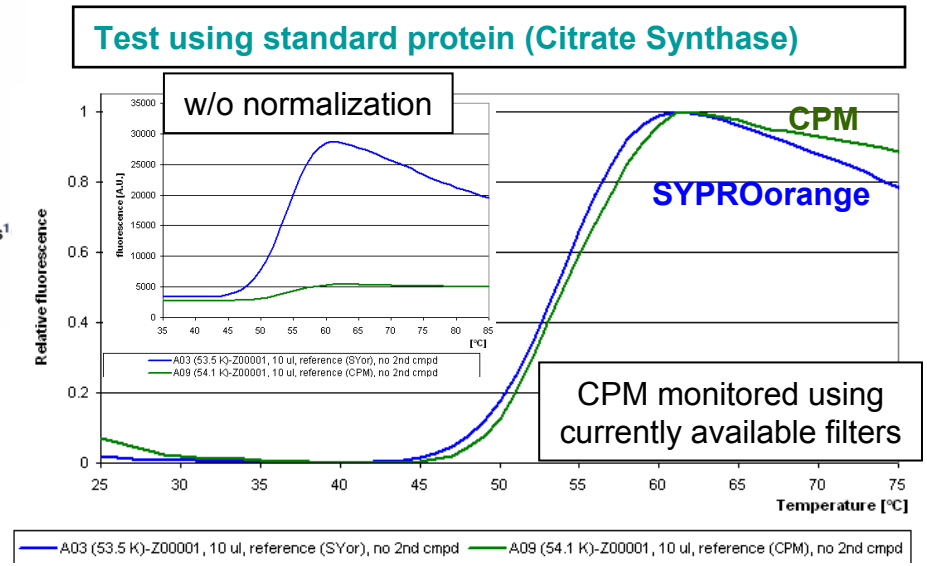
- DSF HTS: motivate suppliers of RT-PCR to build a 384-well instrument that is truly applicable
- Screening of conditions, additives and specific ligands for integral membrane proteins

Structure
Ways & Means

Microscale Fluorescent Thermal Stability Assay for Membrane Proteins

Alexander I. Alexandrov,[1] Mauro Mileni,[1] Ellen Y.T. Chien,[1] Michael A. Hanson,[1] and Raymond C. Stevens[1]
[1]Department of Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037, USA
*Correspondence: stevens@scripps.edu
DOI 10.1016/j.str.2008.02.004

- Assay with GPCR in buffer incl. 0.1 % DDM
- Applied protein concentration: 77 µg/ml (1.8 µM)
  (similar to DSF for soluble proteins)
  (170 µg required for 96 conditions [20 µl/well])



- Challenge: non-two state unfolding; distinctive analysis of 2 or more phases in transitions
- Uncoupling of *DSF Analysis* from Excel and fitting software? – Programmers forward!

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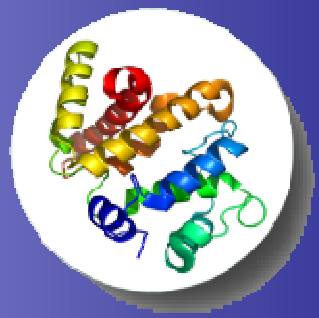

SGC

Thanks to:
Masoud Vedadi
Helena Berglund
Oleg Fedorov
Biophysics/Chemical Biology teams

… and to all other colleagues at the SGC sites in Oxford, Stockholm and Toronto!

Special thanks: Stratagene/Agilent for support, and to numerous DSF users, for their feedback and suggestions!