Protein Interaction studies on Protein Arrays

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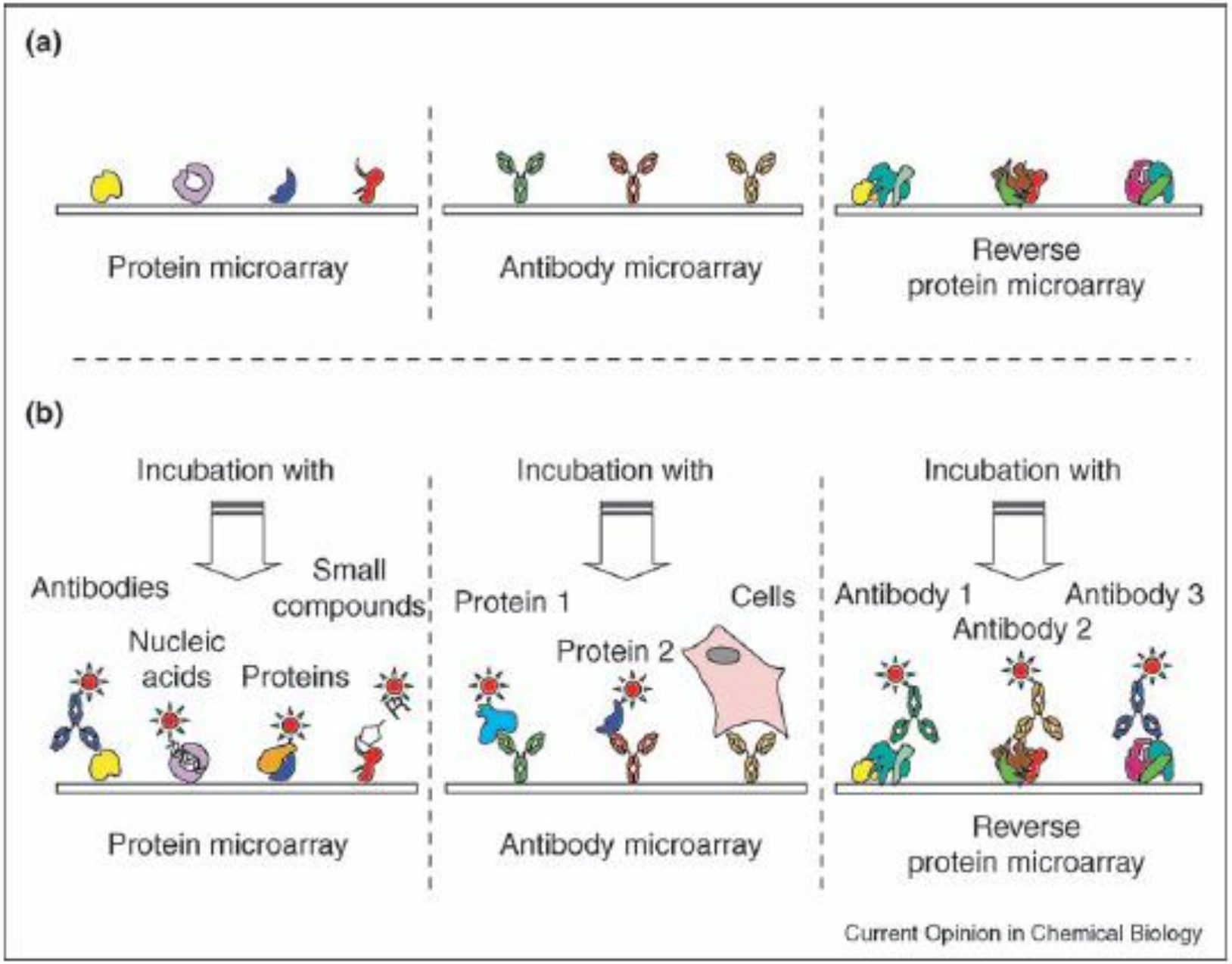
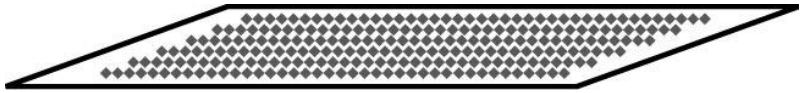

(a)
Protein microarray
Antibody microarray
Reverse
protein microarray
(b)
Incubation with
Antibodies
Small
compounds
Nucleic
acids
Proteins
Protein microarray
Incubation with
Protein 1
Cells
Protein 2
Antibody microarray
Incubation with
Antibody 1
Antibody 3
Antibody 2
Reverse
protein microarray
Current Opinion in Chemical Biology

Protein arrays

Protein array

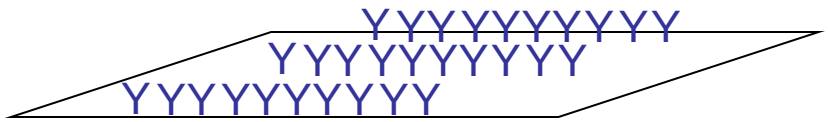


Array of proteins

Assay Protein function
- Antibody characterisation
- Protein interactions
- Small molecule interactions
- Identify substrates

Antibody based arrays

Antibody array



Array of antibodies

Measure levels of proteins or other biomolecules in samples

Overview

Improvements in Protein and Antibody Arrays (Buessow et al., 1998, Lueking et al., 2003; Angenendt et al., 2002, 2003 a,b, 2004a, 2004b, 2006)

Characterisation of antibody specificity and cross reactivity (Lueking et al.,2003, Taussig et al., 2007)

Profiling antibody repertoire in autoimmune disease (Gibson et al., 2010);

Profiling Antibody Repertoire in Autoimmune Disease (Lueking et al., 2003)

Generated disease associated panel of proteins in

- Dilated Cardiomyopathy (DCM) (Horn et al., 2006)
- Alopecia areata (Lueking et al., 2006)
- SLE (Gutjahr et al., 2005)
- Ovarian Cancer (O'Connell et al)
- Alzheimer Disease (O'Kane et al)

Peptide-Protein Interaction Screening (Larkin et al., 2004)

Protein-Protein Interaction Screening (Bauer et al, 2008, O'Connell et al., 2010)

Applications

Horn S., Lueking A., Murphy D., Staudt A., Gutjahr C., Schulte K., Koenig A., Landsberger M., Lehrach H., Felix S. B. and **Cahill DJ**.
Profiling humoral auto-immune repertoire of dilated cardiomyopathy (DCM) patients and development of disease-associated protein chip.
Proteomics, 2006, 6: 605 - 613.

Lueking A, Huber O, Wirths C, SchulteK, Stieler K, Blume-Peytavi U, Kowald A, Hensel-Wiegel K, Tauber R, Lehrach H, Meyer HE, and & **Cahill DJ**.
Profiling of alopecia areata autoantigens based on protein microarray technology. Molecular and Cellular Proteomics 2006, 4:1382-1389

Gutjahr C., Murphy D., Lueking A., Koenig A., Janitz M., O'Brien J., Korn B., Horn S., Lehrach H. and **Cahill DJ**. High-density mouse protein array from T cell cDNA library. Genomics 2005 85(3):285-96.

Steller S, Angenendt P, **Cahill DJ**, Heuberger S, Lehrach H, Kreutzberger J. Bacterial protein microarrays for identification of new potential diagnostic markers for Neisseria meningitidis infections. Proteomics, 2005 5(8):2048-55

Taussig MJ, Stoevesandt O, Borrebaeck CA, Bradbury AR, **Cahill D**, et al. ProteomeBinders: planning a European resource of affinity reagents for analysis of the human proteome. Nature Methods. 2007 4(1):13-7.

Aims

- To probe the human calmodulin neural interactome using protein array technology

- To identify high affinity protein-protein interactions

- To validate the identity of these interactions with sensitive techniques in a high throughput system

- To identify a route for the further study of calcium regulated signalling in the brain

Advantages of Interaction Screening on Arrays

1) Affinity chromatography may lead to identification of the more abundant proteins and the capture of secondary proteins that bind to primary calmodulin targets.
On protein arrays the proteins are presented in distinct locations and secondary targets are not likely to be identified.

2) array screening is effective in identifying interactions with trans-membrane proteins, including receptors and ion channels, which are typically not available in tissue homogenate used for identification through affinity chromatography

3) Using Arrays - ability to return to the protein expressing clone of an identified target protein and express it for further characterization.

Interaction Screening

Aim of the screen to find high affinity (KD ≤ 1 mM) binding partners of calmodulin

Identified 76 human proteins from all intracellular compartments, of which 72 are novel.

Measured the binding kinetics of 74 targets with calmodulin using a high throughput surface plasmon resonance assay.

Most of the novel calmodulin-target complexes identified have low dissociation rates (koff ≤ 103 s-1) and high affinity (KD ≤ 1 mM), consistent with the design of the screen.

Interaction Screening

- Many of the identified proteins are known to assemble in neural tissue, forming assemblies such as the spectrin scaffold and the postsynaptic density.
- Developed a microarray of the identified target proteins with which we can characterise the biochemistry of calmodulin for all targets in parallel.
- Four of the novel targets were selected for exploration of the calmodulin-binding regions.
- Using synthetic peptides and isothermal titration calorimetry, calmodulin binding motifs were identified in the potassium voltage gated channel Kv6.1, (residues 474-493), CaM kinase-like vesicle-associated protein (302-316), EF-hand domain family member A2 (202-216) and phosphatidylinositol-4-phosphate 5-kinase, type I, gamma (400-415)

UCD DUBLIN

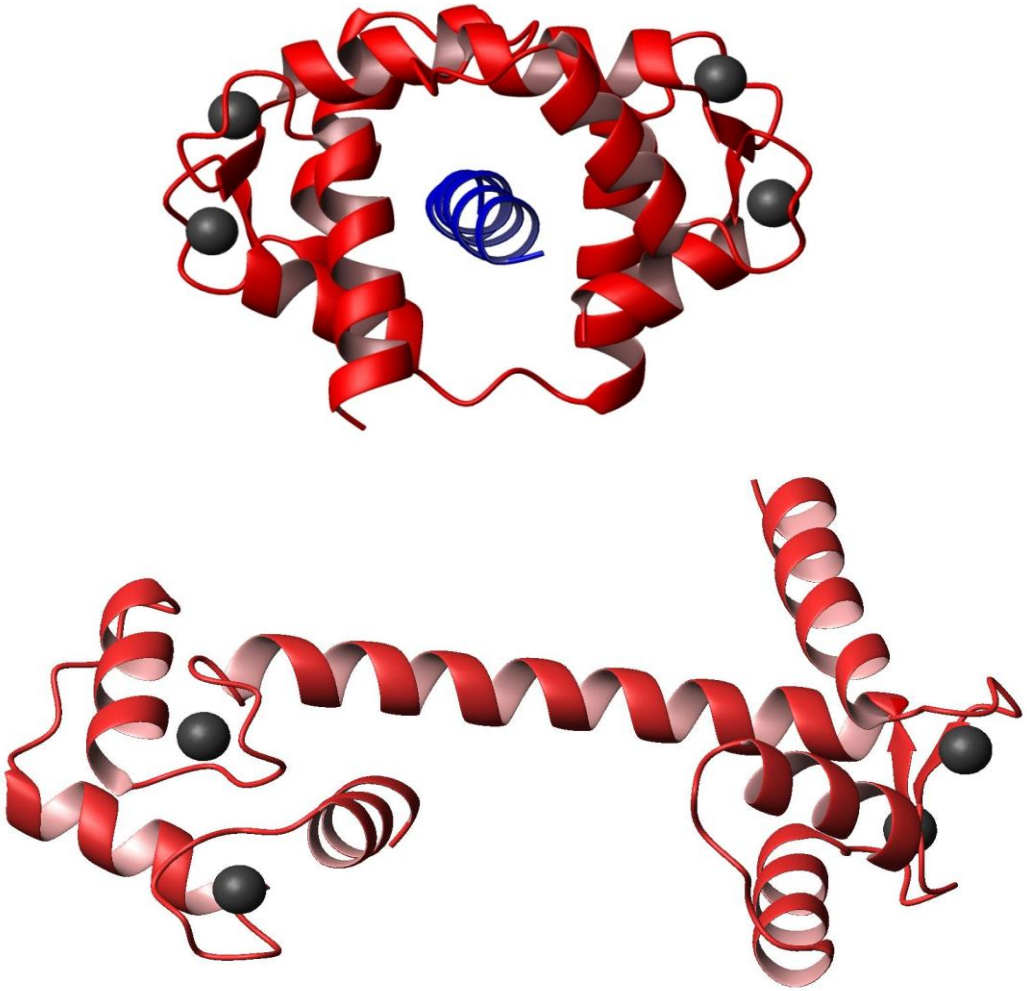
Protein – Protein Interaction

O'Connell D, Bauer MC, O'Brien J, O'Connell D, Bauer MC, O'Brien J, O'Kane S, Berggård T, Merino A, Åkerfeldt KS, Linse S, Cahill D.J
Integrated protein array screening and high throughput validation of 70 novel neural calmodulin binding proteins.
Molecular Cellular Proteomics (2010) in press M900324-MCP200

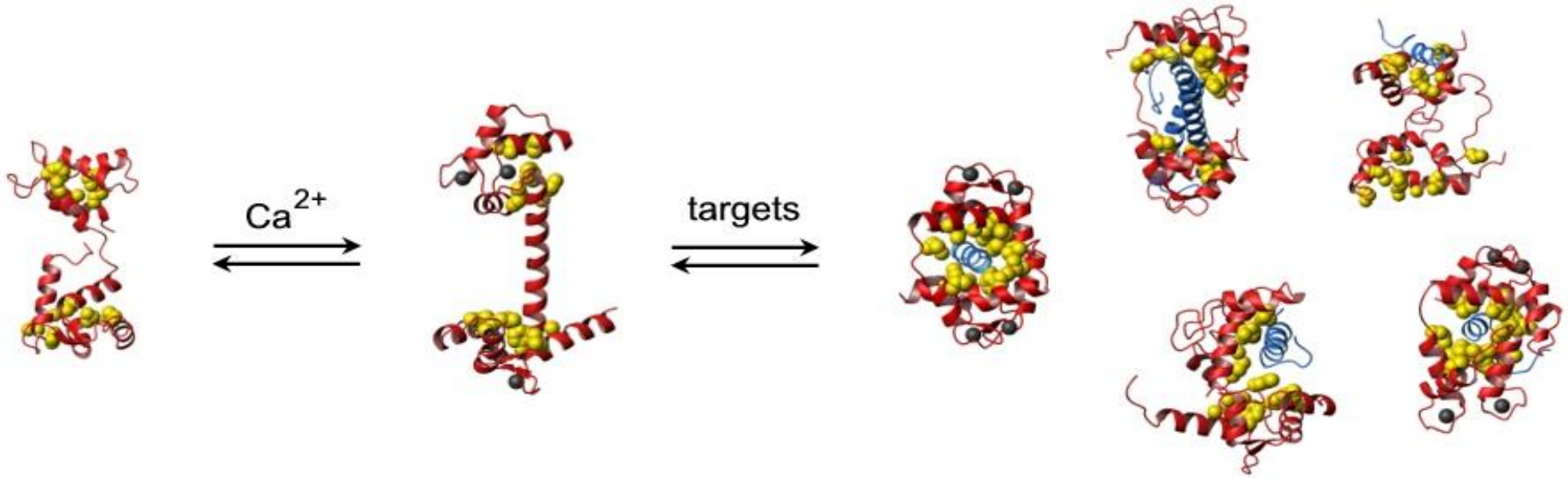
Bauer M., O'Connell D., Cahill, D. J. and Linse S.
Calmodulin binding to the polybasic C-termini of STIM Proteins Involved in Store-Operated Calcium Entry.
Biochemistry (2008) 47:6089-6091.

UCD DUBLIN

Calmodulin



Calmodulin Interactome



Human Protein Array

• A human foetal brain cDNA library, generated by 3' RACE - directionally cloned in a bacterial expression vector that allows IPTG-inducible expression of His6-tagged fusion proteins

• Using robot technology, the library was arrayed in microtitre plates and gridded onto high-density in situ filters

• A monoclonal antibody recognising the N-terminal RGSH6 sequence of espressed proteins detected 20% of the library as putative expressing clones

• Approximately 37,830 non-redundant proteins expressed on the arrays

Protein-Protein Interaction Screening

• Calmodulin is a ubiquitous protein that is expressed in all eukaryotic cells.

• It participates in signaling pathways that regulate many crucial processes such as growth, proliferation and movement

• Regulation of these events is exerted via direct interactions with a large number of cellular proteins

• Calmodulin constitutes at least 0.1% of the total protein in cells and it is expressed at even higher levels in brain and in rapidly growing cells, especially those undergoing division and differentiation

• The protein is strongly conserved and the same sequence is found in all vertebrates.

•Ca2+ binds to calmodulin in a cooperative fashion, a small change in the level of cytosolic Ca2+ leads to a large change in the level of active protein.

Protein-Protein Interaction Screening

Calmodulin research can be classified into three general areas:

(1) elucidating the calmodulin structure and dynamics of Ca^{2+} interaction with other proteins

(2) determining the expression pattern and regulation of calmodulin mRNA levels in various organisms, and

(3) ***discovering novel calmodulin targets***.

Bergarrd et al., J Proteome Res, 2006

Protein Arrays

- **PVDF format**
  Büssow et al., Nucleic Acids Research (1998): 26: 5007-5008

- **Protein chip slide format**

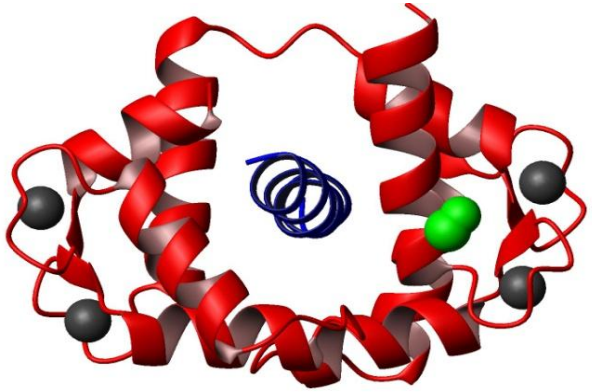
  Lueking, et al., Molecular and Cellular Proteomics (2003) 2(12):1342 – 1349

Advantages

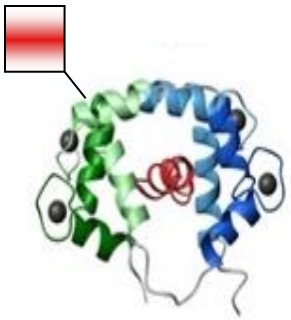
Economical, low sample consumption
Rapid, automated, miniaturised
High sensitivity
Highly parallelised - multiplexed
Same software and hardware tools as DNA arrays

Alexa Flour 488 labelling

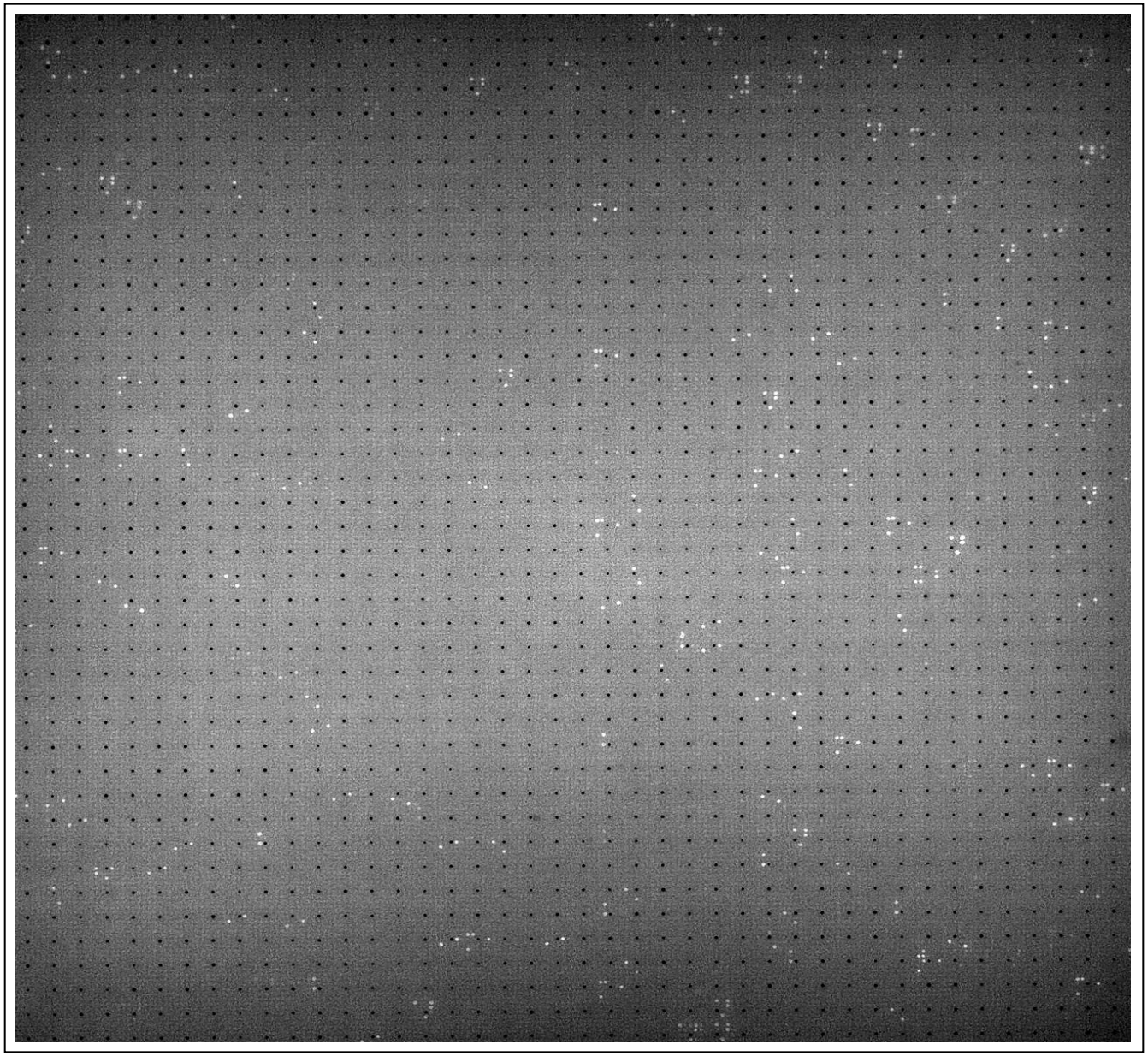
Calmodulin labelled at pos 17 (Ser17->Cys)



Calmodulin Interactome Identification on Human Protein Macroarrays



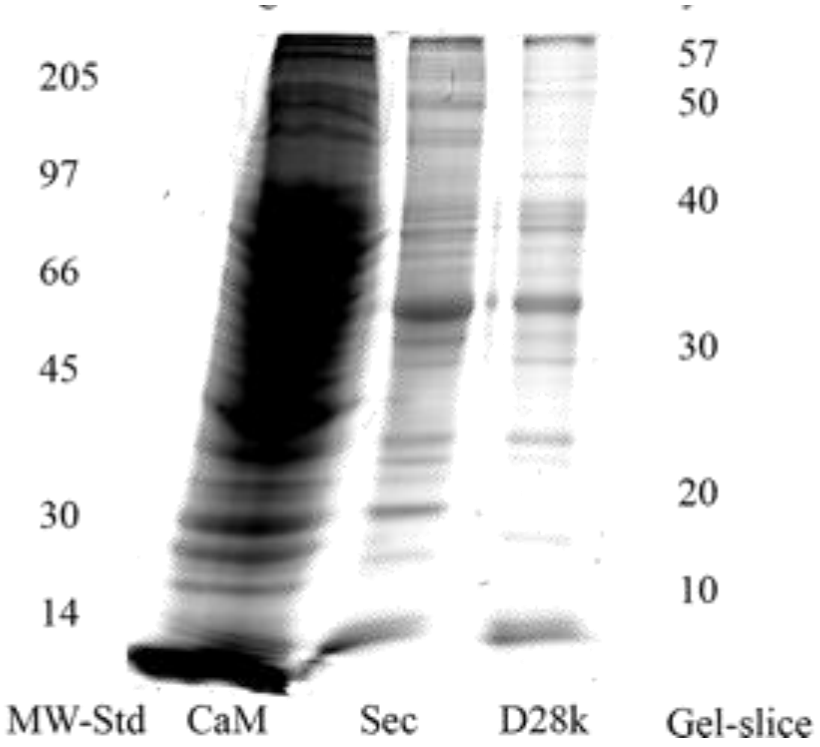
Calmodulin Alexa Flour 488nm



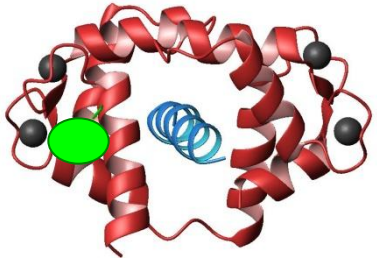
Control experiments

Proteins of similar charge and structure but unrelated function

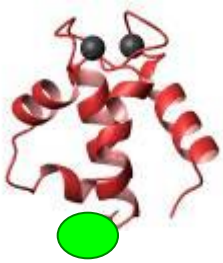
Calbindin D9k (no known targets)
Calbindin D28k (other targets)
Secretagogin (other targets)

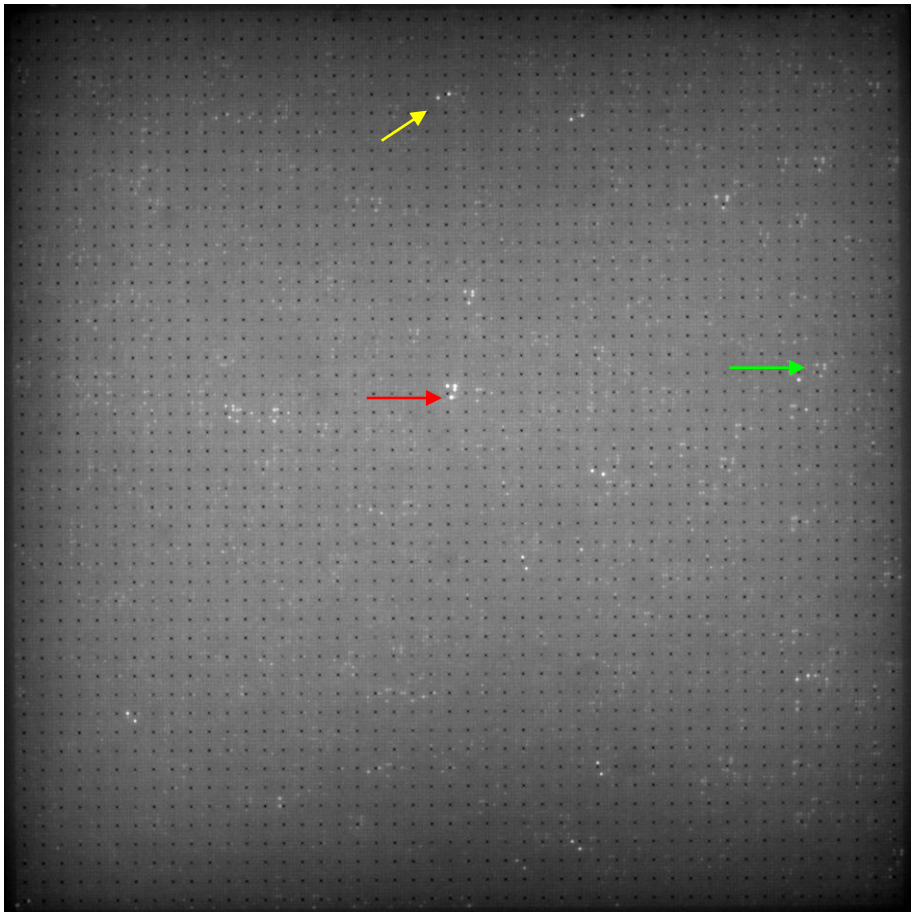


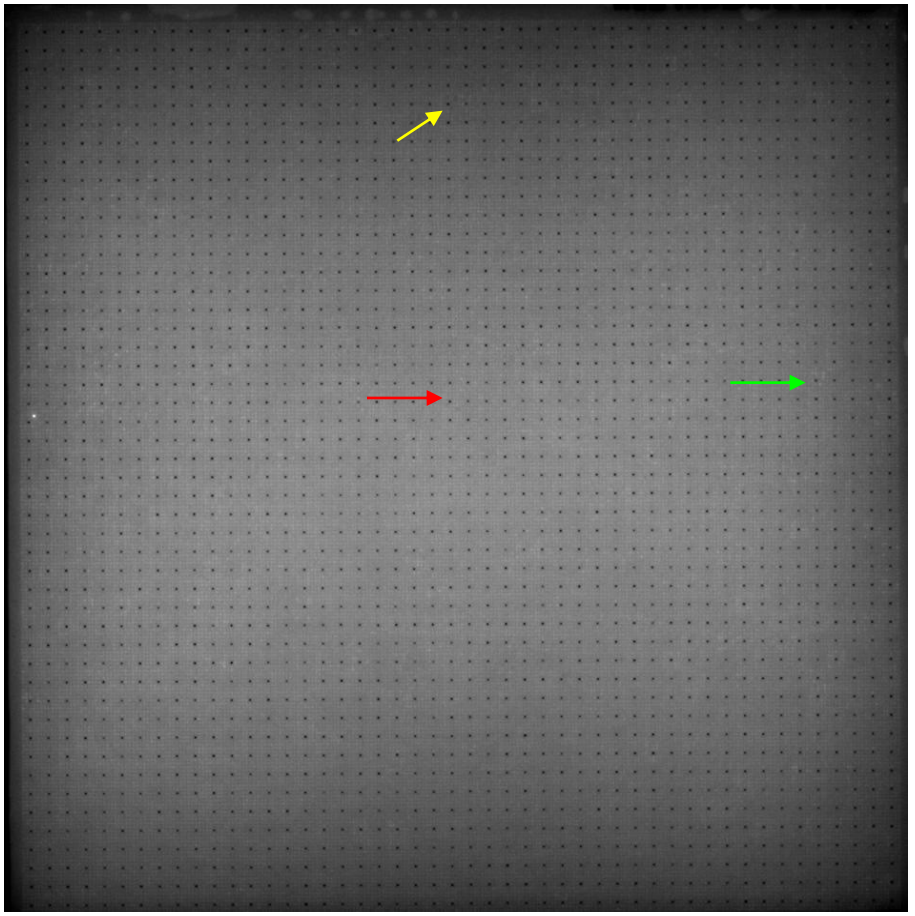
Calmodulin



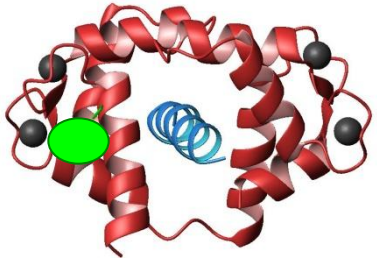
Calbindin D9k



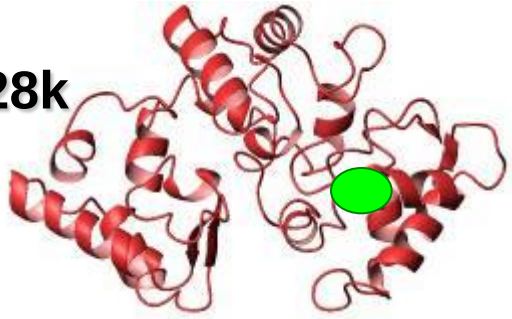


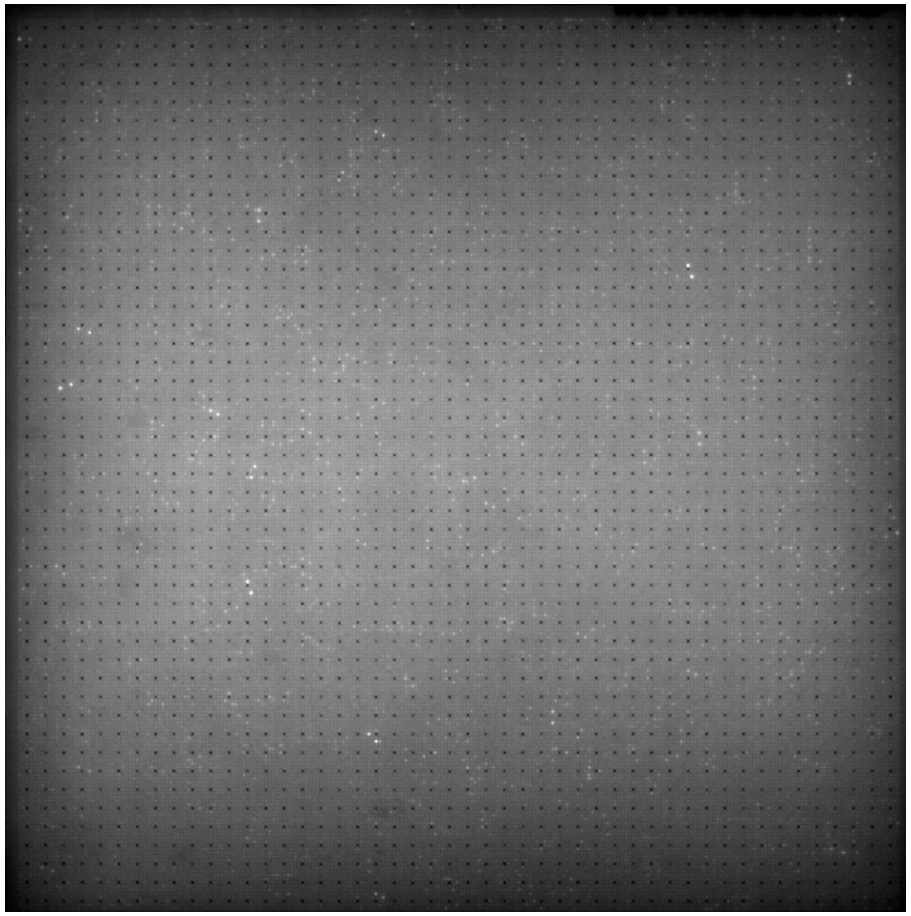


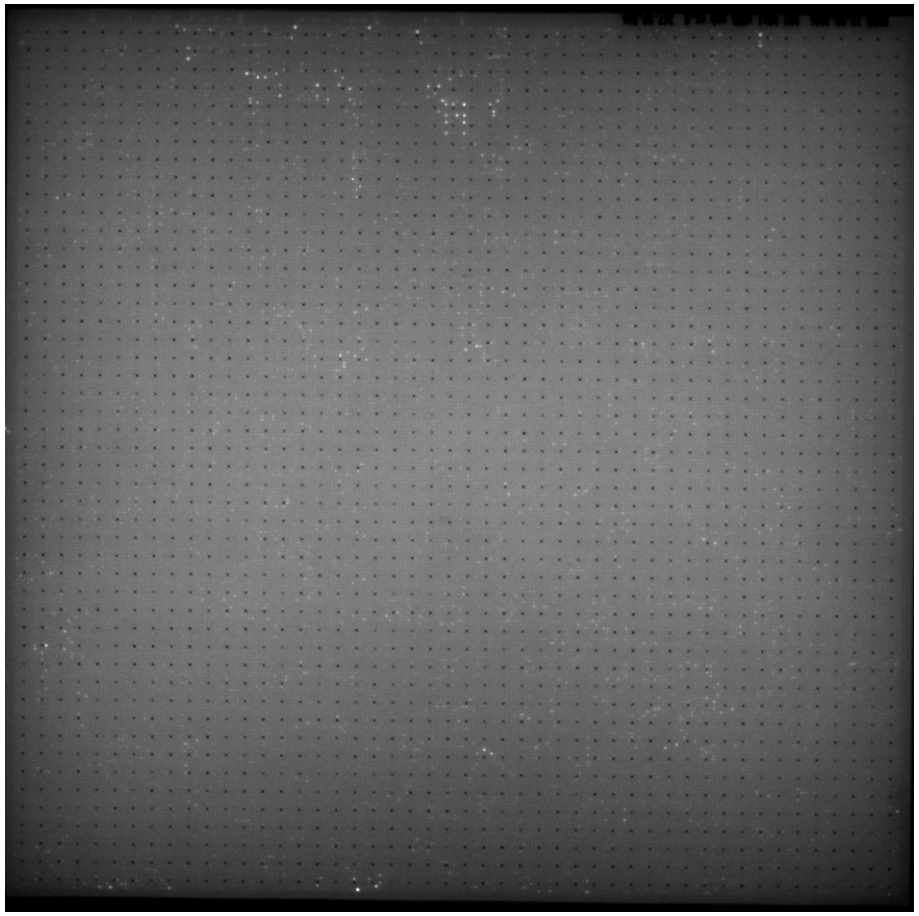
Calmodulin



Calbindin D28k

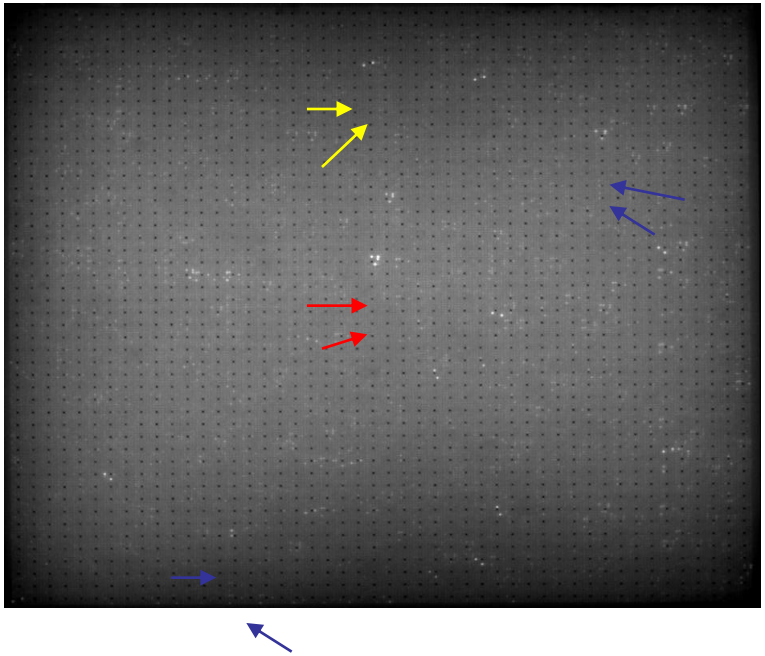




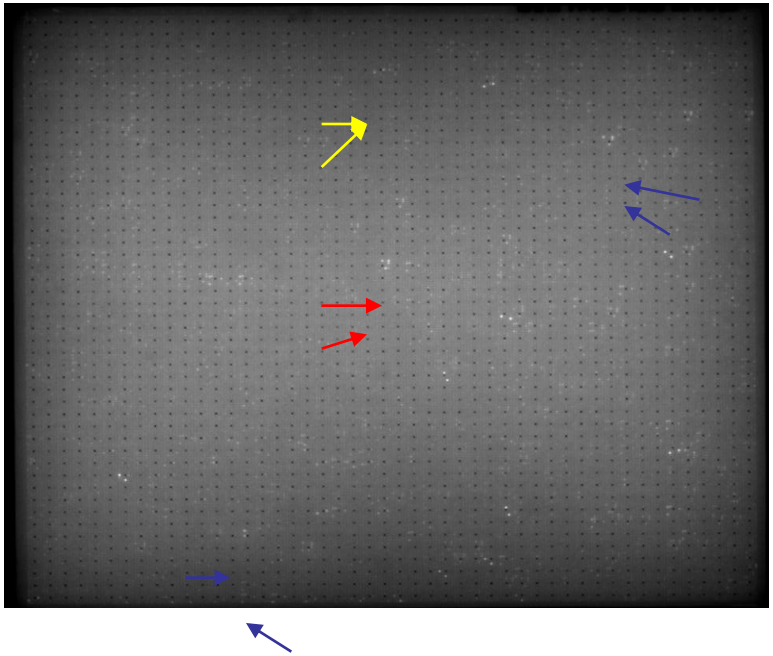


Effect of calcium concentration on alexaflor 488-calmodulin-binding

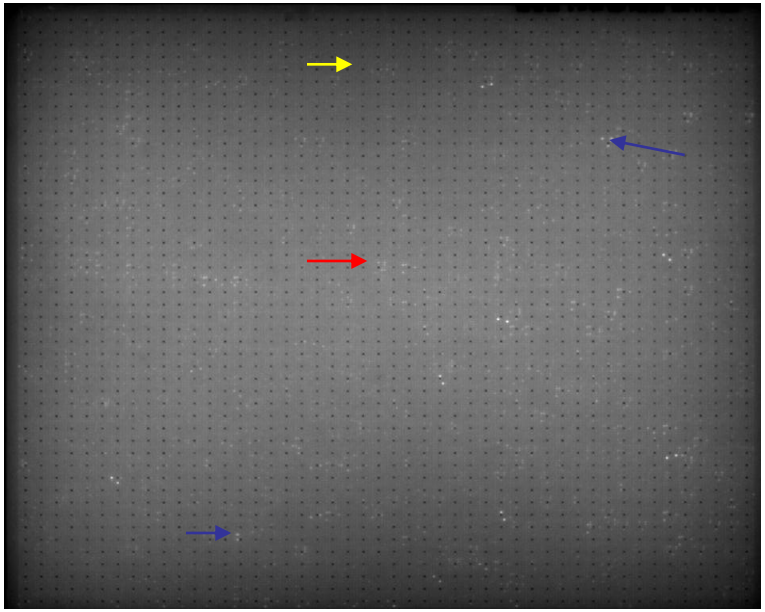
1mM



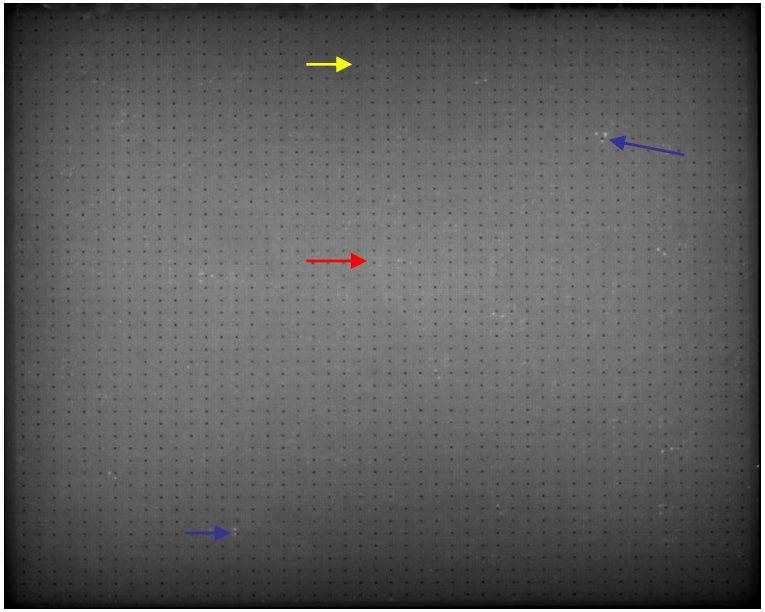
100μM



1μM

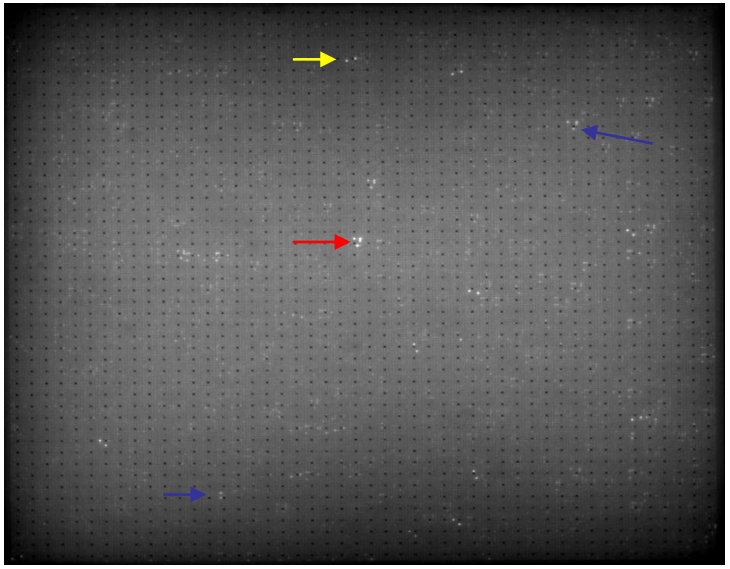


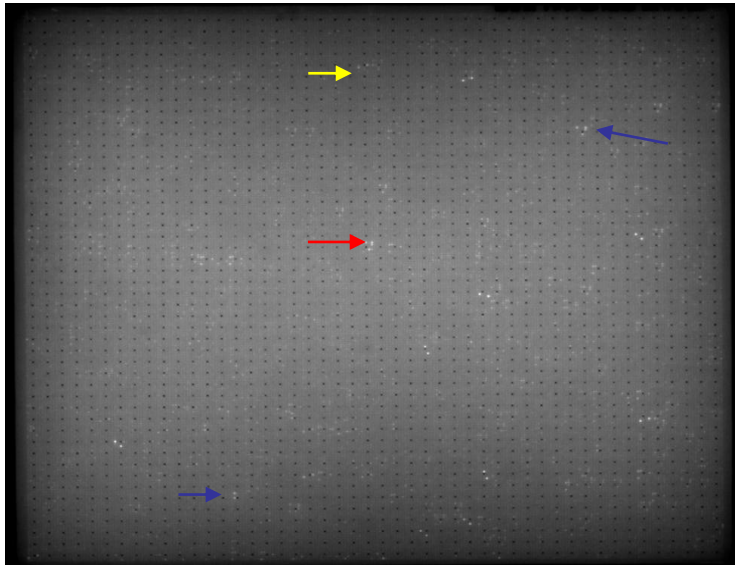
1mM EDTA



Effect of Ca^{2+} concentration on protein binding by calmodulin alexa flour 488

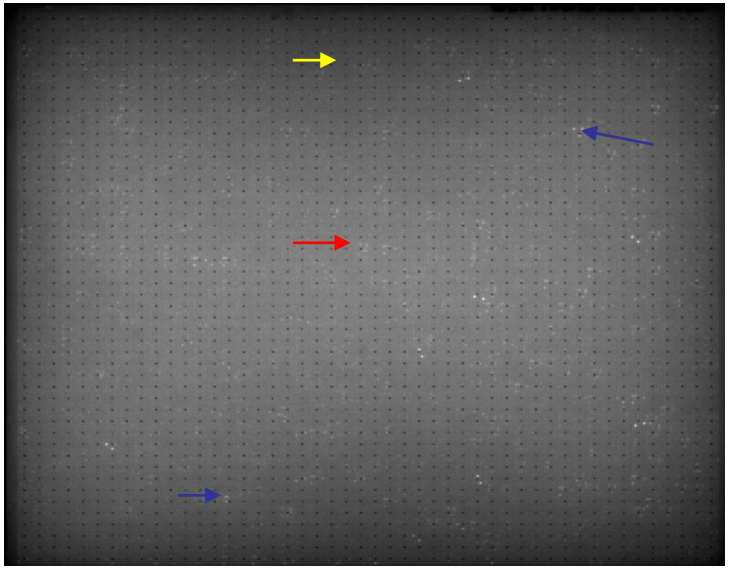
1mM

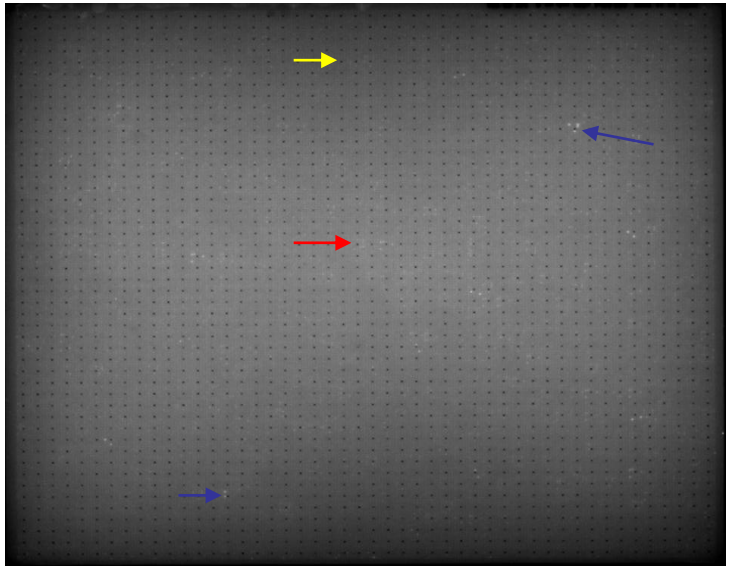




100μM

1μM

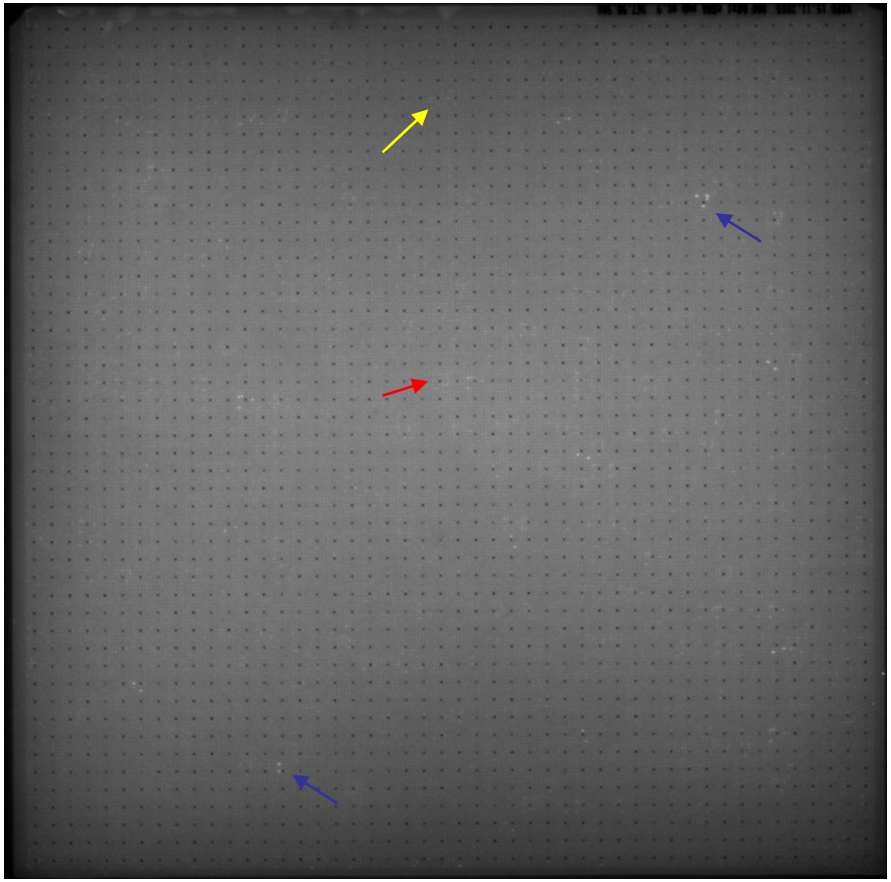




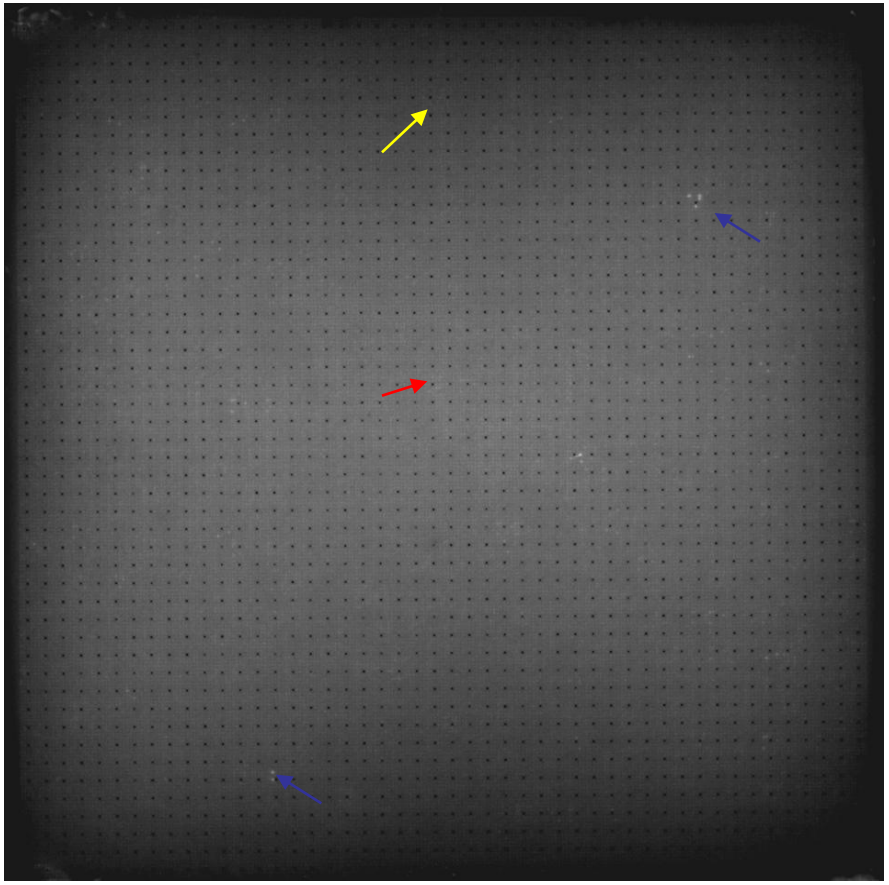
1mM EDTA

Effect of Ca^{2+} concentration on protein binding by calmodulin alexa flour 488

1mM EDTA 1hr

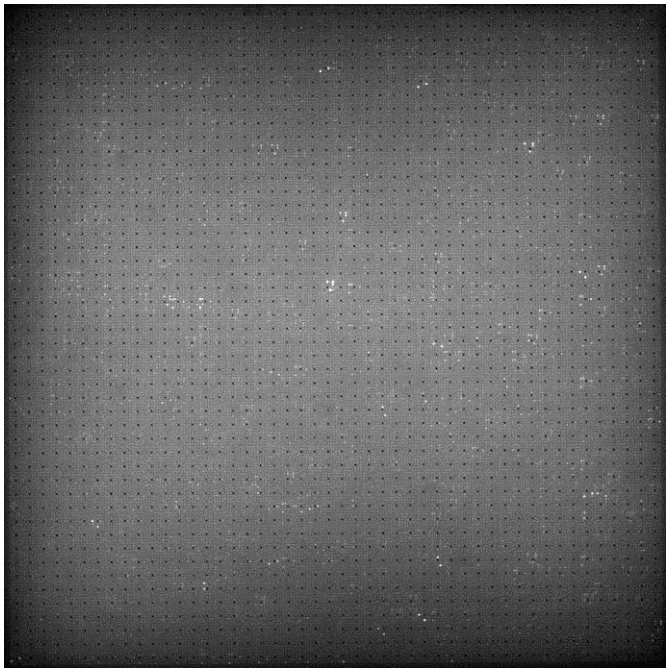


1mM EDTA 24hr

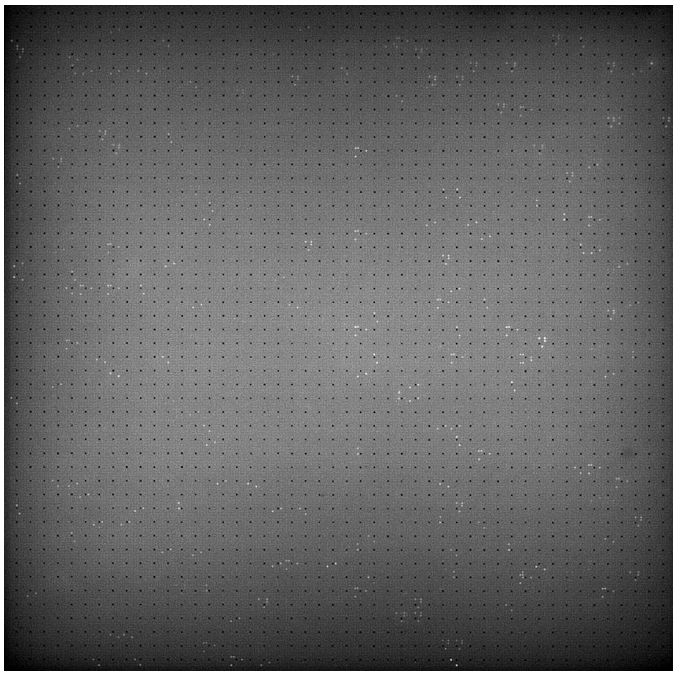


Stringent Washing – High Affinity Binders

TBS + Ca^{2+}



TBS-Tween + Ca^{2+}



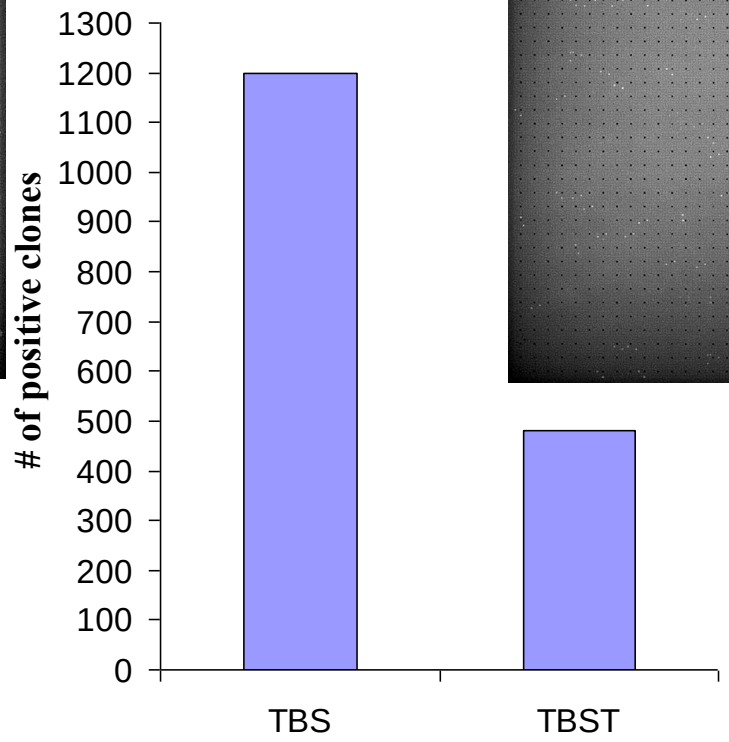
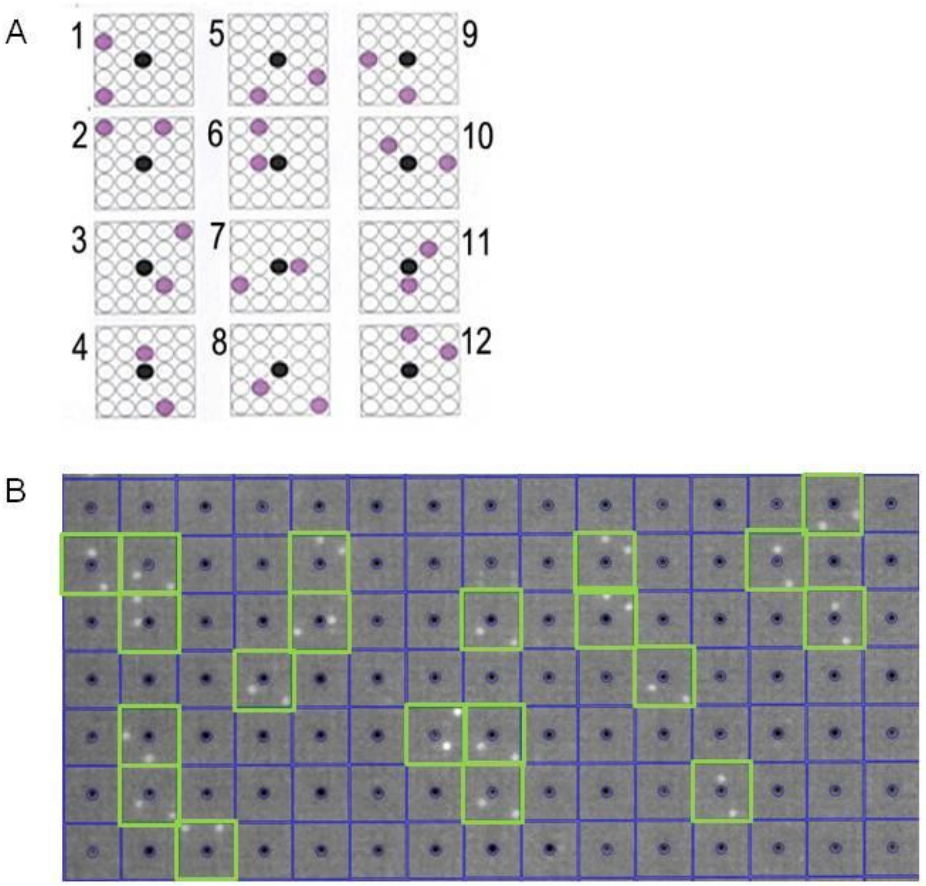
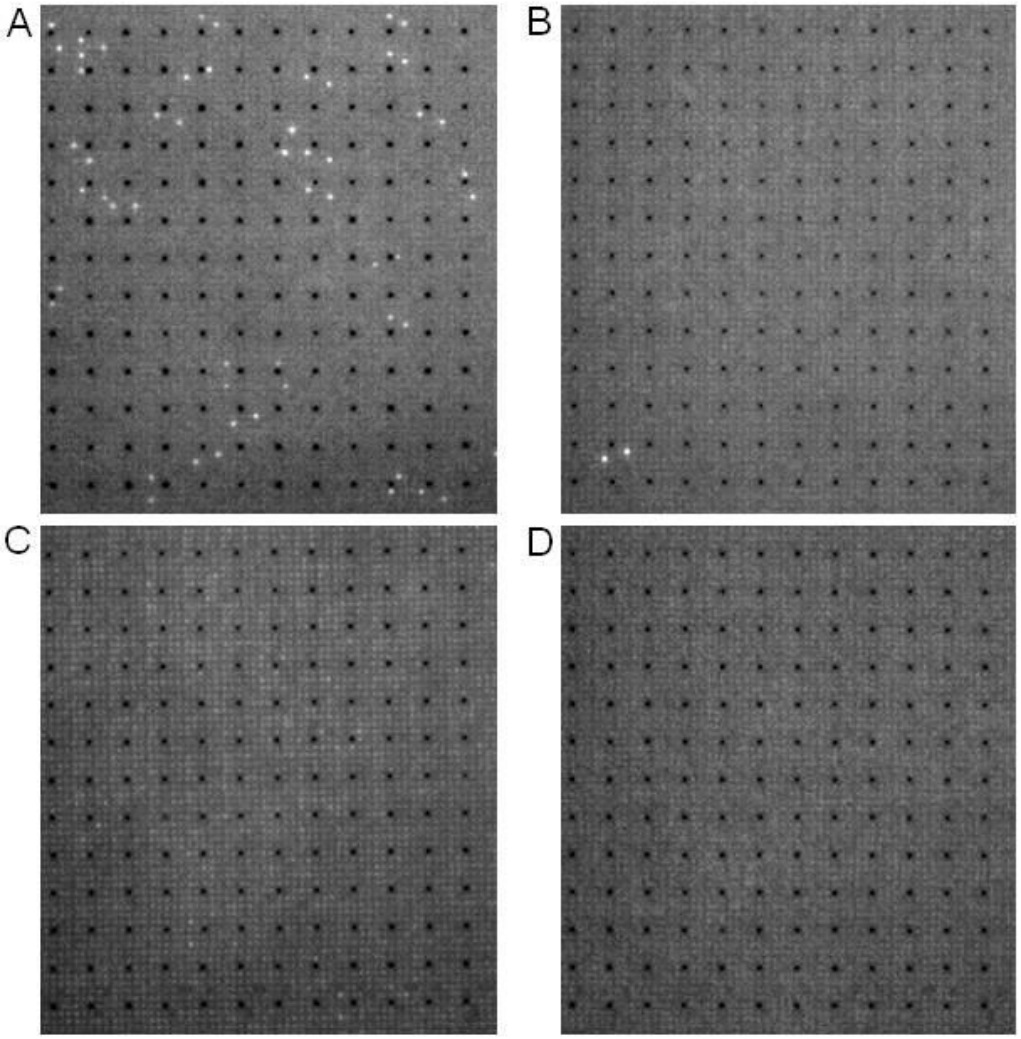


Image Analysis and Scoring of Human Arrays



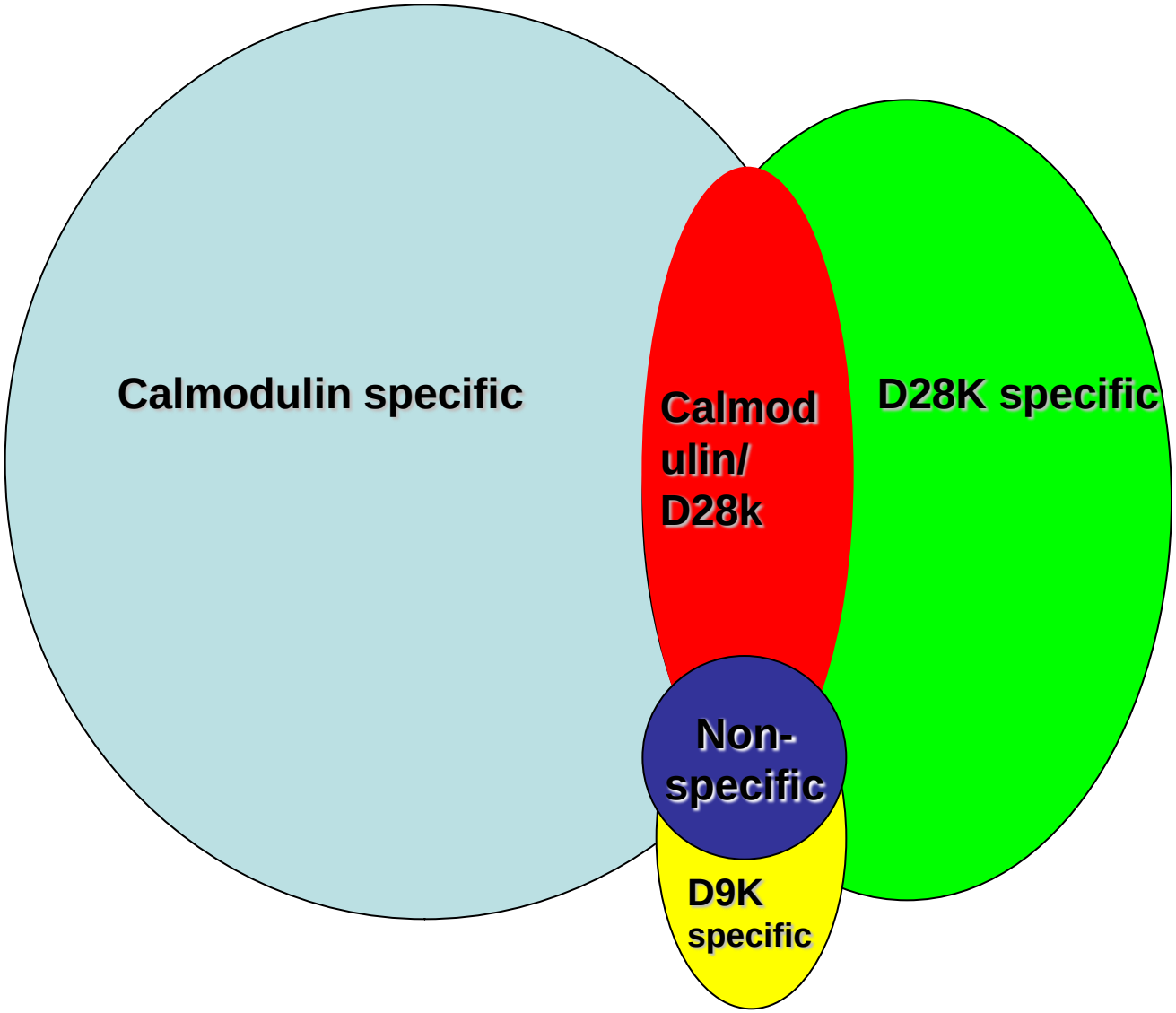
(B) 1 mM CaM-Alexa488 binding to a field of array with positive clones highlighted in green squares

(A) calmodulin (B) secretagogin (C) calbindin D28k, (D) calbindin D9k.



protein array incubated overnight with 1 mM calmodulin protein labelled with Alexa Flour488 in TBS buffer with 1 mM CaCl2, followed by 6 x 10 min washes in TBST buffer

Distribution of positive clones from protein array screening



Calmodulin Interacting proteins

Used stringent washing to retain only high affinity interactions, we identified 76 calmodulin interactome proteins. 74 of the 76 interactions were validated by SPR.

70 were previously unidentified and include:
28 transmembrane proteins,
-15 nuclear proteins,
-3 cytoskeleton proteins,
-3 ribosomal proteins,
-1 mitochondrial protein,
-2 uncategorised proteins and
-11 cytosolic proteins.

68/70 of these novel proteins were validated by high throughput surface plasmon resonance technology and the binding kinetics of the interactions were quantified.

Most of the novel calmodulin-target complexes identified have low dissociation rates and high affinity, consistent with the design of the screen.

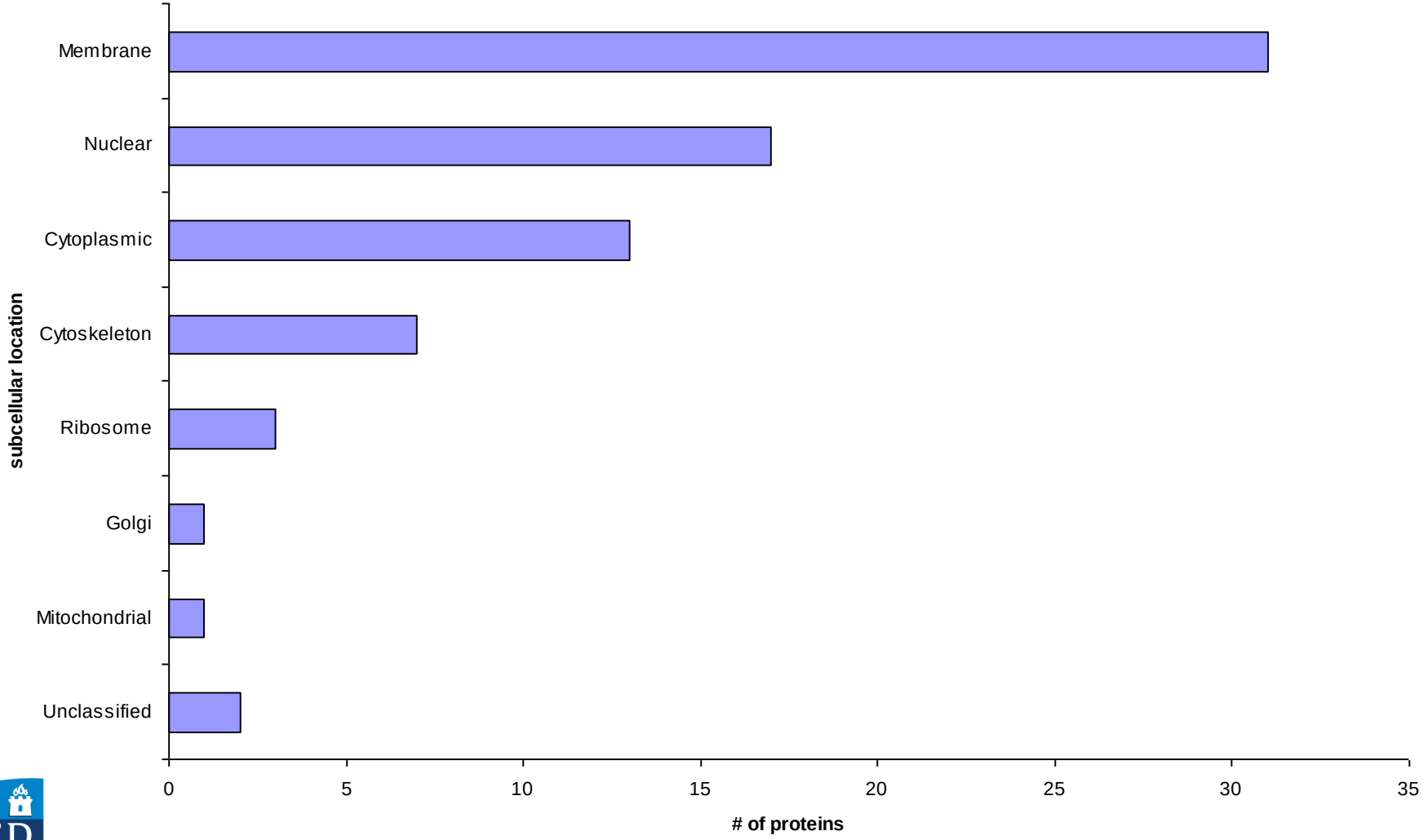
The identification of 70 novel calmodulin interacting proteins on screening a high content protein array has expanded the calmodulin interactome

Distribution of the 76 identified calmodulin interactome proteins over subcellular location

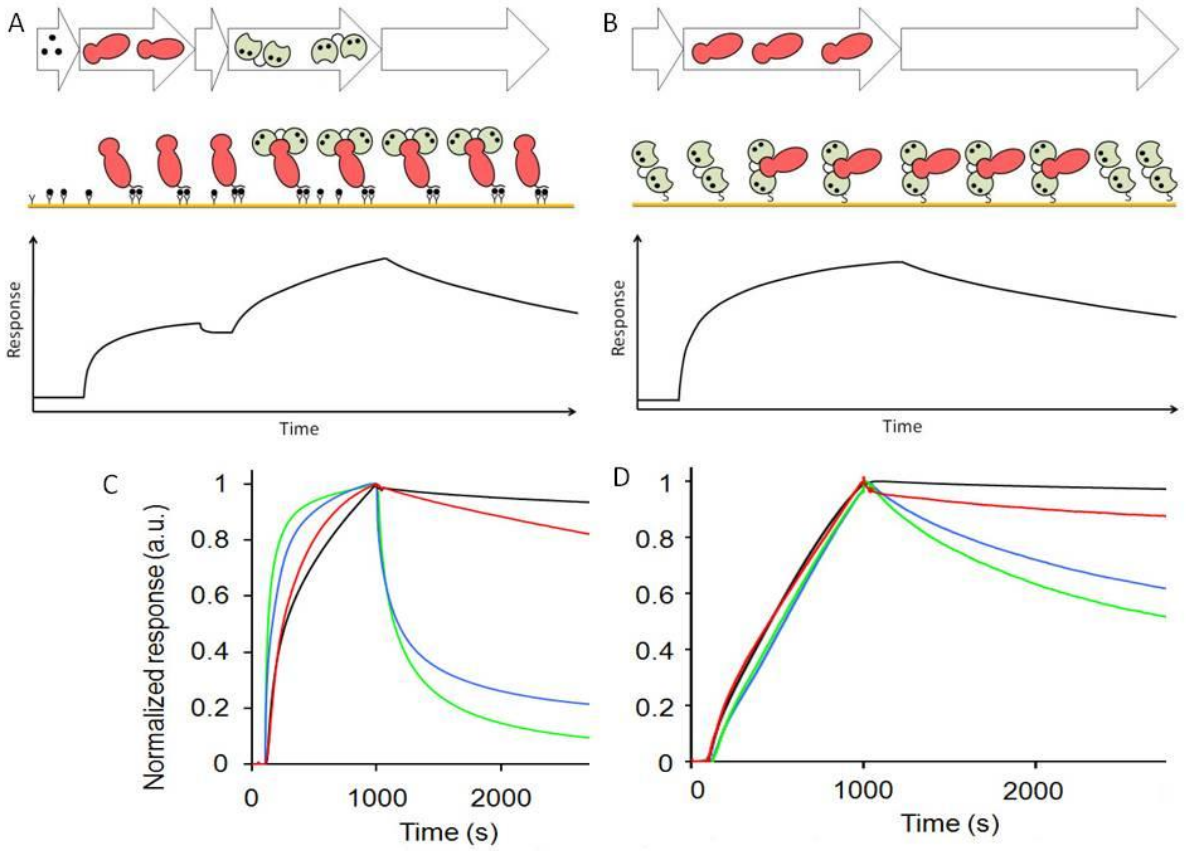
Subcellular location	Interactome proteins	Known CaM binding proteins	Novel interactome proteins
Membrane	31	3	28
Nuclear	17	2	15
Cytoplasmic	13	2	11
Cytoskeleton	7	4	3
Ribosome	3	0	3
Mitochondrial	1	0	1
Golgi	1	1	0
Unclassified	2	0	2

UCD DUBLIN

Distribution of interactome proteins by subcellular location.

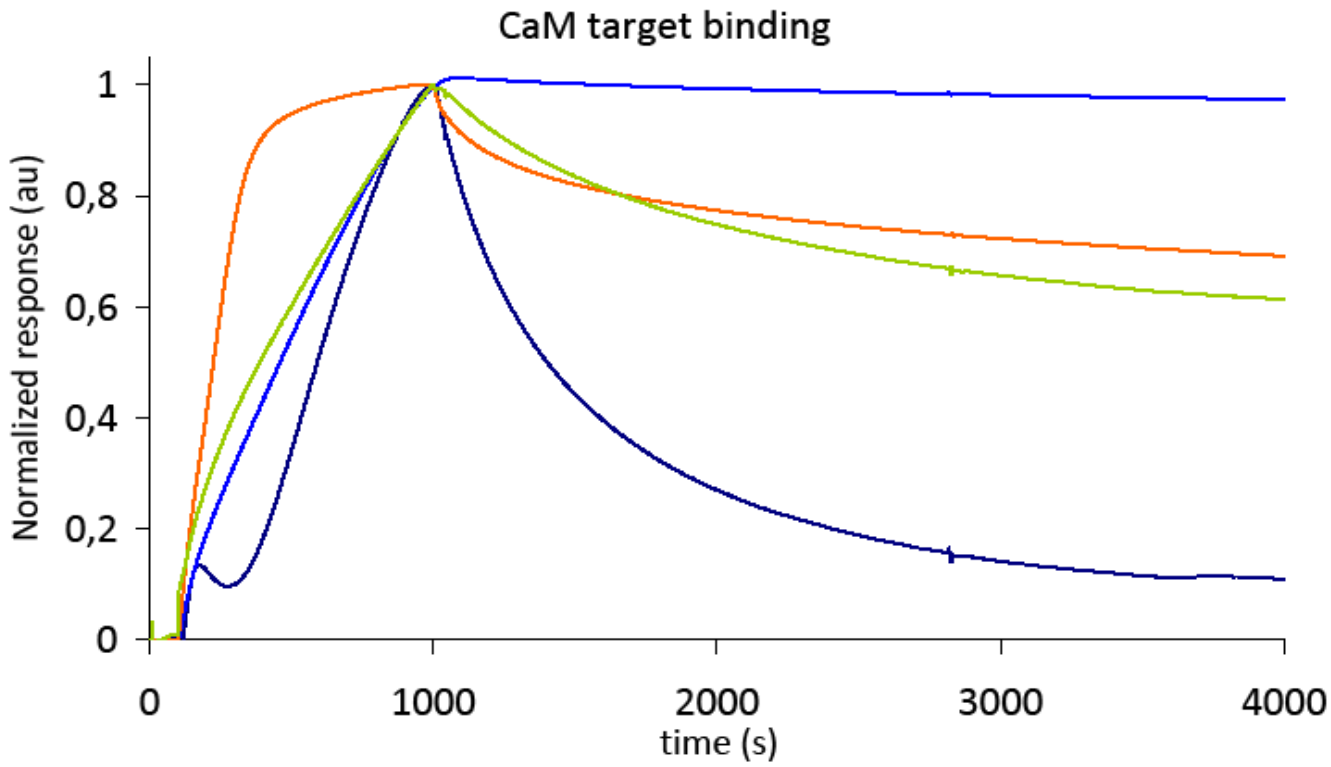


Surface plasmon resonance studies

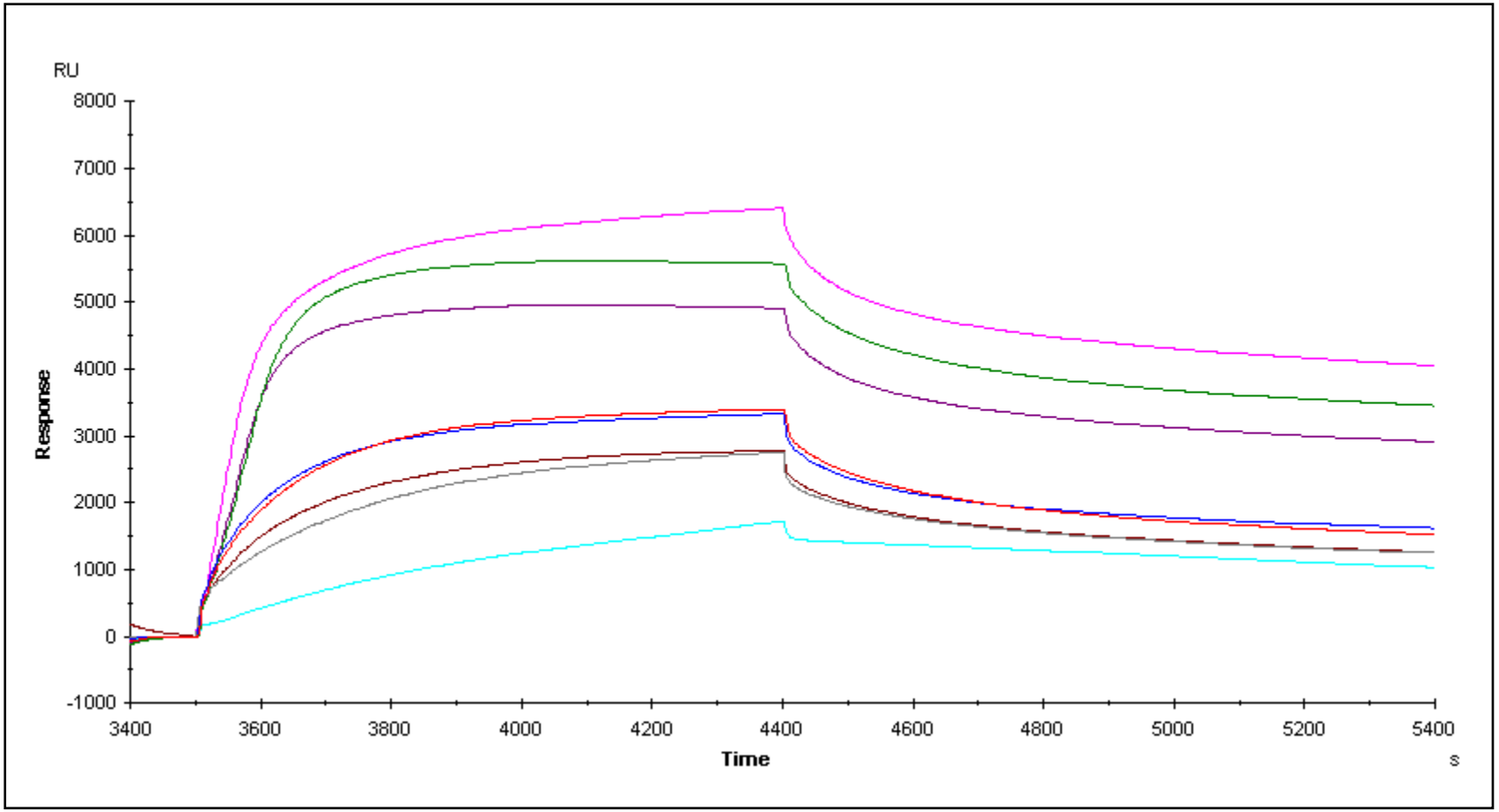


A and B: cartoons outlining the two SPR approaches with target proteins immobilised via His-tag to Ni2+-NTA sensorchips (A) or calmodulin immobilized via thiol linker to CM5 sensorchips (B)
Representative sensorgrams from SPR studies of calmodulin-target interactions in different kinetic ranges for (C) calmodulin binding to His-tag-immobilized ribosomal protein S2 (black), APLP1 (red), dynein (blue) and TFIIIA (green)
(D) target protein binding to immobilised calmodulin for ZHX2 (black), elongation factor 2 (red), Solute carrier family 16, member 8/MCT3 (blue) and semaphorin 4C (green).

Injection of Label Free Calmodulin



Representative Binding Curves for Calmodulin [700nM] to His-Immobilised Interactome Proteins



Calmodulin interacting proteins

Protein	Accession	K_D
Membrane proteins		
Solute carrier family 16, member 8/MCT3	O95907	1 nM
Solute carrier family 7, member 5	Q01650	100 nM
Neuron-specific protein family member 2	Q9Y328	1 nM
plasticity-related protein 2 *(1)*	Q6T4P5	100 pM
Potassium voltage-gated channel Kv6.1	Q9UIX4	100 nM
Glutamate [NMDA] receptor subunit zeta 1 precursor	Q05586	10 nM
Tetraspanin-7	P41732	1 nM
Lysophospholipid acyltransferase 7	Q96N66	100 pM
Semaphorin 3A *(1)*	P51805	1 nM
Transmembrane protein 9B precursor	Q9NQ34	10 nM
Semaphorin-4C precursor	Q9C0C4	10 nM
Cleft lip and palate associated transmembrane protein 1	O96005	1 nM
Receptor accessory protein 2	Q9BRK0	10 nM
Fibroblast growth factor receptor 3 precursor	P22607	100 pM
Yip1 interacting factor homolog B isoform 2	Q5BJH7	10 nM
Stromal interaction molecule 1 (STIM1)	Q13586	100 nM
Similar to Double C2-like domain-containing protein beta *(1)*	Q14184	1 µM
Ras-related protein Rab-11B *(1)*	Q15907	10 nM
Syntaxin-18	Q9P2W9	10 nM

Calmodulin Interactome Study

- Identification of a high affinity non-redundant set of interactome proteins (n=76)

- 85% not previously identified as calmodulin interacting proteins

- 74 of the 76 protein:protein interactions confirmed and quantified using SPR

- Protein microarrays printed to provide a quantitative tool to further explore the interactome with respect to calcium sensitivity, pharmacology, peptide mapping, nanoparticle perturbations and beyond

Interaction Screening

Aim of the screen to find high affinity (KD ≤ 1 mM) binding partners of calmodulin

Identified 76 human proteins from all intracellular compartments, of which 72 are novel.

Measured the binding kinetics of 74 targets with calmodulin using a high throughput surface plasmon resonance assay.

Most of the novel calmodulin-target complexes identified have low dissociation rates (koff ≤ 103 s-1) and high affinity (KD ≤ 1 mM), consistent with the design of the screen.

Interaction Screening

- Many of the identified proteins are known to assemble in neural tissue, forming assemblies such as the spectrin scaffold and the postsynaptic density.
- Developed a microarray of the identified target proteins with which we can characterise the biochemistry of calmodulin for all targets in parallel.
- Four of the novel targets were selected for exploration of the calmodulin-binding regions.
- Using synthetic peptides and isothermal titration calorimetry, calmodulin binding motifs were identified in the potassium voltage gated channel Kv6.1, (residues 474-493), CaM kinase-like vesicle-associated protein (302-316), EF-hand domain family member A2 (202-216) and phosphatidylinositol-4-phosphate 5-kinase, type I, gamma (400-415)

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Protein and Antibody Chip Surfaces

Poly-L-lysine coated slides

Polysine™ slides (Menzel Gläser)

SuperAldehyde slides (Telechem International Inc.)
Epoxy slides (Telechem International Inc.)
Euray Immobilizer™ slides (Exiqon A/S)
Reflective Microarrays (Amersham Biosciences AB)
FAST™ Slides (Schleicher & Schuell)
MaxiSorb (Nunc A/S)
MaxiSorb black (Nunc A/S)
Amine slides (Telechem International Inc.)
Silanated slides (Telechem International Inc.)
Nexterion™ Slide A (Schott Nexterion AG)
Dendrimer slides (Chimera Biotech GmbH)
PEG-Epoxy slides (Jens Sobek, Functional Genomics Center, Zurich)
Polyacrylamide
Polyacrylamide coated slides with:
Immobilines / Streptavidin / PEG / Amino acids
HydroGel™ coated slides (Perkin Elmer Life Sciences Inc.)

Improvements of Protein and Antibody Arrays

P. Angenendt, P., J. Glökler, D. Murphy, H. Lehrach and D. J. Cahill.
Towards optimised antibody microarrays: A comparison of current microarray support materials.
Analytical Biochem. (2002) Oct 15;309(2):253-60

P. Angenendt, P., J. Glökler, J. Sobek, H. Lehrach, and D. J. Cahill.
The Next Generation of Protein Microarray Support Materials:
an Evaluation for Protein and Antibody Microarray Applications'
Journal of Chromatography A (2003) 1009: 97 - 104

P. Angenendt, P., J. Glökler, Z. Konthur, H. Lehrach, D. J. Cahill.
3D protein microarrays: performing multiplex immunoassays on a single chip.
Analytical Chemistry, (2003) 75:4368 – 4372

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Improvements of Protein and Antibody Arrays

Lueking, A., Possling, A., Huber, O.,
Horn, M., Eickhoff, H., Schuchardt, J., Lehrach, H., and Cahill, D. J.
A non-redundant protein microarray suitable for antibody screening and serum profiling.
Molecular and Cellular Proteomics (2003) 2(12):1342 - 1349

P.Angenendt, J. Wilde, G. Kijanka, S. Baars, D.J.Cahill,
J. Kreutzberger, H. Lehrach, Z. Konthur, J. Glokler
Seeing better through a MIST: evaluation of monoclonal recombinant antibody fragments on microarrays.
Analytical Chemistry (2004) 15:76 (10):2916-21

P. Angenendt, L.Nyarsik, W. Szaflarski, J. Glokler, K.H. Nierhaus, H. Lehrach, D.J. Cahill, A. Lueking
Cell-free protein expression and functional assay in nanowell chip format.
Analytical Chemistry (2004) 1:76(7):1844-9.

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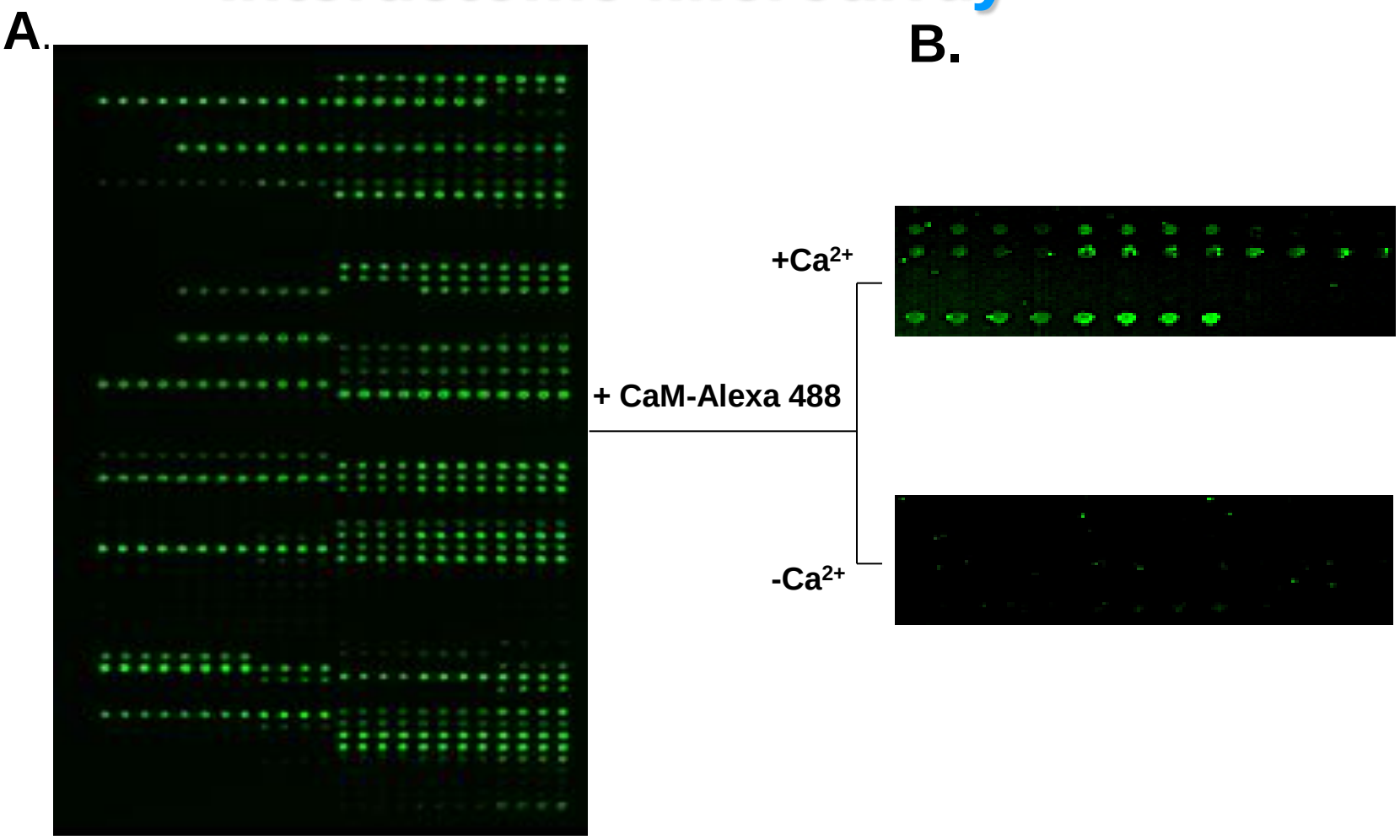
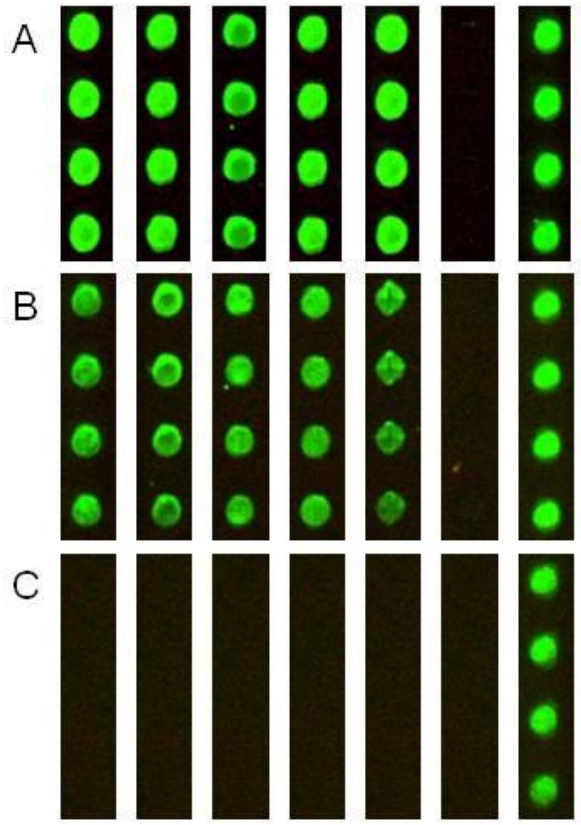
Interactome Microarray



(**A**) A field of a Calmodulin Interactome microarray with proteins highlighted with anti-His tag MAb and labelled secondary antibody.
(**B**) Effect of Ca2+ on the binding of CaM-Alexa to a subset of Interactome proteins

Ca2+ sensitivity of binding of calmodulin to calmodulin interacting proteins on a protein microarray

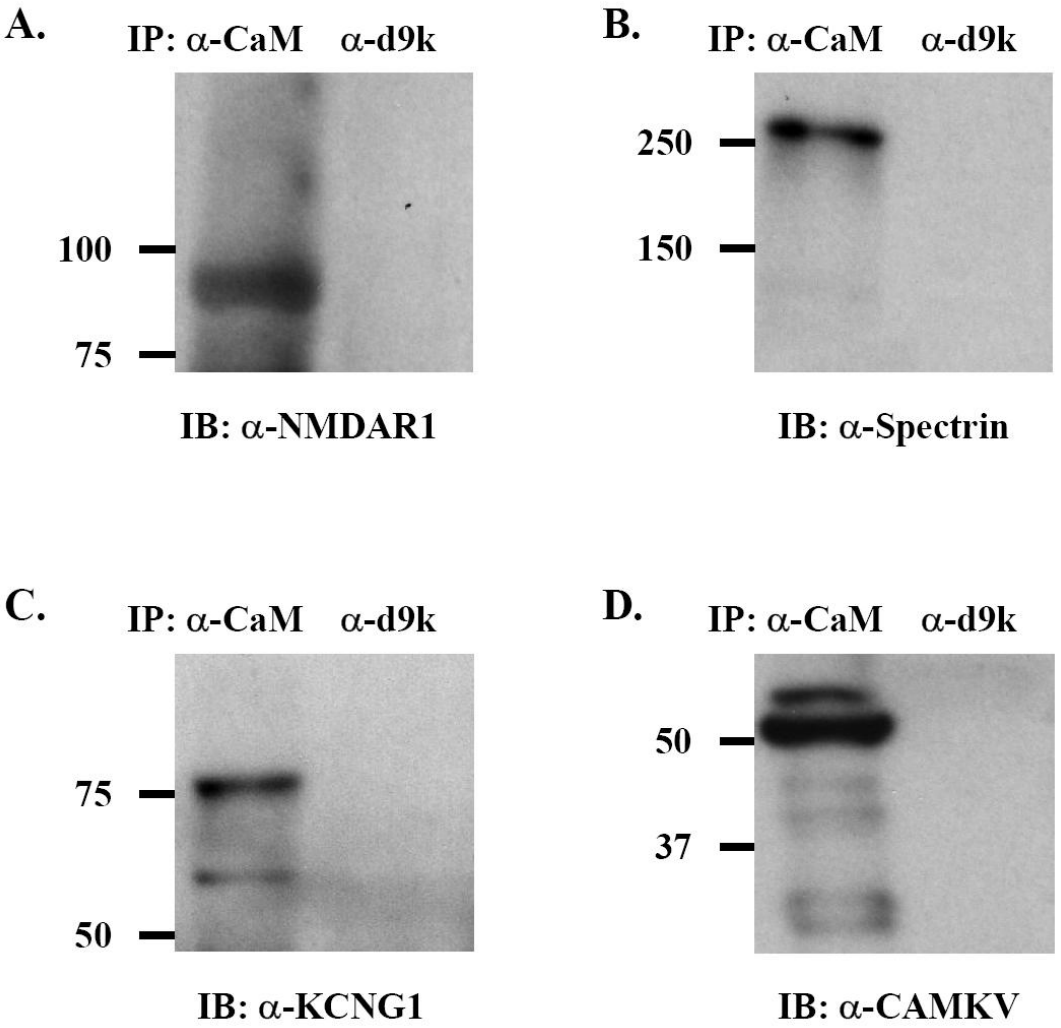


The microarray is incubated with
A) anti-RGS6His and Cy3 labeled anti-mouse IgG,
B) 1 mM CaM-Alexa546 in 1 mM CaCl2 and
C) 1 mM CaM-Alexa546 in 1 mM EDTA.

Lane (1) diphospho mevalonate, (2) ribosomal protein S2,

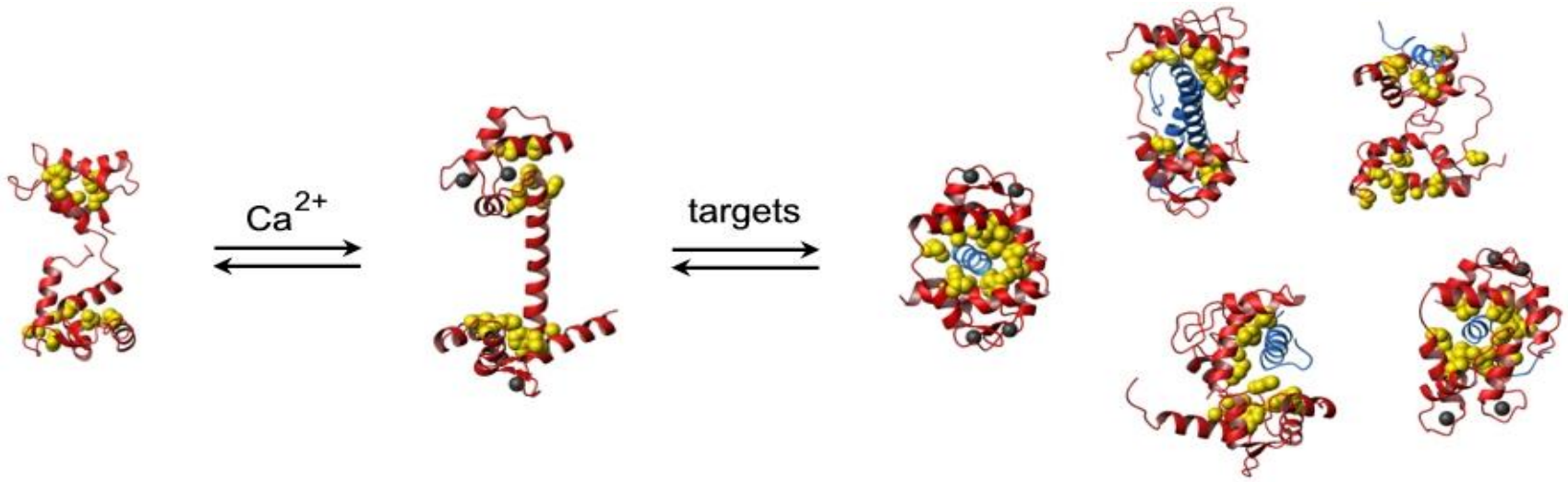
- **(3) dynein, (4) ZNF358, (5) CaM kinase II a, (6) buffer, (7) CaM-Alexa546.**

Immunoprecipitation from hippocampal cell lysates



Western blots of immunoprecipitates from hippocampal cell lysates using either anti-calmodulin IgG (left lane in each panel) or anti-calbindin D9k IgG (right lane in each panel) in the immunoprecipitation (IP) step and (A)anti-NMDAR1 (B)anti-spectrin a-chain (C)anti-potassium voltage gated channel Kv6.1 or (D) anti-vesicle associated CaM-kinase (CaMKV) IgG in immunoblotting (IB) detection step

Exploring the Interactome



Calmodulin binding to the polybasic C-termini of STIM proteins involved in store operated calcium entry

Mikael Bauer, David O'Connell, Dolores Cahill, Sara Linse

Biochemistry Rapid Report 2008

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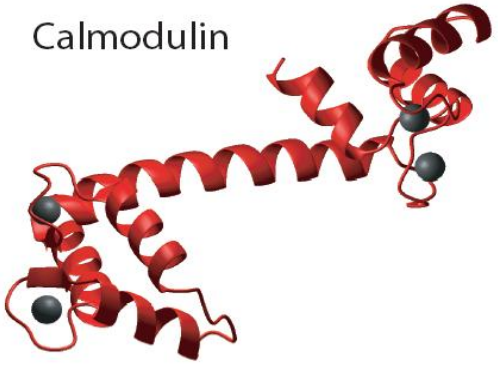
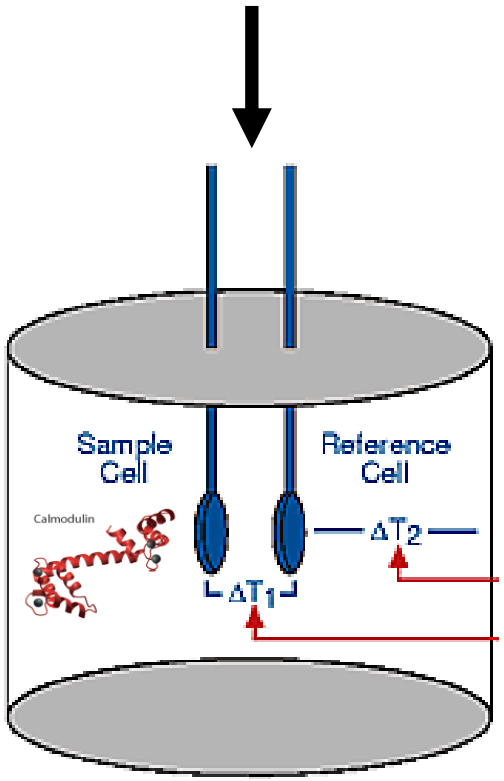
Peptide binding - Isothermal Calorimetry

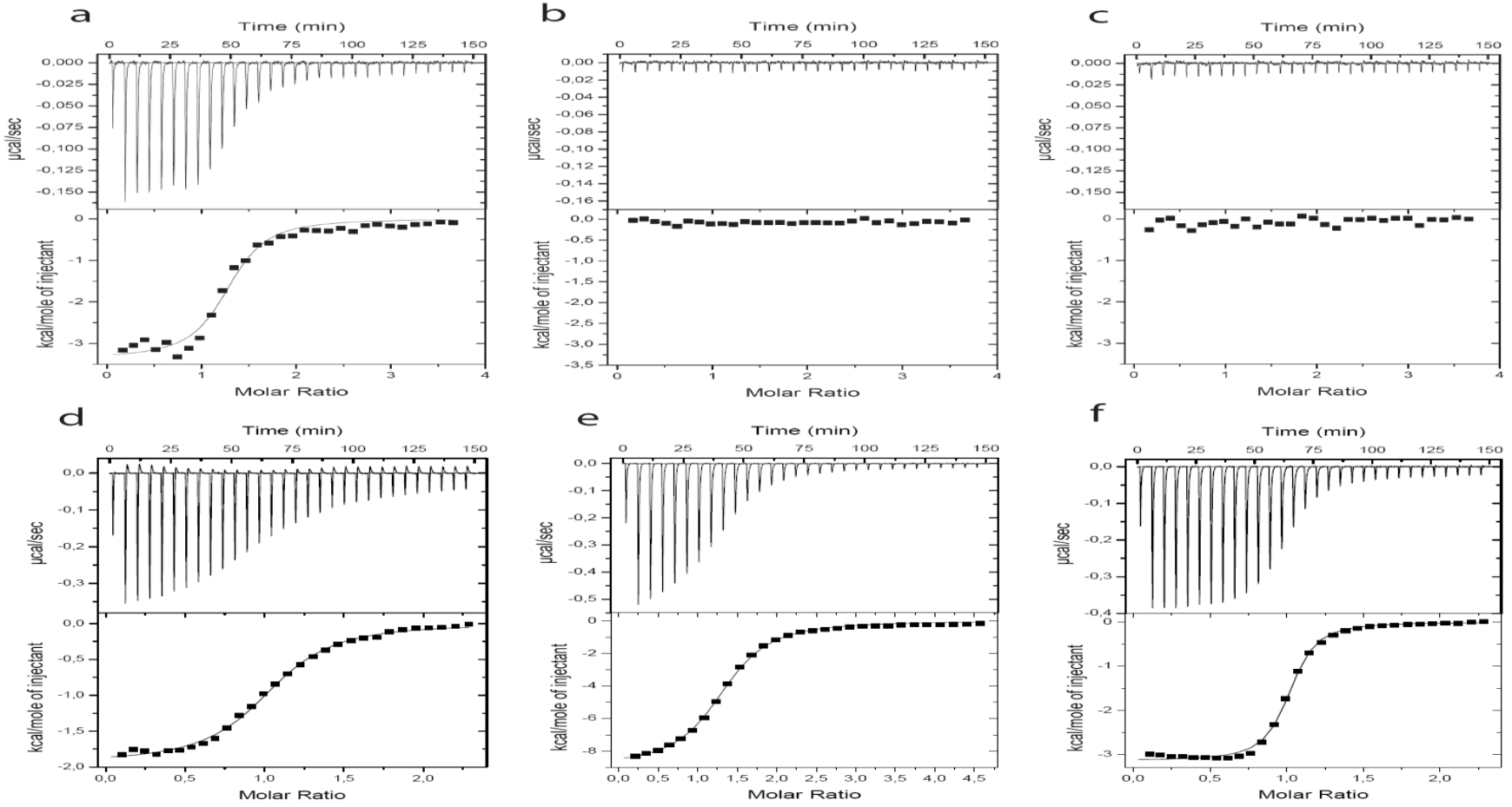
Polybasic C-termini

STIM1: SSPGRKKFPLKIFKKPLKK
STIM2: KSKKPSKIKSLFKKKSK



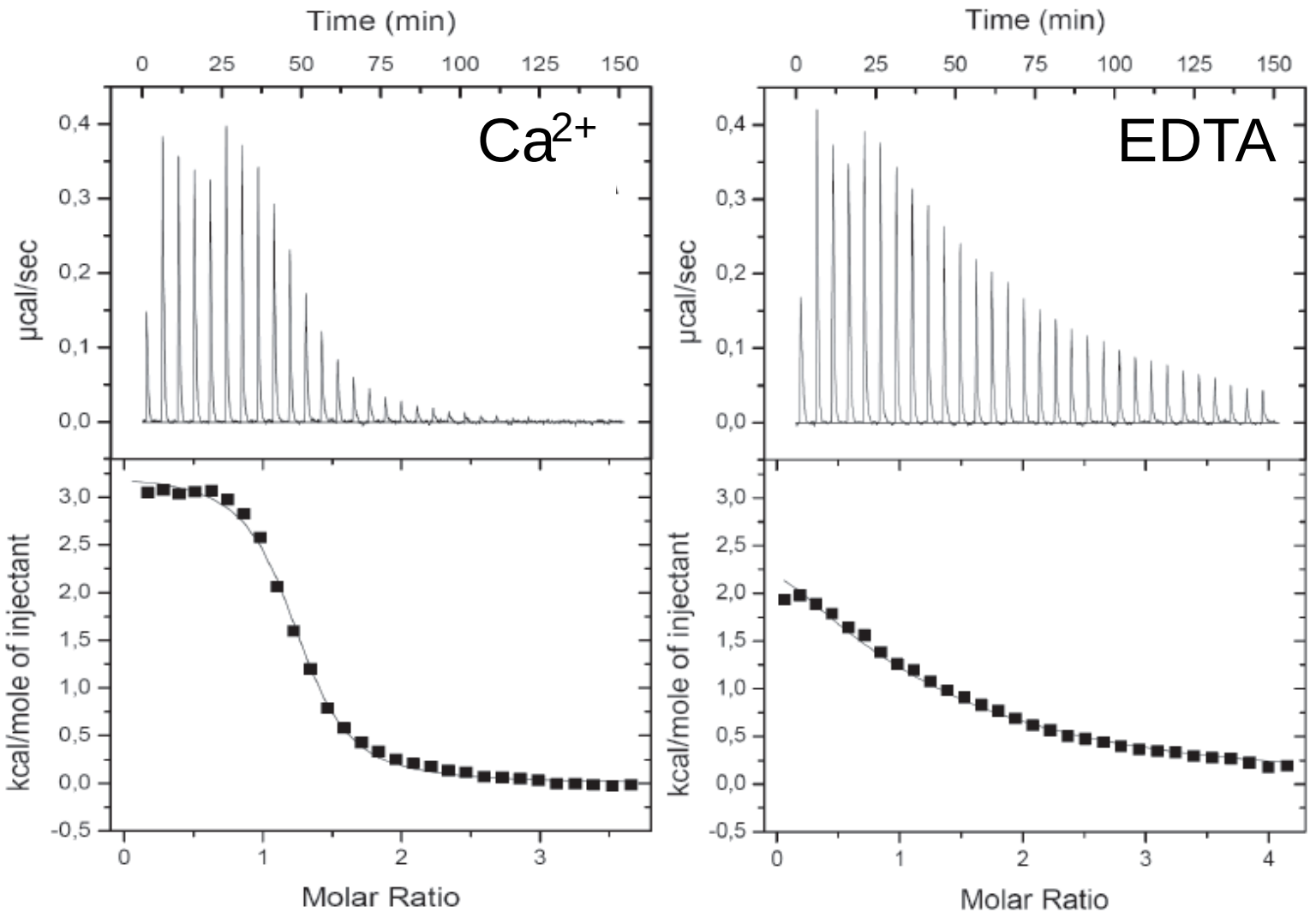


Isothermal titration calorimetry



ICT of peptides (25C) titrated from 200 or 400 µM solutions into 10 µM calmodulin in 10 mM Tris, 150 mM KCl, pH 7.50 with either 1 mM CaCl2 (A,D,E,G) or 1 mM EDTA (B), or peptide titrated into buffer (C).
Upper panels show the raw data, lower panels show integrated heats versus molar ratio of peptide to protein.Solid lines represent the best fit to the data using a 1:1 binding model.

Peptide binding by ITC



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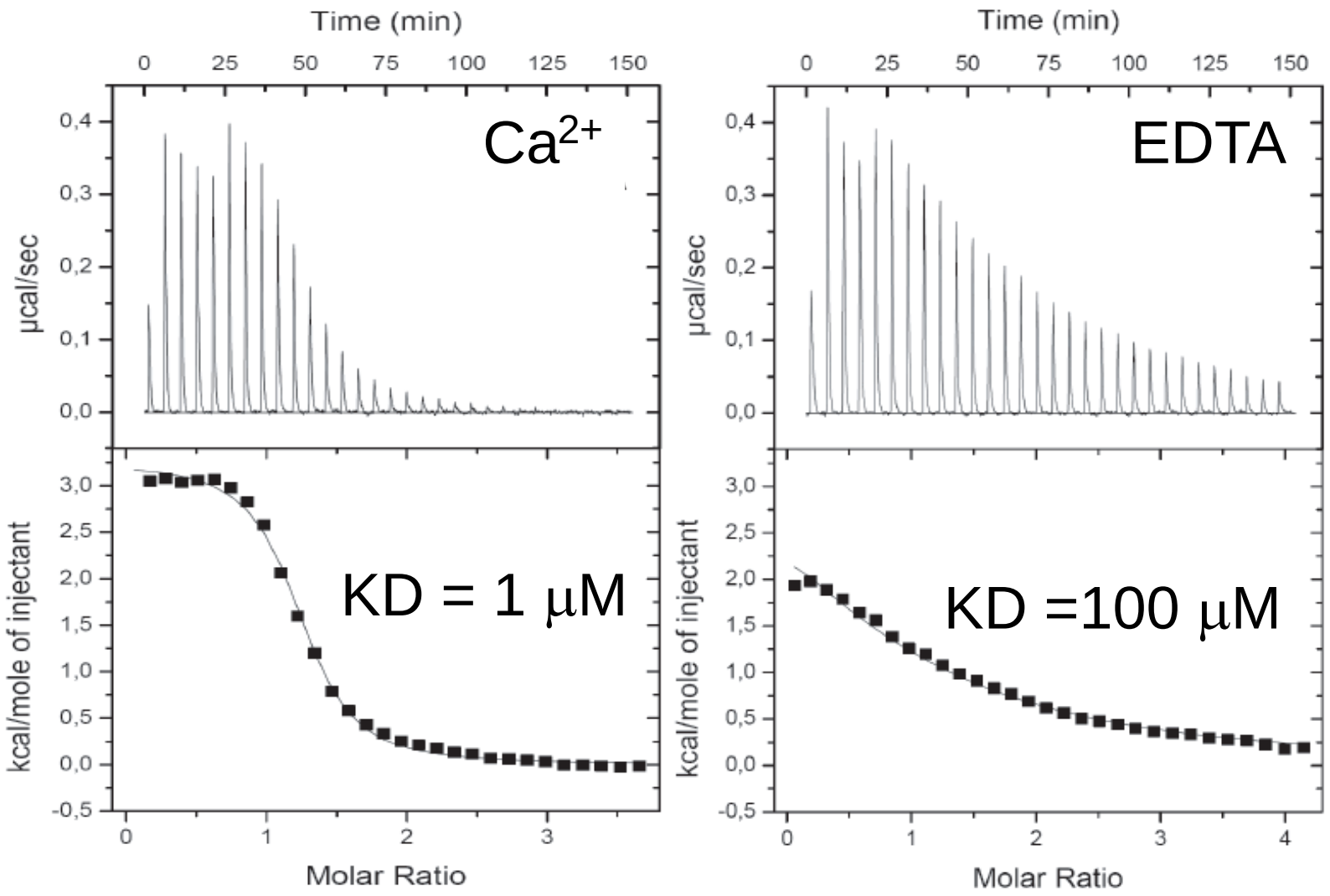
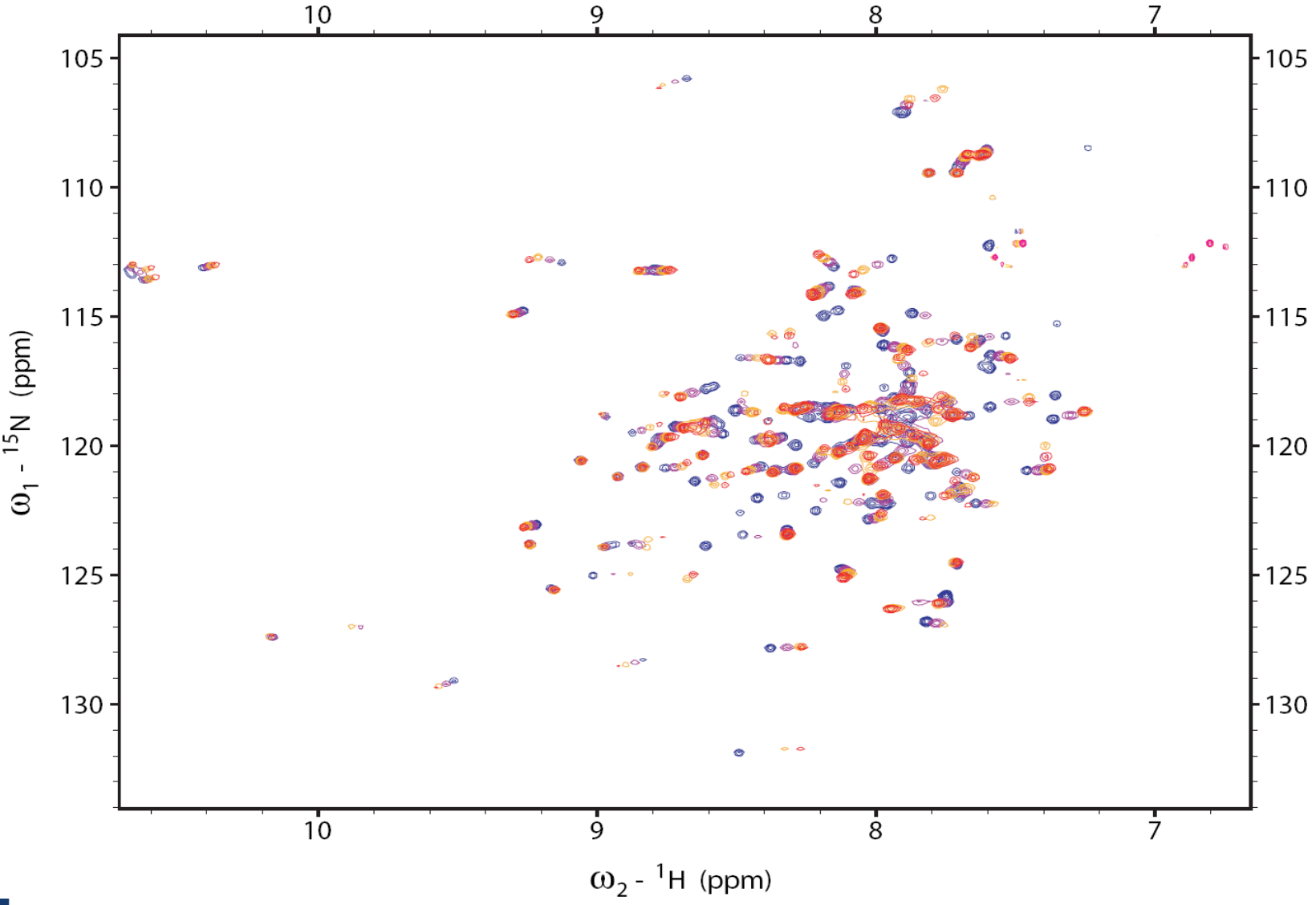
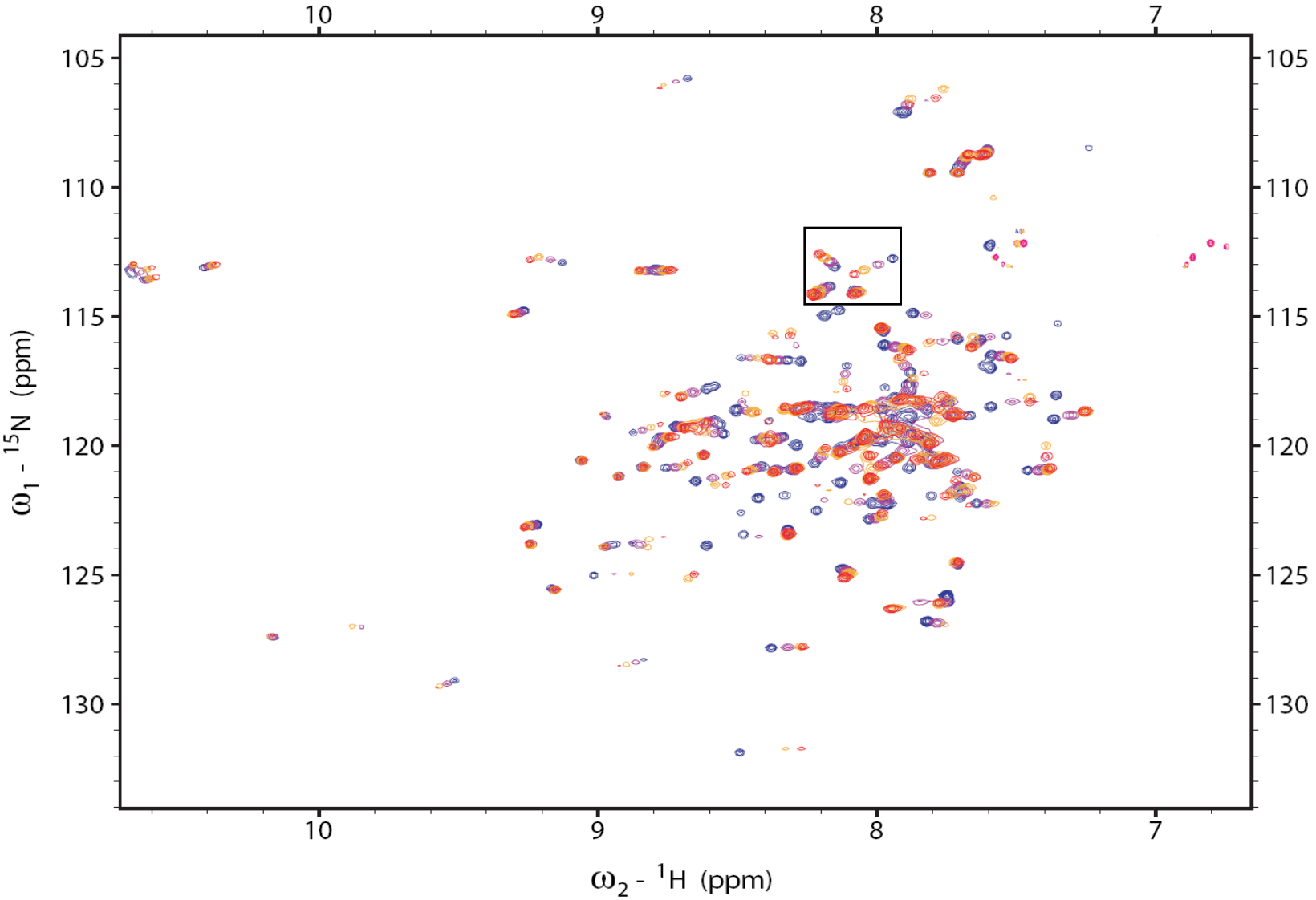
Peptide binding by ITC



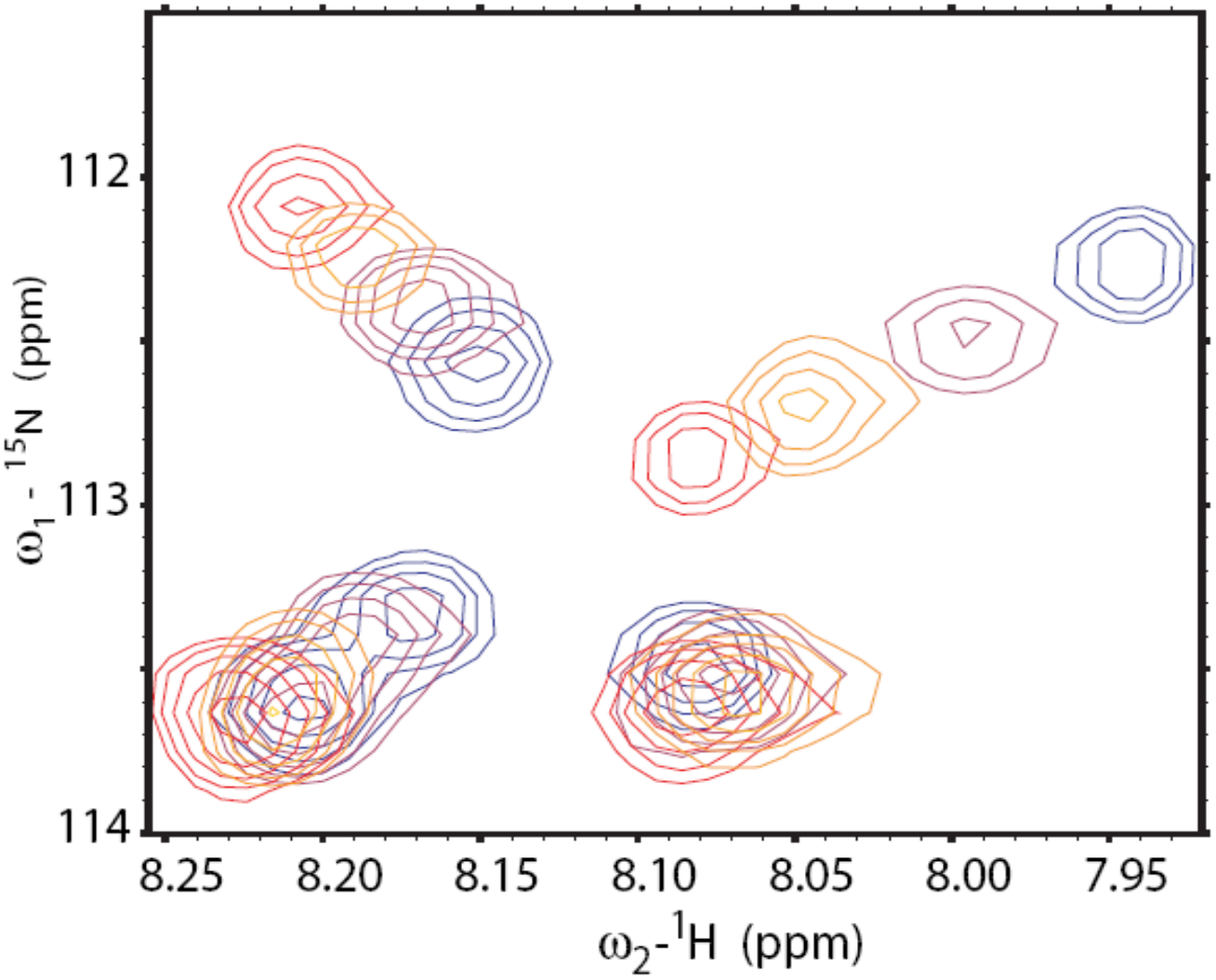
Peptide binding by HSQC NMR



Peptide binding by HSQC NMR



Peptide binding by HSQC NMR

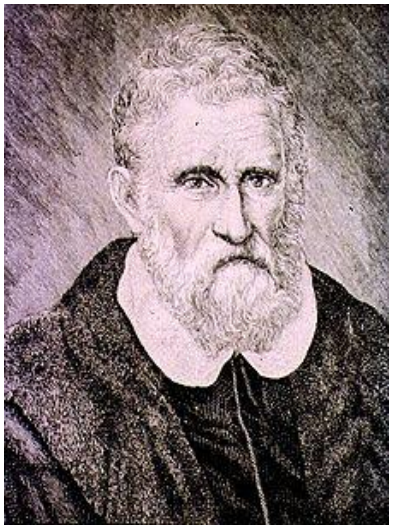


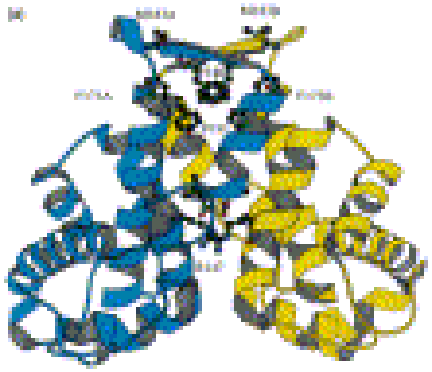
Conclusions from STIM study

• Calmodulin, being dependent on store refilling for its role as the major cytosolic mediator of Ca^{2+} signalling events, binds to STIM1 and STIM2 which are major players in this refilling event

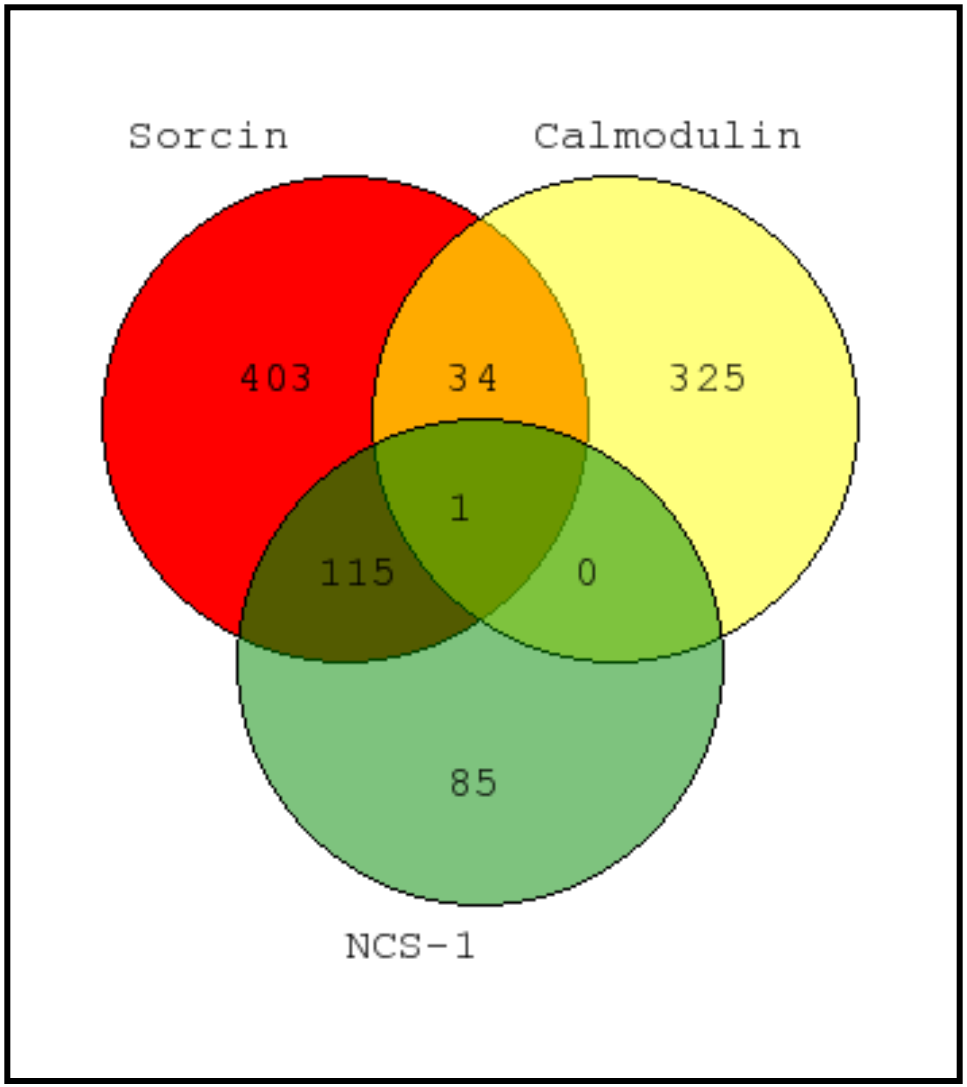
• We speculate that calmodulin thereby is directly involved in regulating this fundamentally important function to ensure the continuation of its own action in intracellular signalling.

Exploring the Sorcin Interactome





Calcium Binding Protein Interactome Analysis



http://mcbc.usm.edu/genevenn/genevenn.htm

Protein – Protein Interaction

O'Connell D, Bauer MC, O'Brien J, O'Connell D, Bauer MC, O'Brien J, O'Kane S, Berggård T, Merino A, Åkerfeldt KS, Linse S, Cahill D.J
Integrated protein array screening and high throughput validation of 70 novel neural calmodulin binding proteins.
Molecular Cellular Proteomics (2010) in press M900324-MCP200

Bauer M., O'Connell D., Cahill, D. J. and Linse S.
Calmodulin binding to the polybasic C-termini of STIM Proteins Involved in Store-Operated Calcium Entry.
Biochemistry (2008) 47:6089-6091.

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Summary

Improvements in Protein and Antibody Arrays (Buessow et al., 1998, Lueking et al., 2003; Angenendt et al., 2002, 2003 a,b, 2004a, 2004b, 2006)

Characterisation of antibody specificity and cross reactivity (Lueking et al.,2003, Taussig et al., 2007)

Profiling antibody repertoire in autoimmune disease;

Proof of concept in Arthritis (Lueking et al., 2003)

Generated disease associated panel of proteins in

- Dilated Cardiomyopathy (DCM) (Horn et al., 2006)
- Alopecia areata (Lueking et al., 2006)
- SLE (Gutjahr et al., 2005)

Peptide-Protein Interaction Screening (Larkin et al., 2004)

Protein-Protein Interaction Screening (Bauer et al, 2008, O'Connell et al., 2010)

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