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Introduction

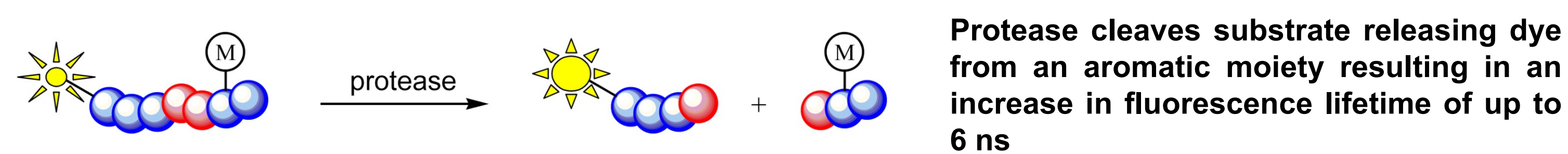
The development of biochemical and cell based assays that use fluorescence lifetime (FLT) as the reporting modality is highly attractive. This intrinsic fluorescence property is generally independent of probe concentration and volume, and is unaffected by auto-fluorescence, light scattering and inner filter effects. Consequently, the inherent properties of this method should lead to more robust assays and enable background interference from fluorescent compound libraries to be minimised, leading to fewer false positives in drug screening applications.

To realise the benefits of this approach, we have recently developed the **FLEXYTE™** assay platform technology, which uses fluorescence lifetime as the reporting modality. Key to the success of this approach has been the development and application of 9-aminoacridine (9AA) as a long lifetime fluorescent reporter ($\tau = 17$ ns). By exploiting the fluorescence properties of this dye, 9AA labelled substrates have been developed, which produce significant changes in fluorescence lifetime upon modification by a variety of target enzymes.

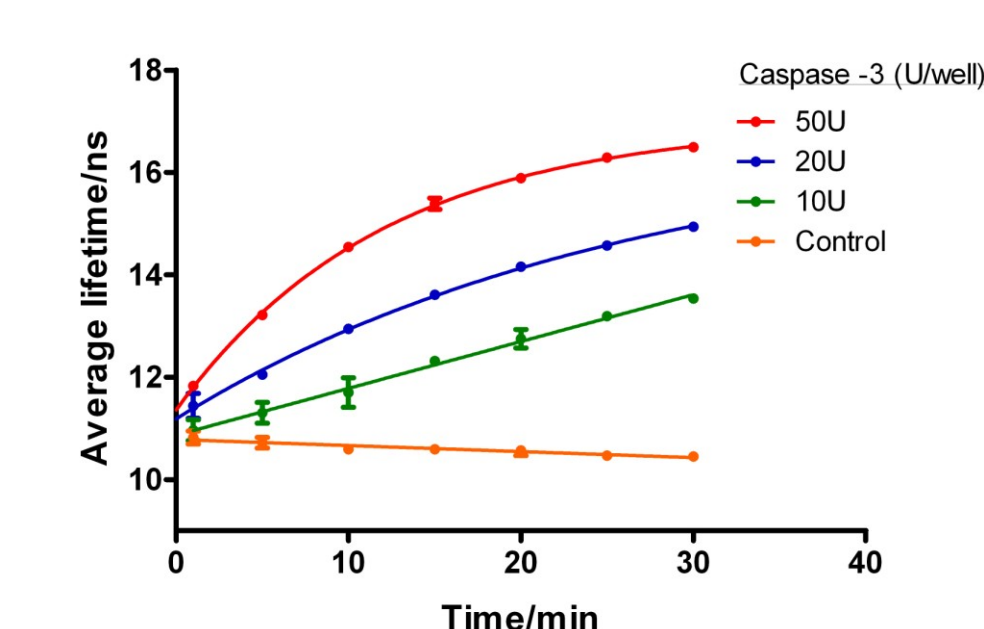
In one application, the **FLEXYTE™** technology has been developed to provide a generic platform for assaying proteases. Using 9AA labelled peptide substrates, homogeneous assays have been configured for a number of different proteases. The substrates are designed so that cleavage of the substrate by the enzyme causes a significant increase in fluorescence lifetime. These assays are robust, with high Z' factors and enable enzyme activity to be monitored in real-time.

Our recently launched **FLEXYTE™** Ser / Thr protein kinase platform uses three generic peptide substrates to configure assays for a broad panel of kinases and the use of tailored substrates for specific kinases as required. This homogeneous, antibody-free approach uses a phosphoserine or phosphothreonine chelating agent to induce a change in the fluorescence lifetime of the 9AA label. We have now configured this technology for assaying Ser / Thr phosphatases using bespoke 9AA-labelled phosphorylated peptide substrates. In addition, we now report the utility of 9AA-labelled peptide substrates for configuring fluorescence lifetime tyrosine kinase assays. These homogeneous, real-time assays can be configured for a broad range of tyrosine kinases using appropriate 9AA-labelled peptide substrates.

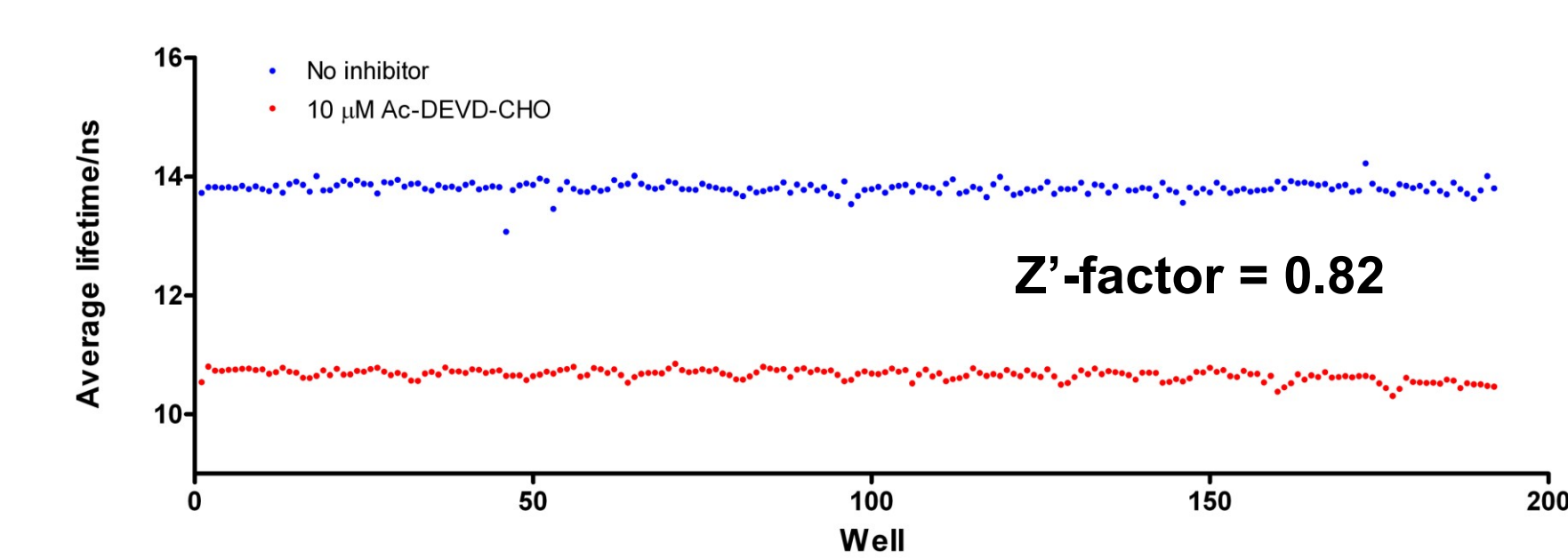
Real-Time Protease Assay



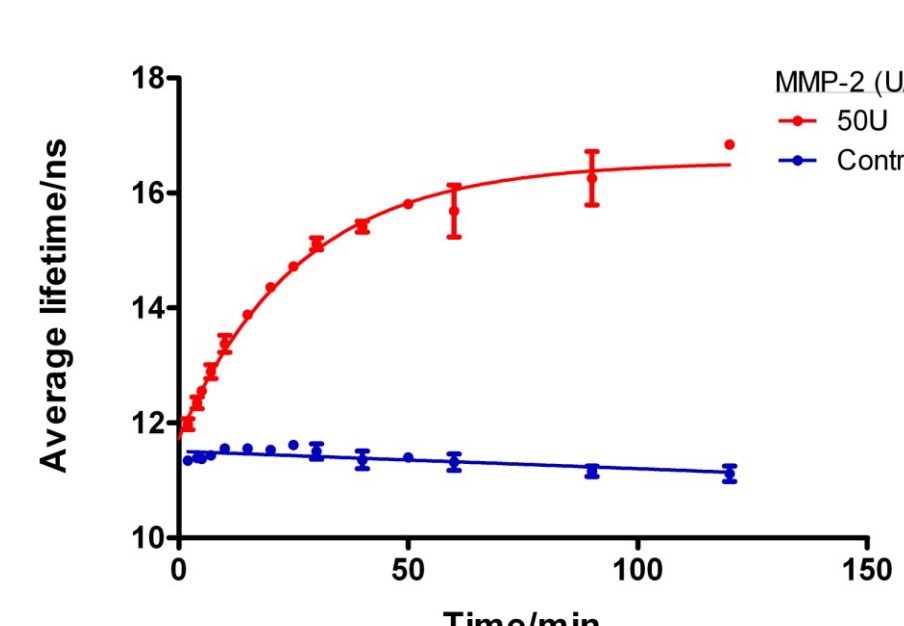
Caspase-3 – Enzyme Titration



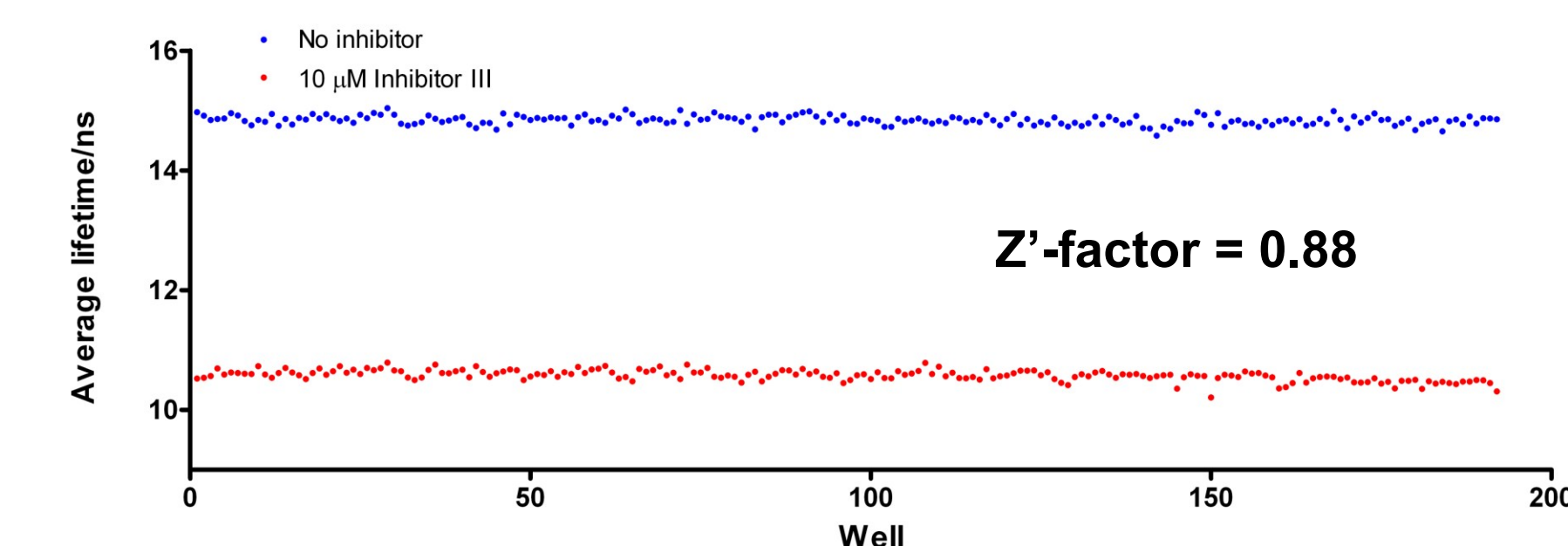
Caspase-3 Assay Z'-Factor



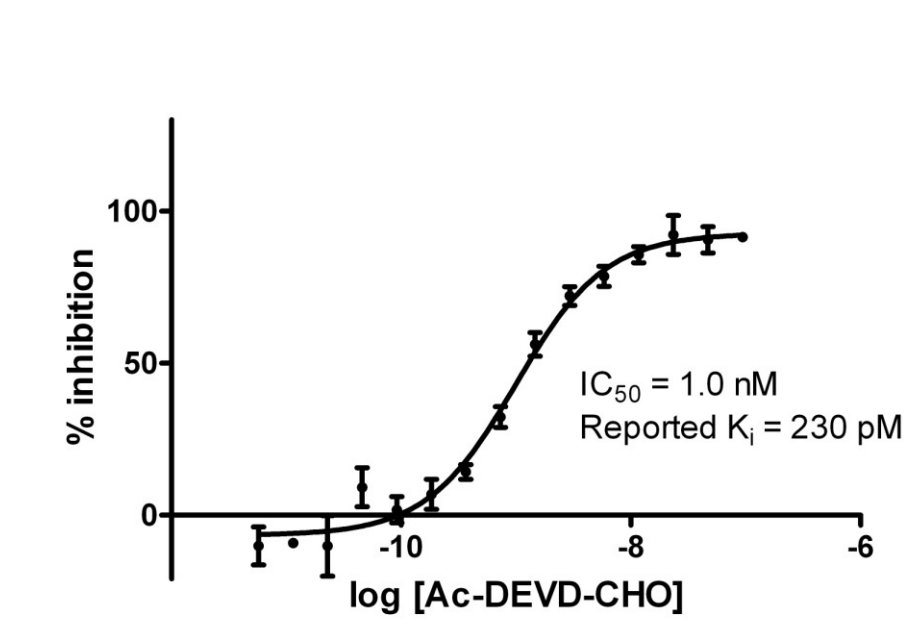
MMP-2 – Enzyme Titration



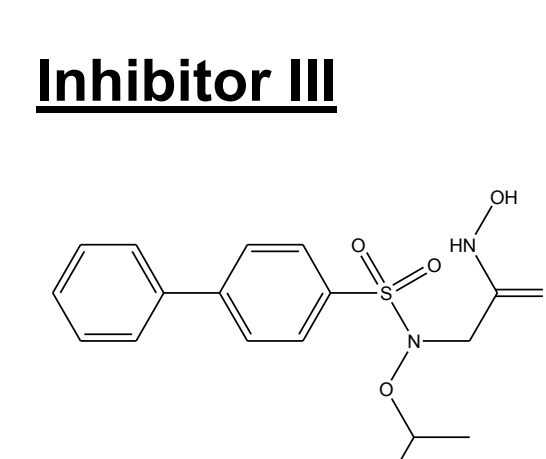
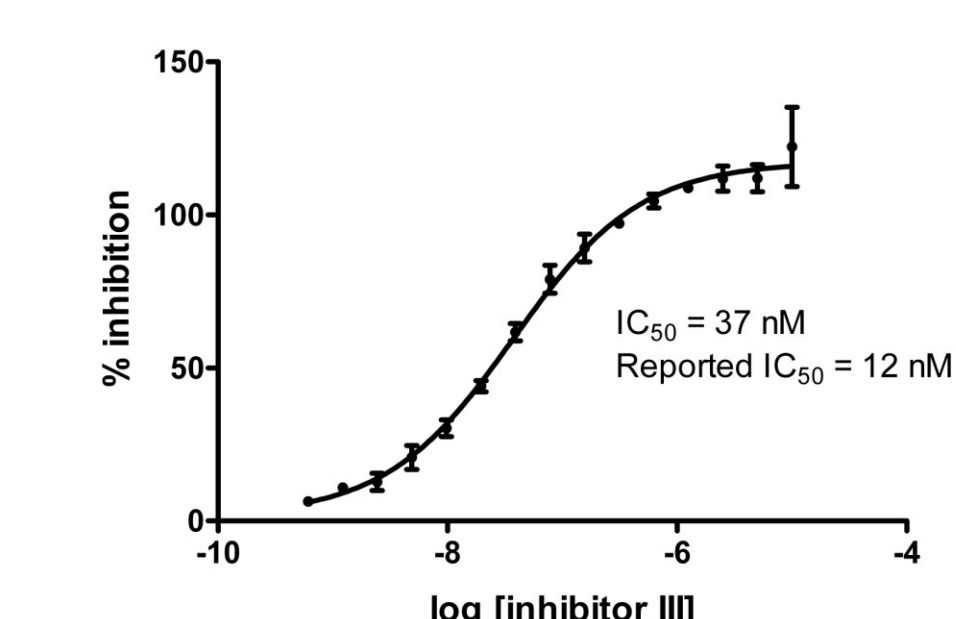
MMP-2 Assay Z'-Factor



Caspase-3 - Inhibitor Assay

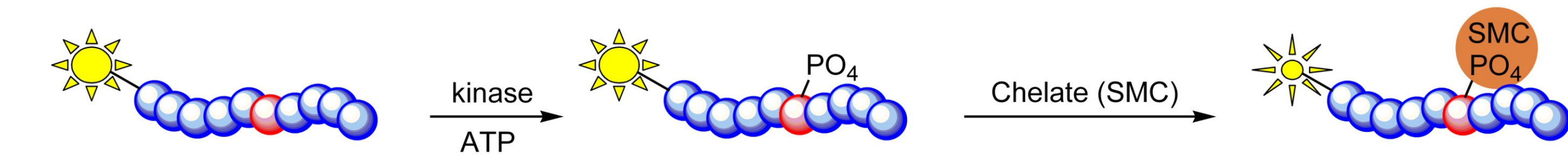


MMP-2 - Inhibitor Assay



- **FLEXYTE™** protease assays are homogeneous real-time assays that allow for kinetic measurements to be performed
- Excellent Z'-factors (>0.8) demonstrate that **FLEXYTE™** protease assays are suitable for HTS and profiling applications
- Bespoke labelled peptides can be configured for a broad range of proteases

Universal Ser / Thr Protein Kinase Assay

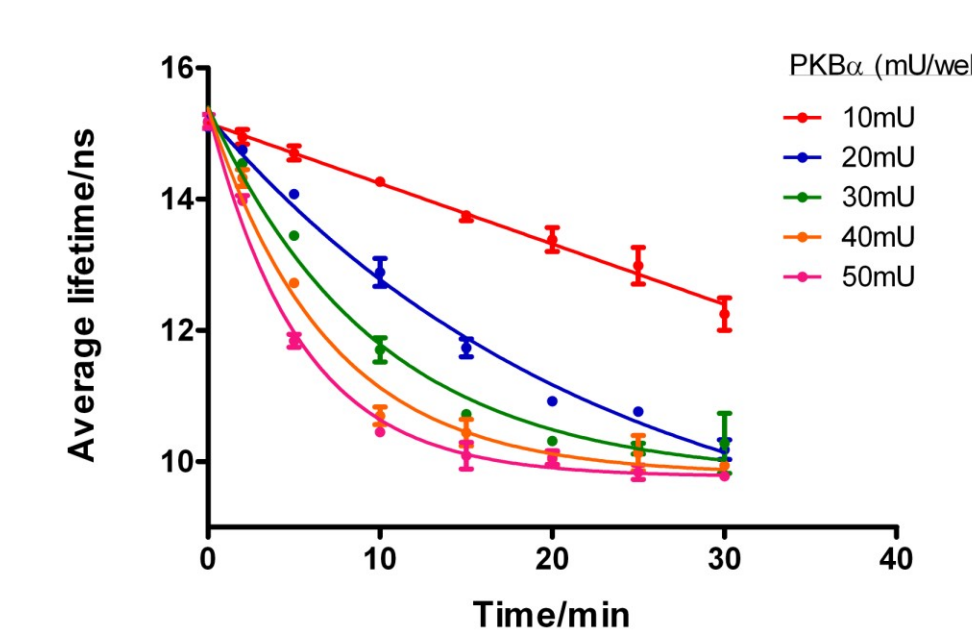


Dye labelled generic peptide substrate with long fluorescence lifetime

Kinase phosphorylates substrate on Ser or Thr

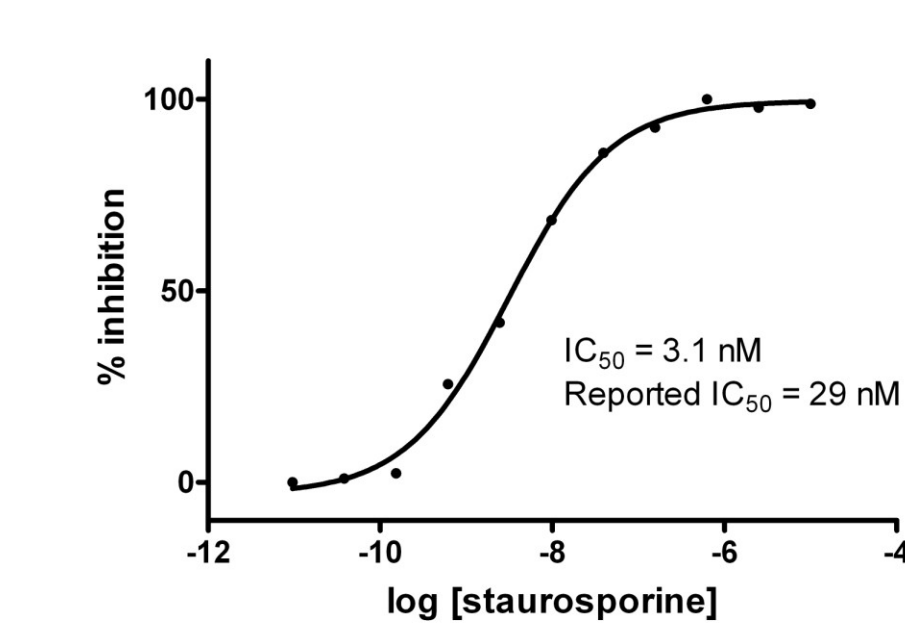
SMC complexes the phosphate group and effects a reduction in fluorescence lifetime of up to 5 ns

PKB α Assay – Enzyme Titration

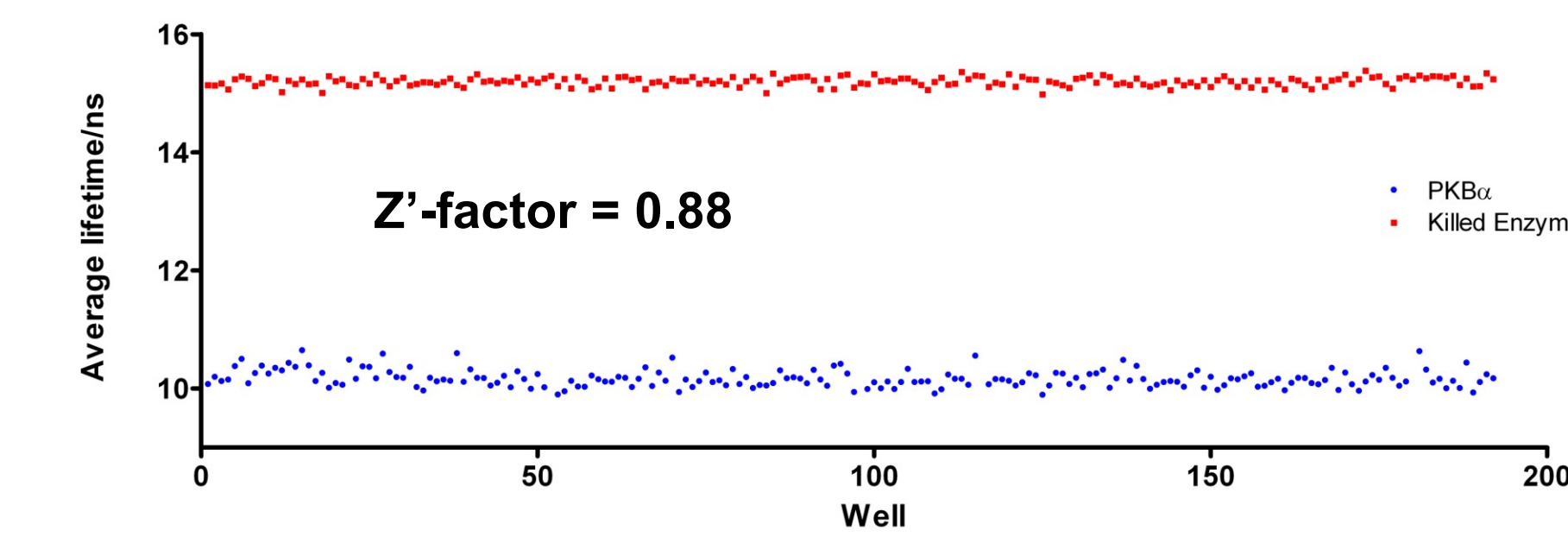


- Generic substrates (KS 1-3) configured for assaying a broad panel of Ser / Thr protein kinases (>100). Tailored substrates developed to meet the requirements of other kinases.
- **FLEXYTE™** protein kinase assays give excellent Z'-factors (>0.8)
- Compatible with a wide range of ATP and substrate concentrations
- Assays are easily miniaturised to low volumes. Application to HTS demonstrated

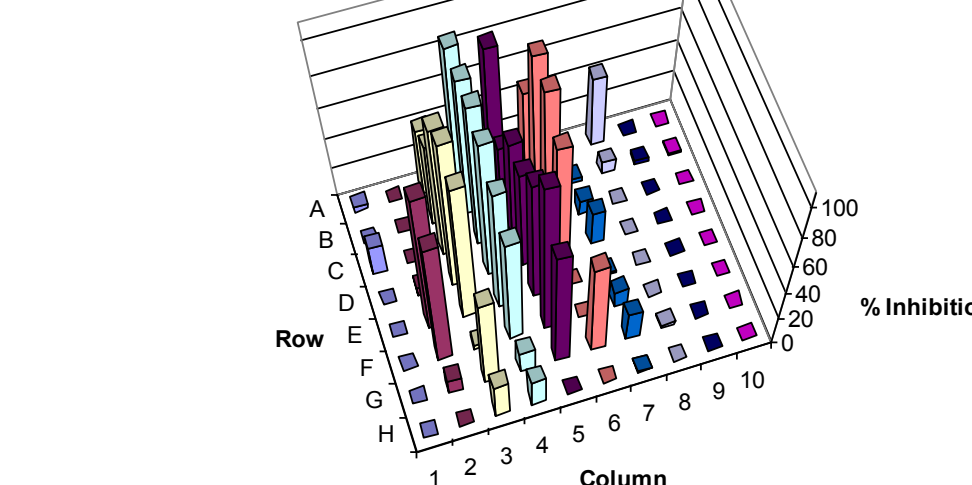
Staurosporine Inhibition



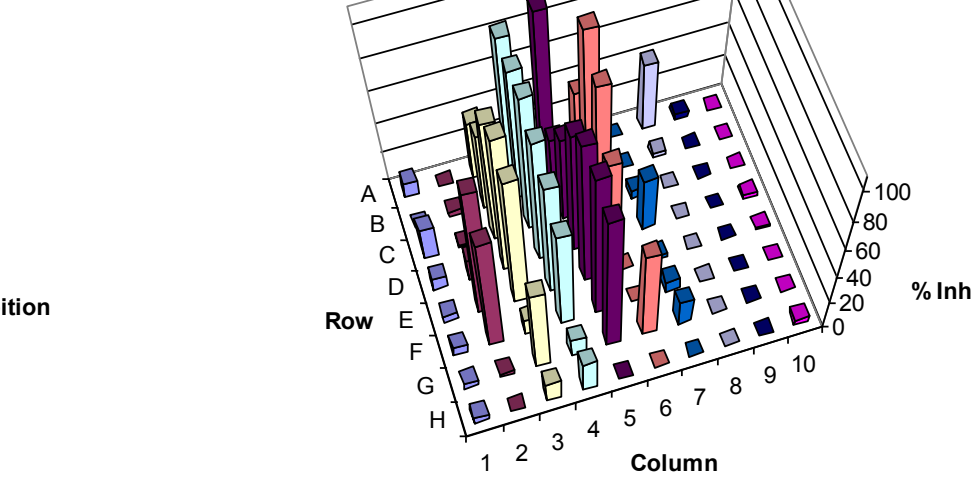
PKB α Assay Z'-Factor



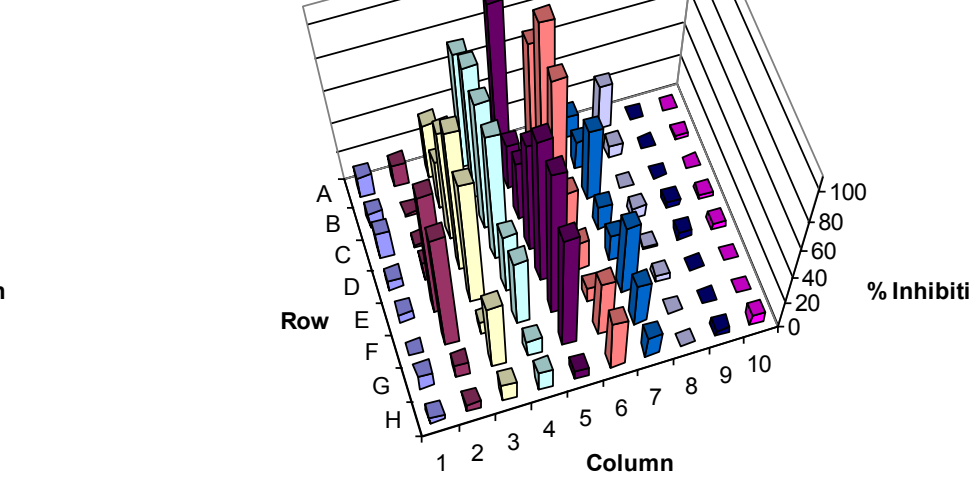
FLEXYTE™



Radiometric

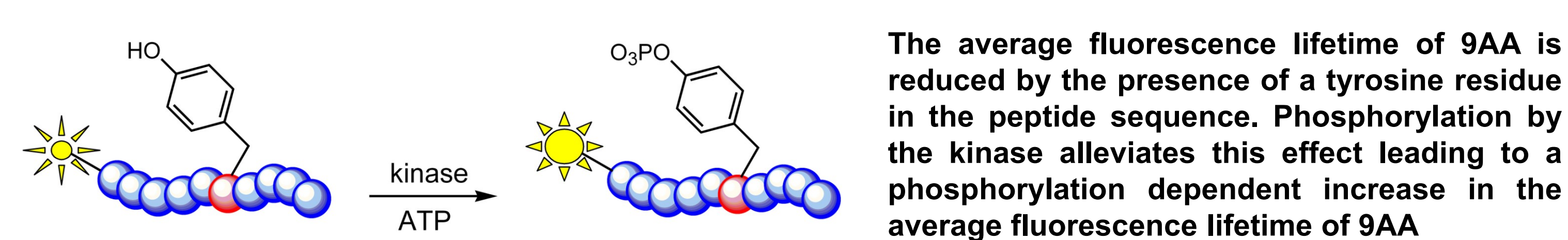


TR-FRET

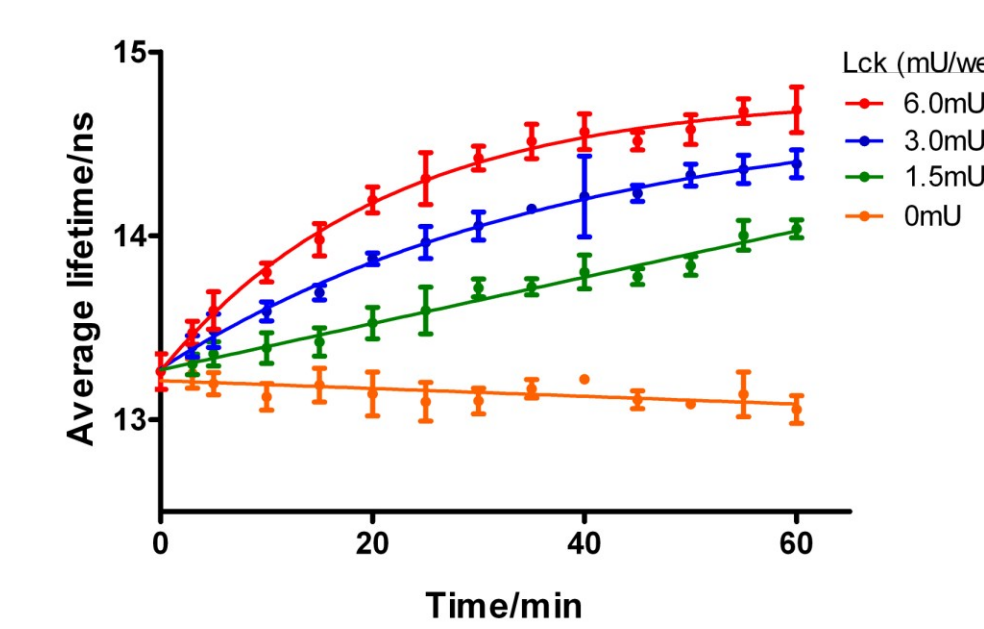


FLEXYTE™ PKB α assay shows excellent comparison with gold-standard radiometric assay and TR-FRET for hit finding (Pearson correlations: 0.98 and 0.93 respectively)

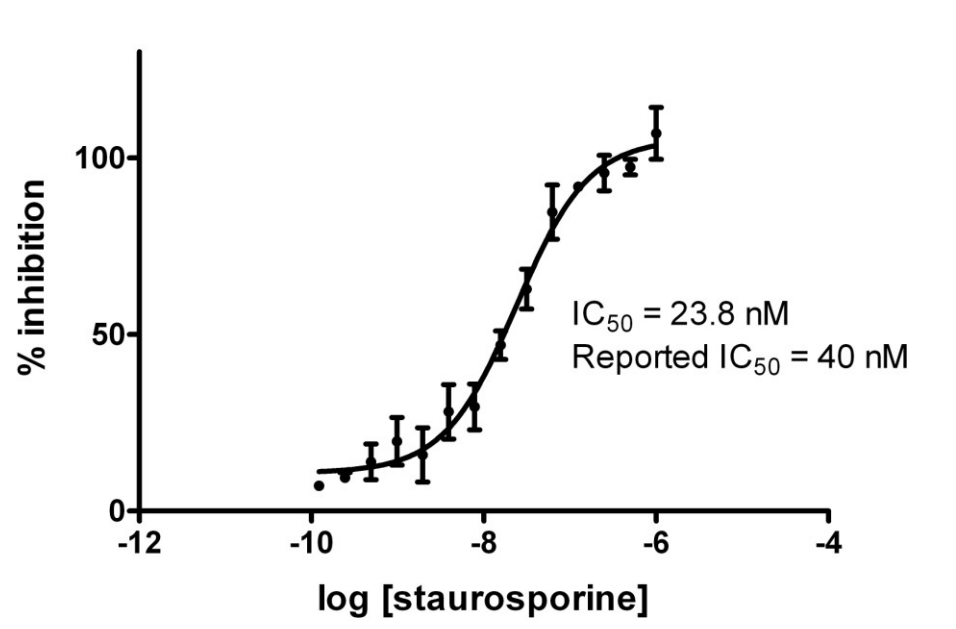
Real-Time Tyrosine Kinase Assay



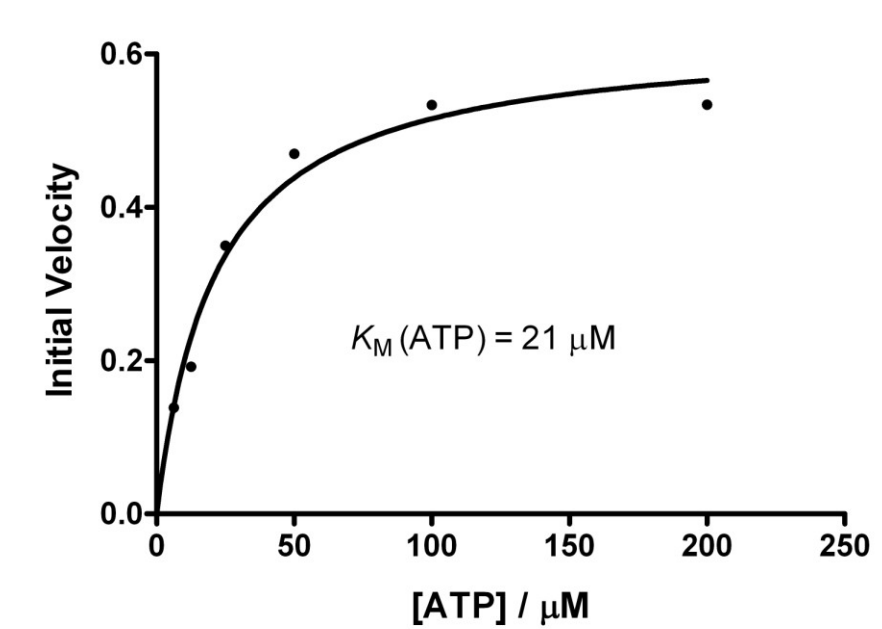
Lck Assay – Enzyme Titration



Staurosporine Inhibition



ATP K_M



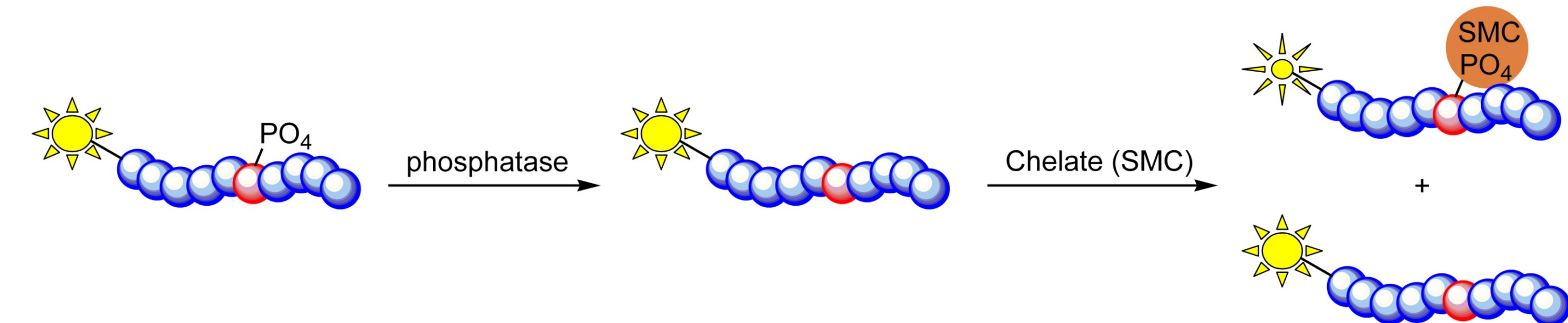
FLEXYTE™ Protease Assays

- **FLEXYTE™** assays have now been developed for proteases, with enzyme activity reported directly by an increase in fluorescence lifetime
- This universal platform technology provides for homogeneous, reliable, real-time monitoring of protease activity
- Generic approach enables **FLEXYTE™** assays to be configured for a broad range of proteases
- B. A. Maltman, C. J. Dunsmore, S. C. M. Couturier, A. E. Tirnaveanu, Z. Delbederi, R. A. S. McMordie, G. Naredo, R. Ramage, and G. Cotton, *Chem. Commun.*, 2010, **46**, 6929-6931.

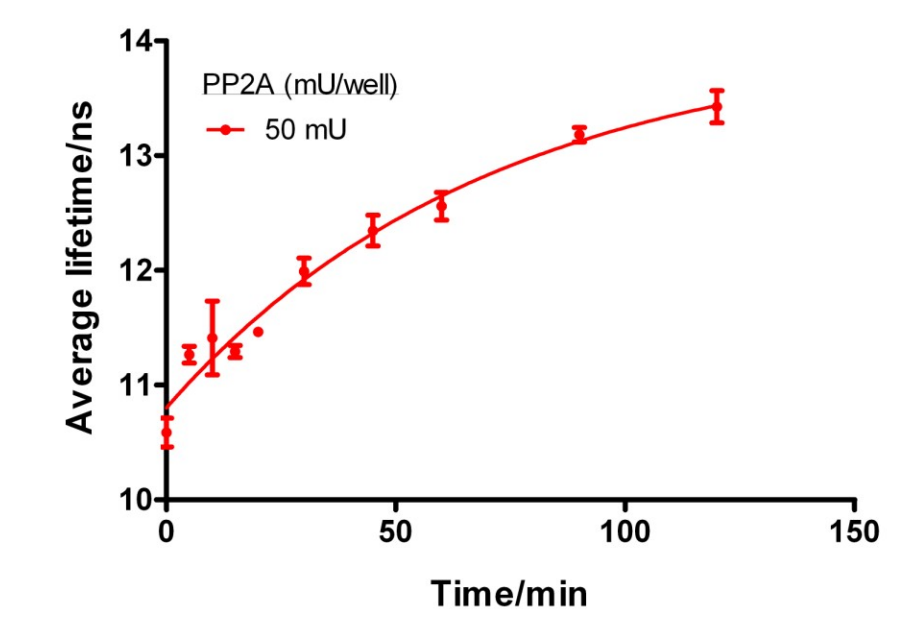
FLEXYTE™ Protein Kinase and Phosphatase Assays

- A universal **FLEXYTE™** Ser / Thr protein kinase assay has been developed based on fluorescence lifetime as the reporting modality
- Generic substrates enable **FLEXYTE™** assays to be configured for a broad panel of Ser / Thr protein kinases in a rapid fashion (covering > 100 different kinases)
- The technology was shown to be extendable to phosphatase assays using bespoke 9AA-labelled peptide substrates
- The **FLEXYTE™** assay platform provides an antibody free, non-radioactive, and cost effective solution for protein kinase and phosphatase screening
- M. J. Paterson, C. J. Dunsmore, R. Hurteaux, B. A. Maltman, G. J. Cotton and A. Gray, *Anal. Biochem.*, 2010, **402**, 54-64.
- In addition, FLT assays have now been configured for tyrosine kinases utilising 9AA-labelled peptide substrates

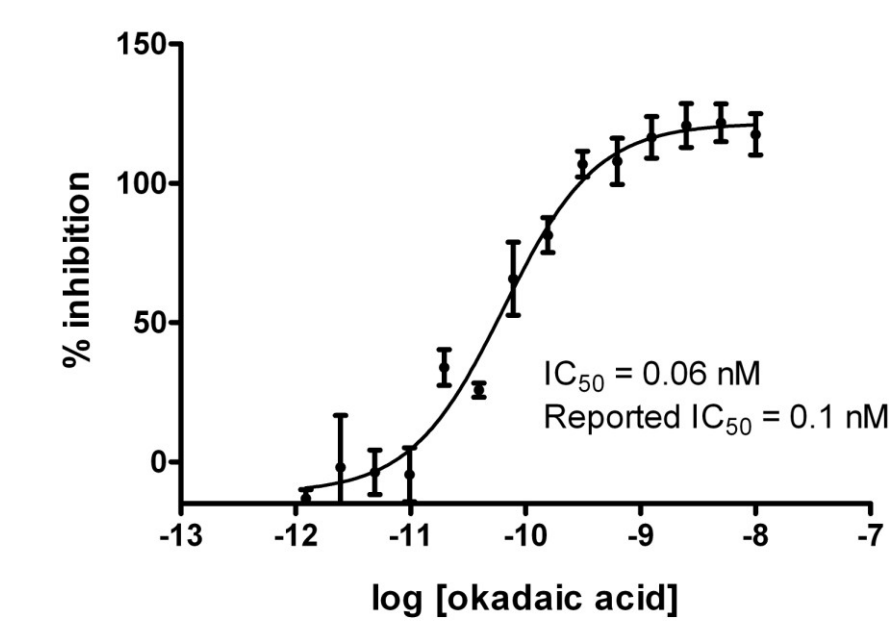
Ser / Thr Phosphatase Assay



PP2A - Enzyme Time-Course



Okadaic Acid Inhibition



FLEXYTE™ phosphatase assays. Phosphatase dephosphorylates peptide substrate and addition of SMC reduces the fluorescence lifetime of phosphorylated substrate only. Hence, reaction is monitored by an increase in fluorescence lifetime of the dephosphorylated product