**Improved Akt reporter reveals intra- and inter-cellular heterogeneity and oscillations in signal transduction**

Dougall M. Norris¹,⁴, Pengyi Yang²,⁴, James R. Krycer¹, Daniel J. Fazakerley¹, David E. James¹,³ and James G. Burchfield¹.

¹ Charles Perkins Centre, School of Life and Environmental Sciences, The University of Sydney, Sydney, 2006, Australia
² Charles Perkins Centre, School of Mathematics and Statistics, The University of Sydney, NSW 2006, Australia
³ Sydney Medical School, The University of Sydney, Sydney, 2006, Australia
⁴ These authors contributed equally to this work

Corresponding author email: james.burchfield@sydney.edu.au

**Key words:** Akt, recruitment, oscillation, adipocyte, insulin, signalling

**Summary Statement:**
Plasma membrane recruitment of full-length Akt2 in 3T3-L1 adipocytes displays both polarity and oscillations in membrane association following insulin stimuli.
Abstract

Akt is a key node in a range of signal transduction cascades and plays a critical role in diseases such as cancer and diabetes. Fluorescently-tagged Akt reporters have been used to discern Akt localisation, yet it has not been clear how well these tools recapitulate the behaviour of endogenous Akt. Here we observed that fusion of eGFP to Akt impaired both insulin-stimulated plasma membrane recruitment and its phosphorylation. Endogenous-like responses were restored by replacing eGFP with TagRFP-T. The improved response magnitude and sensitivity afforded by TagRFP-T-Akt over eGFP-Akt enabled monitoring of signalling outcomes in single cells at physiological doses of insulin with subcellular resolution and revealed two previously unreported features of Akt biology. In 3T3-L1 adipocytes, stimulation with insulin resulted in recruitment of Akt to the plasma membrane in a polarised fashion. Additionally, we observed oscillations in plasma membrane localised Akt in the presence of insulin with a consistent periodicity of 2 minutes. Our studies highlight the importance of fluorophore choice when generating reporter constructs and shed light on new Akt signalling responses that may encode complex signalling information.
Introduction

Imaging of signal transduction in live single cells is essential for achieving both the spatial and temporal resolution required for dissecting complex patterns of information that can be encoded within signals (Kurakin, 2005; Sonnen and Aulehla, 2014). The success of the live cell imaging approach is reliant on stable, sensitive reporter constructs that recapitulate the behaviour of their endogenous surrogates as faithfully as possible. To achieve this, careful design of the reporter is essential and genetically encoded reporters need to be carefully validated. In particular, it is becoming increasingly clear that choice of fluorophore is critical in obtaining optimal functionality. Here we describe an improved reporter of Akt and demonstrate that eGFP tagging of this protein impairs its biological function.

The Ser/Thr kinase Akt serves as a master signalling switch (Manning and Cantley, 2007), whose recruitment to the plasma membrane (PM) and subsequent activation is essential for many distinct cellular processes including metabolism, cell growth, survival and apoptosis (Kandel and Hay, 1999). There are 3 Akt isoforms (Akt1, Akt2, Akt3) that have distinct tissue distribution profiles and reportedly distinct functions (Dummler and Hemmings, 2007). Akt possesses an N-terminal pleckstrin homology (PH) domain, a kinase domain and a C-terminal hydrophobic motif. The PH domain has a high affinity for phosphatidylinositol (3,4,5) triphosphate (PIP3), a phospholipid that is produced by PI-3-kinase (PI3K) in response to growth factor stimulation and degraded by phosphatase and tensin homolog (PTEN) and SH2 domain containing inositol 5-phosphatase 2 (SHIP2). Recruitment of Akt to the PM via its PH domain is critical for its activation. The PH domain-PIP3 interaction is thought to cause a conformational change that allows Akt to be activated by phosphorylation on Thr309 (in Akt2, T308 in Akt1; T305 in Akt3) and Ser474 (in Akt2, S473 in Akt1; S472 in Akt3) residues by the kinases PDK1 and mTORC2, respectively (Alessi et al., 1997; Sarbassov, 2005).

Subcellular fractionation and immunofluorescence imaging of endogenous Akt suggest that it is strongly recruited to the PM in response to growth factor stimulation (Carvalho et al., 2000; Currie et al., 1999; Wang, 2006). Further, live cell imaging of fluorescent Akt reporter constructs have revealed important insights such as isoform specificity, chemotaxis, phospholipid binding, conformational changes in Akt, nuclear activation and membrane diffusion rates, across a range of cell lines (Calleja et al., 2003; Gonzalez and McGraw, 2009; Kontos et al., 1998; Lasserre et al., 2008; Servant et al., 2000; Wang and Brattain, 2006). Numerous groups have assessed Akt recruitment to the PM in response to various stimuli in live cells using GFP-tagged full-length Akt and Akt PH domain
constructs. The extent of Akt membrane recruitment in these studies was highly variable. Furthermore, none of these studies appear to have compared the activity and/or function of these constructs to the endogenous protein (Asano et al., 2008; Calleja et al., 2003; Carpten et al., 2007; Cenni et al., 2003; Currie et al., 1999; Du et al., 2014; Feng et al., 2014; Gonzalez and McGraw, 2009; Huang et al., 2011; Imazaki et al., 2010; Kontos et al., 1998; Lasserre et al., 2008; Parikh et al., 2012; Rodríguez-Escudero et al., 2005; Servant et al., 2000; Terashima et al., 2005; Watton and Downward, 1999; Zhang et al., 2009). While GFP remains the most commonly used fluorescent protein, there are a growing number of publications that report fusion protein dysfunction (Goto et al., 2003; Huang and Shusta, 2006; Kalatskaya et al., 2006; Liu et al., 1999; Yantsevich et al., 2009) and mislocalisation (Skube et al., 2010; Zhu et al., 2013), which one group has attributed to the affinity of eGFP for the nucleus (Seibel et al., 2007). Cellular stress responses have also been reported in cultured cells stably expressing GFP (Zhang et al., 2003), and eGFP expression has also been reported to increase production of superoxide and hydrogen peroxide (Ganini et al., 2017). Adverse effects have also been observed in transgenic GFP animals (Devgan et al., 2004; Huang et al., 2000; Mawhinney and Staveley, 2011). Therefore, despite GFP tagging of proteins driving huge advances in our understanding of biological processes, GFP is not as inert as previously assumed.

We report that eGFP-Akt2 exhibits impaired insulin-regulated recruitment and phosphorylation when compared to the endogenous protein. With the availability of brighter, more photostable fluorophores that have a reduced propensity to oligomerise (Cranfill et al., 2016) and have distinct structural features (e.g. electrostatic charge), we proposed that an alternate fluorophore may allow us to develop a more accurate Akt reporter. Indeed, we observed a markedly enhanced response in both recruitment and phosphorylation of Akt when fused to TagRFP-T (Shaner et al., 2008) compared to eGFP-tagged Akt. The improved dynamics of this reporter enabled us to detect intricacies of Akt recruitment to the PM, including oscillations at the cell surface, which could be important with regard to complex information transmission.
Methods and materials

Materials

3T3-L1 murine fibroblasts were sourced from the American Type Culture Collection (ATCC, Manassas, VA) and recently tested and proven to be free of contamination. High glucose Dulbecco’s Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Glutamax, Trypsin/EDTA, MEM amino acids and FluoroBrite DMEM were from Gibco (11965, 16000-044, 35050, 15400-054, 11130051 and A1896701). 100mm culture dishes were sourced from Corning (430167) and 6-well plates were from Costar (3516). NP40, Sodium Deoxycholate, SDS, Glycerol, Sodium Orthovanadate, Sodium Pyrophosphate, Ammonium Molybdate, Dexamethasone, Biotin, Isobutyl-1-methyl-xanthine (IBMX) and Saponin were from Sigma Aldrich (13021, D6750, L4509, G5516, S6508, S6422, A7302, D4902, B4639, G5516, I5879 and S7900). Glycine was sourced from Univar (1083). Insulin was from Calbiochem. 35 mm glass bottom dishes were from Ibidi (81158). Matrigel was sourced from Corning (356234). The electroporator used was an ECM 830 Square Wave Electroporation System produced by BTX Molecular Delivery Systems and the 0.4 cm Electroporation Cuvettes were sourced from BioRad (16520181). EDTA was sourced from Amresco (0105). Protease inhibitors were from Roche (11873580001) and Sodium Fluoride was from Fluka (71522). Pierce Bicinchoninic assay, Alexa Fluor 488 goat anti-rabbit SFX and Wheat Germ Agglutinin Alexa Fluor 647 conjugate were from ThermoFisher Scientific (23224, A31628 and W32466). Polyvinylidene difluoride membranes were sourced from Merck Millipore (IPVH00010). Phospho-Akt Thr308, phospho-Akt Ser473, Pan Akt (rabbit), Pan Akt (mouse) and phospho-AS160 Thr642 antibodies were sourced from Cell Signalling Technology (9275, 4051, 4685, 2920 and 4288) and the antibody for 14-3-3 was from Santa Cruz Biotechnology (sc-629). (Paraformaldehyde 16% was sourced from Electron Microscopy Sciences (15710). Dabco 1,4-Diazabicyclo(2,2,2)Octane was from Polysciences (15154).

Cloning

pDEST53-Cycle3_GFP-Akt2 was cloned using the gateway technique where human Akt2 in the pDONR221 backbone was inserted into pcDNA-DEST53 vector.

pDEST53-eGFP-Akt2 was cloned using Gibson assembly from pDEST53-Cycle3_GFP-Akt2 (Gibson et al., 2009). The pDEST53-Akt2 fragment was cloned using the primers TCTTCGCCCTTAGACACCATGTCTCCCTATAGTGAGTC and TAATGGCATGGACGAGCTGTAGTGAGTC. The eGFP
fragment was cloned from pEGFP-C1 (Clonetech) using the primers CCAAGCTGGCTACATCGCTAGAGAAGAAGGGAAGAGGA and CTTTGATGACAGCCTCTATTCTTGTAACGACTCTCTGCTCCATGCCATTA. TagRFP-T-Akt2 was cloned using Gibson assembly from pDEST53-eGFP-Akt2. The pDEST53-Akt2 fragment was cloned using the same primers as above. TagRFP-T fragment was cloned from pGEM-T-TagRFP-T using the primers GACTCACTATAGGGAGACATGGTGTCTAAGGGCGAAGA and CTTTGATGACAGCCTCTATTCTTGTAACGACTCTCTGCTCCATGCCATTA.

**Preparation of matrigel coated dishes**

Pipettes were pre-chilled, and matrigel was diluted 1:50, with sterile ice-cold PBS. 6-well plates and 35mm glass bottom dishes were coated with the diluted matrigel and then incubated for 2 hours at room temperature. The dishes were then washed twice with room temperature PBS prior to use.

**Cell culture and electroporation.**

3T3-L1 fibroblasts were cultured in DMEM, with 10% FBS and Glutamax at 37°C and 10% CO2 in 100mm dishes. Each confluent 100mm dish of fibroblasts was then reseeded into 6-well plates and then differentiated 5 days post-seeding in culture medium described above, supplemented with 0.22uM Dexamethasone, 100 ng/mL Biotin, 2 μg/mL Insulin and 500 μM IBMX for 3 days. Differentiation media was then replaced with post-differentiation medium, which is made up of culture medium and 2 μg/mL Insulin for a further 3 days. Adipocytes were then refreshed daily with culture medium. 7 days post-differentiation, adipocytes were trypsinised with 5x trypsin/EDTA for 5-10 minutes at 37°C. Trypsin was quenched with the addition of culture medium and the cells were centrifuged at 150 x g for 5 min and then washed with PBS and centrifuged twice more. The cell pellet was then resuspended in electroporation solution (20mM Hepes, 135mM KCl, 2mM MgCl$_2$, 0.5% Ficol 400, 1% DMSO, 2mM ATP and 5mM glutathione, pH 7.6) and 5-10 ug of plasmid DNA. Cells were then electroporated at 200 mV fir 20 ms and then plated onto 35 mm glass bottom dishes for imaging, or 6-well plates coated with Matrigel. Western blot analysis and imaging experiments were performed either 24 and 48 hours post electroporation.

**Western blotting analysis**

48 hours after electroporation, adipocytes were serum starved for two hours at 37°C with 10% CO2 and then stimulated with either 1 nM or 100 nM insulin. Cells were dunked in an ice-cold bath of PBS, then harvested on ice using 400ul Radio-Immuno-Precipitation-Assay (RIPA) buffer (50 mM Tris pH 7.5 [neutralised with NaOH], 150 mM NaCl, 1% NP40, 0.5%
Sodium Deoxycholate, 0.1% SDS, 1 mM EDTA, 1% Glycerol) containing phosphatase inhibitors (2 mM Sodium Orthovanadate, 1 mM Sodium Pyrophosphate, 1 mM Ammonium Molybdate and 10 mM Sodium Fluoride) and protease inhibitors. Lysates were scraped and isolated in 1.5 ml tubes, then sonicated for 15 s, 1 s on 1 s off. Lysates were centrifuged at 21,000 x g for 30 min to separate insoluble material (lipids and nuclear content). The milky lipid suspension was carefully removed and the supernatant was transferred to a new tube. Protein concentrations were assessed using the bicinchoninic acid method. Lysates were then separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked in 0.5% skim milk powder in Tris Buffered Saline and 0.1% Tween (TBST) and subsequently blotted for pan-14-3-3, phospho-Akt (Thr308 and Ser473), pan-Akt (total Akt) and phospho-AS160 (Thr642).

**Live cell microscopy**

For live experiments cells were serum-starved in FluoroBrite DMEM (with 0.2% BSA and Glutamax) for 2 hours at 37°C with 10% CO₂ and then stimulated with 1 nM and/or 100 nM insulin. For TIRF microscopy, experiments were performed on a Nikon Ti-Lapps H-TIRF module, equipped with an Okolab cage incubator and temperature control. For spinning disk confocal microscopy, experiments were performed on a Nikon Ti-Lapps spinning disk confocal equipped with an Okolab cage incubator and temperature control. Insulin and drugs were added using a custom made perfusion system. The system was benchmarked by testing the delivery of 3 ng/ml FITC and 100 ng/ml Alexa-647 labelled goat anti-rabbit F(ab’)2 antibody, to mimic the delivery of drugs and large proteins respectively. Delivery to the edge and middle of cells was measured by TIRF. No difference in the delivery to the edge or middle of cells was detected (Fig. S1A).

**Immunofluorescence**

Cells were seeded into 8 well Ibidi u-slides, coated with matrigel, day 7 post differentiation. Cells were then allowed to attach for 2 days. On day 9 post differentiation, cells were serum-starved in DMEM (with 0.2% BSA and Glutamax) for 2 hours at 37°C with 10% CO₂ and then stimulated with 100 nM. The coverslips were then briefly immersed in an ice-cold PBS bath and instantly fixed with 4% Paraformaldehyde at room temperature for 15 minutes. Cells were then washed twice with room temperature PBS and quenched with 100 mM Glycine for 10 minutes. We then washed the cells twice more with room temperature PBS, and incubated in blocking and permeabilising buffer (PBS with 2% BSA and 0.1% saponin) for 30 minutes. Cells were incubated with the anti-Pan-Akt (mouse) primary antibody (1:100) overnight at 4°C. The following day, the cells were washed with blocking and permeabilising buffer 5
times, and then incubated with anti-mouse Alexa-488 (1:500) at room temperature for 30 minutes in the dark. Cells were washed 5 more times with PBS and then stored and imaged in PBS, 5% glycerol and 2.5% Dabco.

Calculation of electrostatic potential

Structure preparation and pKa calculations were performed for eGFP (2y0g), TagRFP-T (3T6H) and the PH domain of Akt2(1p6s) using the PDB2PQR webserver (Dolinsky et al., 2004). Default parameters were used (PARSE forcefield, PH7.0). the Poisson-Boltzmann equation was solved using the Adaptive Poisson-Boltzmann Solver (APBS) webserver (Baker et al., 2001). The +1 and −1 ion species were set to 150 mM to prevent exaggerated electrostatic properties. Structures and electrostatic maps were visualised using the PyMOL Molecular Graphics System, Version 1.8 Schrödinger, LLC.

Image analysis

Image and statistical analyses were performed using custom analysis pipelines developed in FIJI (Schindelin et al., 2012) and R programming environment.

Temporal signal extraction and characterisation. Distinct molecular (e.g. Akt and GLUT4) recruitment patterns in single cells following various treatments (e.g. basal, insulin and MK) were extracted using self-organising maps (SOMs) (Kohonen, 1982), an unsupervised learning procedure that constructs an artificial neural network to scale image data from multi-dimensional space to a two-dimensional space. SOMs not only can characterise pixels based on their temporal signal patterns but also preserve the topological properties of the relative location of individual pixels in a cell image, ideal for visualising the temporal and topological inter-relationships in a single cell. Specifically, for each cell, data were standardised to be unit free before feeding into an SOM with a 5 by 5 hexagonal grid denoted as \( r_{ij} \) \((i = 1 \ldots 5, j = 1 \ldots 5)\). Each “neuron” \( m_k \) is updated by an input data point \( x_t \) as follows:

\[
m_k \leftarrow m_k + \alpha h(||\ell_t - \ell_k||)(x_t - m_k)
\]

where \( \alpha \) is the learning rate, \( \ell \) is an index to a grid position, and \( h \) is a neighbourhood function that assigns more weight to update neuron when it is closer to the input \( x_t \). In our case, we used Euclidean distance in \( h \) to measure the neighbourhood. Individual temporal patterns identified by SOMs from each cell were visualised as bounded line plots by calculating the mean profile of all pixels partitioned in each group and their variance as bounds. Only pixels with signal present for the duration of the time course were included in this analysis. Thus any changes in cell, size or shape resulting from insulin stimulation do not
influence the analysis.

**Oscillation analysis.** To extract the oscillation signal, we fitted locally weighted scatterplot smoothing (LOESS) model (Cleveland and Devlin, 1988) to each individual pixel. The estimated coefficients of LOESS for the $i$th point at iteration $t$ are found by minimising the following quantity:

$$
\sum_{j=1}^{n} \left( Y_j - \left( \beta_{0,i}^{(t)} + \beta_{1,i}^{(t)} \cdot x_j \right) \right)^2 \cdot K_i(x_j)
$$

where $K$ is the tricube kernel function and $\beta$s are the coefficients estimated for a local the regression line. The optimal span for each LOESS model was determined by Akaike information criterion (AIC) and the average of all optimal spans from all pixels in all cells was used as the final span for subsequent LOESS model fitting. We then extracted the residual matrix from fitted LOESS models for oscillation analysis. Single-pixel-resolution periodicities were estimated from each residual profile extracted from the residual matrix using Fourier transformation and plotted as histogram to identify the average periodicity for each single cell. We then visualised the oscillation pattern as heatmaps using LOESS residual matrix extracted from all pixels in a single cell. To quantify relative amplitude of oscillation in each cell, for each pixel in a cell we split its temporal responses extracted from the LOESS fit into non-overlapping windows based on the estimated periodicity in the previous step and calculated the relative amplitude by taking the maximum of absolute values in each window. We then divided this by the average value in that window, giving an amplitude estimate in form of fold change relative to the average in each window. By applying this approach to all pixels in a cell and summarising its distribution, this allowed us to estimate the amplitude for each cell and compare them across multiple cells. To investigate if the oscillation amplitude remains constant or changing across time, we calculated the difference of amplitudes estimated from the second to the second last windows for each pixel in a cell and tested if the distribution of differences is shifted from 0 using Wilcox rank sum test. These two windows were chosen to identify any change of amplitude in early and late time points but avoiding the potential boundary effect on the first and last windows.
**Results**

TagRFP-T-Akt2, but not eGFP-Akt2, is recruited to the plasma membrane in a similar manner to endogenous Akt.

In order to study Akt in live cells it is vital to have Akt reporter constructs that recapitulate the responses of endogenous Akt. The first step in Akt activation is the PIP3-dependent recruitment of Akt to the PM (Ng et al., 2008). We assessed the localisation of endogenous Akt in 3T3-L1 adipocytes by immunofluorescence confocal microscopy (Fig. 1A). Under basal conditions, Akt displayed diffuse cytoplasmic staining and clear nuclear localisation. Insulin resulted in decreased cytosolic staining and the appearance of a strong “rim” signal at the cell periphery, characteristic of accumulation at the PM.

Similar to endogenous Akt, eGFP-Akt2 exhibited cytoplasmic and nuclear localisation under basal conditions when expressed in 3T3-L1 adipocytes (Fig. 1B-D). However, in contrast to endogenous Akt, eGFP-Akt2 displayed poor membrane redistribution in response to insulin (Fig. 1B-D). We hypothesised that poor recruitment of eGFP-Akt was an artefact of eGFP tagging and that recruitment could be improved through the use of an alternate fluorophore. In particular, eGFP has a highly negative electrostatic charge (Fig. S1B). Since Akt PM recruitment is dependent on binding of the positively charged PH domain (Fig. S1C) to the negatively charged PIP3 (Levental et al., 2008), it may be that electrostatic repulsion between eGFP and PIP3 impairs this process or electrostatic attraction between eGFP and the PH domain may sterically interfere with the PH domain. TagRFP-T has a relatively even surface charge distribution and has no net electrostatic charge (Fig. S1D) despite high structural homology to eGFP (Fig. S1E). Further, the brightness, photostability, rapid maturation and distinct origin (*Entacmaea quadricolor* vs *Aequorea victoria*) of TagRFP-T made it an ideal alternative to eGFP. TagRFP-T-Akt2 was expressed in 3T3-L1 adipocytes at similar levels to eGFP-Akt2 and localised similarly in unstimulated cells (Fig. 1B-D). However, stimulation of TagRFP-T-Akt2 with insulin resulted in a robust redistribution to the periphery of the cell (Fig. 1B-D) in a similar manner to endogenous Akt (Fig. 1A).

**TagRFP-T-Akt2 is highly insulin responsive in comparison to eGFP-Akt2**

We next examined the translocation kinetics of the eGFP- and TagRFP-T- tagged Akt2 constructs in response to insulin using total internal reflection fluorescence (TIRF) microscopy. eGFP alone was used as a control and displayed no detectable change in localisation with insulin (Fig. 1B, C, E, F). We also assessed TagRFP-T alone under these...
conditions and observed no insulin stimulated change in localisation (Fig. 1B, C). Insulin stimulation increased the TIRF signal for both eGFP- and TagRFP-T-Akt2 (Fig. 1E-G), and both constructs displayed a graded intracellular dose response to insulin. However, TagRFP-T-Akt2 displayed significantly greater recruitment compared to eGFP-Akt2 with a more than 4.5-fold and 5.8-fold increase in PM levels in response to insulin at doses of 1 and 100 nM, respectively (Fig. 1F). The shape of the curves also differed with the TagRFP-T fusion construct revealing an overshoot in PM recruitment following a 1 nM insulin stimulus (Fig. 1F inset). This response was not evident in cells expressing the eGFP-Akt2 construct. Further, substantial heterogeneity in TagRFP-T-Akt2 recruitment was observed between single cells exposed to the same dose of insulin, which was not as apparent with the eGFP-Akt2 construct (Fig. 1G).

**TagRFP-T-Akt2 is highly phosphorylated in response to insulin**

Following PM recruitment, Akt2 is phosphorylated at Thr309 by PDK1 and Ser474 by mTORC2. To further assess how well the Akt reporter constructs recapitulated the behaviour of endogenous Akt we assessed insulin-stimulated phosphorylation of endogenous Akt, eGFP-Akt2 and TagRFP-T-Akt2 by western blotting with phosphospecific antibodies to phosphoThr309 and phosphoSer474 (Fig. 2A). In addition, we assessed Akt activity by blotting for phosphorylation of the Akt-regulated phosphosite Thr642 in TBC1D4/AS160. Stimulation of adipocytes with 1 and 100 nM insulin dose dependently increased phosphorylation of endogenous Akt at Thr309 and Ser474 (Fig. 2B), and of TBC1D4/AS160 Thr642 (Fig. 2C). Overexpression of TagRFP-T-Akt2 or eGFP-Akt2 had no effect on the phosphorylation of endogenous Akt at either Thr309 or Ser474 (Fig. 2B). There were no significant changes in phosphorylation of AS160 Thr642 with overexpression of either fluorescent Akt construct (Fig. 2C). This is not surprising given that only a small amount of active Akt is required for AS160 to be completely phosphorylated (Tan et al. 2012; Hoehn et al. 2008) and this is easily satisfied by endogenous Akt under these conditions.

Consistent with data on insulin-stimulated translocation to the PM (Fig. 1B-G), TagRFP-T-Akt2 was phosphorylated at both sites to a greater extent than eGFP-Akt2 in response to both 1 nM and 100 nM insulin. For example, at 100 nM insulin TagRFP-T-Akt2 was phosphorylated 14-fold and 9-fold more than eGFP-Akt2 at the Thr309 (Fig. 2D) and Ser474 (Fig. 2E) sites, respectively. Like endogenous Akt, TagRFP-T-Akt2 displayed a dose response from 1 nM to 100 nM. Together, analysis of translocation and phosphorylation suggests that activation of the eGFP-Akt2 fusion construct is impaired in comparison to
endogenous Akt. In contrast, the new TagRFP-T-Akt2 reporter more faithfully recapitulated the behaviour of endogenous Akt.

**Insulin stimulates polarised Akt recruitment to the PM**

The enhanced sensitivity of TagRFP-T-Akt2 permitted detection of subtle changes in the subcellular membrane association of Akt in response to insulin that was not apparent with eGFP-Akt2. These included polarised recruitment of Akt to the PM and oscillations in PM Akt with a highly reproducible frequency are present.

TagRFP-T-Akt2 accumulated on the basal surface of 3T3-L1 adipocytes in response to insulin (Fig. 3A). Subsequently, membrane localisation of TagRFP-T-Akt2 became increasingly heterogeneous and Akt accumulated more intensely at the periphery of the basal surface (Fig. 3B-D). This was often accompanied by a decrease in signal at the centre of the cell (Fig. 3B-D). To define this behaviour more comprehensively we expressed the fluorescence of individual pixels as a function of time and classified all pixels in a single cell using self-organising maps (SOMs). This analysis is presented such that regions exhibiting the same pattern of Akt translocation behaviour in response to insulin are given the same colour (Fig. 3E, S2, S3).

In unstimulated adipocytes, or adipocytes incubated with MK2206 (an inhibitor of Akt membrane recruitment), the patterning of TagRFP-T-Akt2 responses appeared random (Fig. 3E). However, in cells stimulated with 100 nM insulin, there were distinct regions of Akt behaviour. This was most notable in the difference between Akt responses in the central region of the basement membrane, where Akt showed a rapid increase that slowly tapered off with time, and the periphery, where Akt increased with time (Fig. 3E, S2B). This feature of TagRFP-T-Akt2 behaviour was seen in the majority of cells analysed (Fig. S2A). To determine whether these responses were an artifact of imaging or changes in membrane geometry, we next tested whether we observed similar clustering for GLUT4 using the pH-sensitive reporter GLUT4-pHluorin (Burchfield et al., 2013). GLUT4 translocates to the PM with insulin and acts as a membrane reference under these conditions. When we assessed the clustering patterns of GLUT4 and Akt behaviour in cells co-expressing GLUT4-pHluorin and TagRFP-T-Akt2 following a 100 nM insulin stimulus, the Akt responses maintained this grouping of activity described above (Fig. 3E, S3A), whilst the GLUT4 responses were more random (Fig. 3F, S3A). GLUT4 responses were similarly random in cells co-expressing GLUT4-TagRFP-T and eGFP-Akt2, however the Akt2 clustering was not evident with the eGFP tag (Fig. S3B). These data support the concept that this clustering of Akt translocation
behaviour observed with TagRFP-T-Akt2 is a bona fide feature of Akt responses to insulin stimulation.

**Insulin-stimulation induces self-organising oscillations in plasma membrane associated Akt**

Signal transduction pathways utilise temporal signalling patterns such as oscillations to encode information of a greater complexity, with enhanced specificity for downstream substrates and processes (Cheong and Levchenko, 2010; Kubota et al., 2012). Self-organising oscillatory behaviour in PIP3 generation, facilitated by PI3K, has been described for amoeboid cells expressing the PH domain of Akt during random cell migration (Arai et al., 2010), however such oscillations have not yet been demonstrated in response to insulin. Imaging of TagRFP-T-Akt2 in response to insulin in 3T3-L1 adipocytes revealed oscillations in PM Akt that appeared to propagate throughout the cell (Fig. 4A). We applied LOESS based normalisation to extract oscillations from individual pixels. The optimal flexibility (span) of the LOESS model was determined by the average of spans selected by Akaike information criterion (AIC) for individual pixels from all cells. This optimal span was then used for subsequent data analysis (Fig. 4B).

Single-pixel-resolution periodicities were estimated from each residual profile extracted from the LOESS model using Fourier transformation. These data were plotted on a histogram to identify the average periodicity for each single cell (Fig. 4C, D). In the presence of insulin, the average frequency of oscillations was highly consistent between cells, with a periodicity of approximately 2 min (Fig. 4C, S4A). Distinct reproducible oscillations were not observed in the absence of insulin or in the presence of the Akt inhibitor MK-2206, suggesting that the oscillations in PM Akt are a specific feature of insulin signalling (Fig. 4D). The mean amplitude of oscillations varied from cell to cell (Fig. S4B), but did not change within a single cell with respect to time (Fig. S4C). Once established, oscillations were observed to propagate throughout the cell, but the origin of the oscillations and direction of travel appeared stochastic (Fig. 4E, F). Although these analyses were carried out at the single-pixel level, the signals at this level were almost always part of a greater region of synchronised oscillations (Fig. 4F). This behaviour is characteristic of a self-organising stochastic process, which is thought to act as a mechanism to facilitate signal plasticity and robustness in response to dynamic perturbations (Kurakin, 2005).
Discussion

Akt is a highly studied kinase that plays a key role in cellular signalling and in some disease states, most notably cancer. Here we describe an improved Akt reporter construct, TagRFP-T-Akt2, for monitoring Akt localisation in live cells. This new reporter outperformed eGFP-Akt2 in both the magnitude and sensitivity of plasma membrane recruitment in response to insulin in adipocytes. Further, the TagRFP-T-Akt2 construct displayed a dose response that was similar to endogenous Akt at the level of insulin-stimulated phosphorylation of Thr309 and Ser474. Imaging of TagRFP-T-Akt2 revealed polarisation of, and specific oscillations in, PM Akt in the presence of continuous insulin. These behaviours have not previously been described for Akt in response to insulin and highlight the utility of the TagRFP-T-Akt2 reporter in studying Akt response to growth factors.

At face value, poor recruitment of the eGFP construct suggests that eGFP is interfering with the function of the PH domain. The interaction of Akt with the PM is driven by the interaction between the positively charged PH domain and negatively charged PIP3 (Levental et al., 2008). eGFP has an uneven surface charge distribution that is strongly negative overall. We hypothesised that this charge may result in steric interference with the PH domain via electrostatic attraction or alternatively reduce binding to the PM as a result of electrostatic repulsion. This would explain the diminished recruitment and subsequent phosphorylation of eGFP-Akt2. To test this we replaced eGFP with the red fluorescent protein TagRFP-T that carries a net neutral surface charge. This change resulted in a dramatic improvement in the ability of the tagged Akt construct to bind the PM supporting the idea that the electrostatic characteristics of eGFP are impeding the PH domain function. We cannot, however, discount other mechanisms for the difference between TagRFP-T and eGFP. For example, Ganini and colleagues (Ganini et al., 2017) demonstrated that immature eGFP could produce hydrogen peroxide in vitro and it could activate the oxidative stress response when overexpressed in HeLa cells. TagRFP was shown to produce hydrogen peroxide in vitro at a lower rate. It is possible that this may result in oxidation of the PH domain resulting in altered function. The observation that coexpression of TagRFP-T-Akt2 with eGFP-Akt2 did not impair the TagRFP-T-Akt2 response suggests this would need to be an intramolecular phenomenon. Regardless, these data demonstrate that switching the fluorescent protein in fusion constructs can have profound effects on the fidelity of reporters, especially if they undergo localisation changes, and highlight the importance of validating reporters with respect to the endogenous protein.

The TagRFP-T-Akt2 signal to noise ratio allowed for the detection of increased PM
abundance at a physiological dose of insulin (1 nM, Fig. 1). This is, as far as we are aware, the first report of live-cell, full-length Akt membrane recruitment by TIRF microscopy in response to a physiologically relevant dose of insulin. Further stimulation with 100 nM resulted in further Akt recruitment in all cells studied, demonstrating that single cells display an intracellular dose response to insulin and that population dose responses are not driven by switch-like behaviour at the single cell level. These observations of intracellular dose response and intercellular response heterogeneity are consistent with our previous observations (Burchfield et al., 2013), suggesting that differing responses to insulin may be driven by differences in Akt activation.

The increased sensitivity of TagRFP-T-Akt2 revealed new subcellular features of Akt membrane recruitment, including both intra-cellular heterogeneity in the Akt signal along with the appearance of self-organising oscillations in cell surface Akt in the continuous presence of insulin. This behaviour has not been previously reported for Akt in response to insulin. The intensity of the TagRFP-T-Akt2 signal across the plasma membrane in response to insulin was surprisingly variable. The centre of a cell’s basal surface and periphery displayed markedly distinct response profiles, suggesting that PIP3 production and/or Akt membrane dynamics may be tightly regulated at the subcellular level. In further support of this, we detected Akt2 membrane oscillations, which propagated throughout the cell stochastically. Whilst this is yet to be described in response to insulin in adipocytes, other groups have reported similar behaviour for the PH domain of Akt, both in MIN6 β-cells in response to glucose and insulin (Hagren and Tengholm, 2006), and in Dictyostelium cells during random cell migration (Arai et al., 2010). Although oscillations are potentially a feature of overexpression of a protein which contributes to a feedback loop (Cheong and Levchenko, 2010), the fact that it has been observed in cells expressing just the PH domain of Akt, and thus no kinase functionality, suggests that it is a real phenomenon. Furthermore, the period of the oscillations within single cells was conserved in response to the same stimulus. This may enhance the versatility in the way that Akt, as a central node in a number signalling pathways, processes and transmits signals, The ability to alter periodicity, amplitude, fold-change and duration provides additional layers of information encoded by this signal (Sonnen and Aulehla, 2014) that can then be differentially transmitted to downstream effectors. When assessing the oscillatory patterns, pixels of positive or negative signal are typically part of a much larger area of similar intensity (Fig. 4F). This suggests that the oscillations in membrane Akt are not a random state of disorder, but likely a self-organising behavior, where Akt molecules synchronise to form a larger functional arrangement. Self-organisation is a
process thought to endow systems with adaptability and robustness in response to intra- and extra- cellular changes (De Wolf and Holvoet, 2005; Kurakin, 2005). Given the involvement of Akt in numerous dynamic cellular processes, self-organisation is a likely means by which Akt remains dynamic and sensitive to such stimuli and the oscillations that arise as a consequence may facilitate signal transmission to specific substrates.

Our studies demonstrate that the choice of fluorophore in generating a fusion construct can have drastic effects on how well this reporter recapitulates properties of the endogenous protein. It is paramount that novel fusion proteins are validated and compared to the endogenous protein of interest in order to generate the most sensitive and informative reporter constructs. Our new TagRFP-T-Akt2 construct accurately reflects the behavior of endogenous Akt and the improvement in sensitivity of TagRFP-T-Akt2 revealed intricate details of Akt membrane recruitment in response to insulin. These features provide insight into the potential mechanisms by which Akt manages and transmits complex signalling information.
Acknowledgements
We are grateful to the Dr Daniel Hesselson (Garvan Institute of Medical Research, Sydney) for providing the pGEM-T-TagRFP-T construct. The authors acknowledge the facilities and the scientific and technical assistance of the Australian Microscopy & Microanalysis Research Facility at the Australian Centre for Microscopy & Microanalysis at the University of Sydney.

Competing interests
We have no competing interest to declare.

Funding
This work is supported by National Health and Medical Research Council (NHMRC) grants [GNT1061122, GNT1086850 and GNT1120201 to D.E.J.], an Australian Postgraduate Award scholarship [to D.N.], an ARC Discovery Early Career Research Award [DE170100759 to P.Y.], an NHMRC Early Career Fellowship [APP1072440 to J.R.K.], an NHMRC Senior Principal Research Fellowship [APP1019680 to D.E.J.] and a Diabetes Australia Research Program grant [G190680 to J.G.B. and D.J.F. ]. The contents of the published material are solely the responsibility of the authors and do not reflect the views of the NHMRC.
List of Abbreviations

AIC - Akaike information criterion
AS160 - Akt substrate of 160 kDa
eGFP - Enhanced green fluorescent protein
GFP - Green fluorescent protein
GLUT4 - Glucose transporter 4
LOESS - Locally weighted scatterplot smoothing
mTORC2 - Mammalian target of rapamycin complex-2
PDK1 - 3-phosphoinositide-dependent kinase 1
PH - Pleckstrin homology
PI3K - Phosphoinositide 3-kinase
PIP3 - Phosphatidylinositol (3,4,5)-trisphosphate
PM - Plasma membrane
PTEN - Phosphatase and tensin homolog
SHIP2 - SH2 domain containing inositol 5-phosphatase 2
SOMs - Self-organising maps
TIRF - Total internal reflection fluorescence
TBC1D4 - TBC1 domain family member 4
References


Figure 1. TagRFP-T-Akt2, but not eGFP-Akt2, is highly insulin responsive and recruited to the PM in a similar manner to endogenous Akt. (A) Confocal immunofluorescence images of endogenous Akt (anti-pan akt) in 3T3-L1 adipocytes in the absence and presence of 100 nM insulin. (B) Confocal images of adipocytes co-expressing eGFP-
Akt2 and TagRFP-T (upper panels), and adipocytes co-expressing TagRFP-T-Akt2 and eGFP and (lower panels) in the presence and absence of 100 nM insulin. The fluorescent proteins TagRFP-T and eGFP were expressed as markers for the cytoplasm. Alexa 647 labelled Wheat Germ Agglutinin (WGA) was added 6 minutes before the insulin stimulus and was used as a plasma membrane marker. Scale bars are 10 microns. (C) Enlarged regions of interest (white boxes in panel B) demonstrate the extent of co-localisation of each Akt construct with the cytoplasm or plasma membrane. The images are 7x7 microns. (D) Representative images of eGFP-Akt2 and TagRFP-T-Akt2 co-expression in a 3T3-L1 adipocyte imaged before and after stimulation (10 min) with 100 nM insulin using spinning disk microscopy. Scale bar is 20 microns. (E) Representative images displaying the recruitment of eGFP alone, eGFP-Akt2 and TagRFP-T-Akt2 into the TIRF zone in response to 1 and 100 nM insulin and after inhibition with MK2206. Scale bars are 20 microns. (F) Quantification of TIRF responses from 3T3-L1 adipocytes expressing expressing eGFP, eGFP-Akt2 and TagRFP-T-Akt2 (n= 27, 41 and 41 cells respectively). Data are expressed as mean + SEM. (G) Heatmap of the recruitment response for cells (as in F) expressing either eGFP-Akt2 or TagRFP-T-Akt2. Each row represents a single cell’s average TIRF fluorescence normalised to basal fluorescence.
Figure 2. TagRFP-T-Akt2, but not eGFP-Akt2, is highly phosphorylated in response to insulin. 3T3-L1 adipocytes expressing either eGFP, eGFP-Akt2 and TagRFP-T-Akt2 were stimulated with 1 or 100 nM insulin for 10 min. (A) Representative western blot analysis of adipocyte lysates (n=3). The upper bands in the phospho and total Akt blots are the exogenous Akt fluorescent fusion constructs (Ex.), which run at approximately 85 kDa. The lower bands are the endogenous Akt (End.), which run at approximately 60 kDa. (B-E) Densitometry of blots in (A). Quantification of endogenous Akt phosphorylation (B) and
AS160 phosphorylation (C) from cells overexpressing the Akt fusion constructs. Quantification of eGFP-Akt2 and TagRFP-T-Akt2 phosphorylation at sites Thr309 (D) and Ser474 (E). Data are presented as mean ± SEM; ** p<0.01 vs eGFP-Akt2 equivalent.
**Figure 3. Insulin stimulates polarised Akt recruitment to the PM.** Live 3T3-L1 adipocytes expressing TagRFP-T-Akt2 were imaged by TIRF microscopy. Cells were imaged for 10 min prior to stimulation with 100 nM Insulin for 20 min. This was followed by the addition of 10μM MK-2206. **(A)** Representative image of a single cell imaged by TIRF microscopy. Red-line indicates the plane of the kymograph **(B)** displayed for 100 nM only. Each pixel has been normalised to the pixel mean across the 100 nM stimulus. **(C)** Single frames at timepoints indicated during the 100 nM stimulus response from the same cell as in **(A) and (B). (D)** Time course of Akt recruitment to the PM for the whole cell and sub-regions demarcated in **(A). (E)** Self-organising map (SOM) analysis results for the same cell either unstimulated, 100 nM stimulated or inhibited with MK2206. Colours indicate a clustered response. Panels below show examples of the cluster profiles **(F)** SOM analysis applied to a cell co-expressing TagRFP-T-Akt2 and pHluorin-GLUT4 and stimulated with insulin, showing the distinct polarisation of Akt but not GLUT4.
Figure 4. Insulin-stimulation induces self-organising oscillations in plasma membrane associated Akt. (A) Oscillations in PM Akt for a 1 μM² region of a cell (Fig. 3A) after 100 nM stimulation, relative to the local mean. (B) Violin plot showing distribution and density of LOESS spans selected for individual pixels based on Akaike information criterion (AIC). The median value of LOESS spans selected for individual pixels was then used as optimal span in the subsequent analysis. (C) Histograms showing the average periodicity of oscillations in individual cells (n=6) in the presence of 100 nM insulin. (D) Histograms of the average periodicity of oscillations in Cell 1 during the basal and MK periods. (E) Kymograph of a slice of a cell after loess normalisation across the entire time course. (F) Representative images from the same cell demonstrate the self-organising nature of the Akt membrane oscillations in response to 100 nM insulin, whereby a given pixel is typically part of a greater, similar region, which continues to propagate stochastically across the cell surface.
Supplementary Figure 1. (A) Custom perfusion system performance. Delivery of 3 ng/ml FITC and 100 ng/ml Alexa-647 labelled goat anti-rabbit F(ab’)2 antibody to the edge and middle of cells measured by TIRF. **eGFP has a strongly negative electrostatic charge, TagRFP-T does not.** (B) eGFP, (C) the PH domain of Akt2 and (D) TagRFP-T overlaid with their respective electrostatic potential calculated using the Adaptive Poisson-Boltzmann Solver (APBS) at 150 mM ionic strength with a solute dielectric of 2 and a solvent dielectric of 78.5 and rendered using PyMOL Volume visualisation. Highly negative regions are displayed as opaque red and highly positive regions as opaque blue. Borders are equivalent to isocontours at +/-1 kT/e. (E) Aligned cartoon representations of eGFP and TagRFP-T.
Supplementary Figure 2. Insulin stimulates polarised Akt recruitment to the PM. (A) Self-organising map (SOM) analysis results for cells either unstimulated, 100 nM stimulated or inhibited with MK2206. Colours indicate differing intracellular response profiles. (B) All twenty-five cluster profiles for the cell in Figure 3A-E in response to 100 nM insulin. Diagonally opposing (e.g. red and pink or yellow and purple) cluster profiles are most dissimilar to one another.
Supplementary Figure 3. Insulin stimulated membrane polarisation is specific for Akt but not GLUT4. (A) Self-organising map (SOM) analysis for 5 cells co-expressing GLUT4-pHluorin and TagRFP-T-Akt2 stimulated with 100 nM insulin. (B) Self-organising map (SOM) analysis for a cell co-expressing GLUT4-tagRFP-T and eGFP-Akt2 stimulated with 100 nM insulin. Colours indicate differing intracellular response profiles.
Supplementary Figure 4. Oscillation frequency, but not amplitude, is consistent across multiple cells. (A) Comparison of oscillation frequency for six cells in addition to those in Figure 4C (from 2 independent experiments) across a 100 nM insulin-stimulated timecourse. (B) Comparison of oscillation amplitude for each individual cell in Figure 4C across the 100 nM-stimulated timecourse. (C) Oscillation amplitude variability within each cell across the timecourse.