AuPS Meeting - Melbourne 2008

Free communications: Ion Channels

Monday 1 December 2008 − King Theatre

Chair: Alan Bretag

Effect of volatile anaesthetics on the calcium release channel in the heart

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Although volatile anaesthetics serve a crucial role in preventing pain they continue to have a number of serious side effects. One of these is their ability to excite Ca^{2+} release from the intracellular Ca^{2+} stores (sarcoplasmic reticulum, SR) *via* calcium release channels named ryanodine receptors (RyR). This can result in fatal episodes of malignant hyperthermia in otherwise normal patients harbouring mutations in the skeletal muscle RyR isoform (RyR1, MacLennan & Chen, 1993). The cardiac isoform of the RyR (RyR2) plays a key role in cardiac muscle contraction, pacemaking and rythmicity (Vinogradova *et al.,* 2005). During periods of ischemia, changes in the intracellular milieu cause a decrease in RyR activity and consequently an increase in SR Ca^{2+} load. Upon reperfusion of the heart tissue, recovery of RyR2 activity in the presence of abnormally high store loads leads to cardiac arrhythmias. Evidence now indicates that activation of RyR2 by volatile anaesthetics protects against myocardial injury and arrhythmias following ischemia and reperfusion (Yang *et al.*, 2005). Here we report the first detailed investigation on the effects of volatile anaesthetics on the function of cardiac RyRs.

RyRs were isolated from sheep hearts and incorporated into artificial lipid bilayers that separated baths corresponding to the cytoplasm and SR lumen. The activity of RyRs was measured using single channel recording. Volatile anaesthetics were added to the baths by injection of solutions from sealed reservoirs. These solutions contained the desired concentration of anaesthetic (either halothane or isoflurane) and were carefully titrated for various levels of free Ca^{2+} and Mg^{2+}. We found that clinical doses of these anaesthetics increased RyR2 open probability (P_o) *via* increases in the channel mean open time and opening frequency. The K_as for halothane and isoflurane were 1 mmol/l and 3 mmol/l, respectively. However, the maximal effect of halothane (5-fold increase in P_o) was ~3-fold larger than that for isoflurane. These agents were shown to activate RyRs by interacting with their cytoplasmic domains. Furthermore, anaesthetic site of action was found to be distinct from the adenine nucleotide activating sites, contrary to previous suggestions (Yang *et al.*, 2005).

The effects of halothane on RyR2 regulation by cytoplasmic and luminal Ca^{2+} and Mg^{2+} were accurately fitted by a model based on a tetrameric RyR structure with four Ca^{2+} sensing mechanisms on each subunit (Laver, 2007; Laver & Honen, 2008); two activation sites (the luminal *L*-site with 40 μmol/l affinity and the cytoplasmic *A*-site with 1 μmol/l affinity) and two cytoplasmic inactivation sites (I_1-site with 10 mmol/l affinity and the I_2-site with 1 μmol/l affinity). Halothane did not appear to alter the ion binding affinities for these sites. Rather, it increased channel opening rate and decreased the channel closing rate associated with Ca^{2+} binding to the two activation sites.

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The role of depolarising and repolarising currents in the induction of early afterdepolarisations during acute hypoxia in ventricular myocytes

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Ventricular arrhythmia is a major cause of death in patients suffering from myocardial infarction. Arrhythmias typically occur as a result of re-entrant excitation or increased automaticity in and around the infarct zone. Early afterdepolarisations (EADs), thought to be responsible for re-entry like activity, are depolarisations of the membrane during phase 2 or 3 of the cardiac action potential and require an inward current large enough to increase total inward membrane current. Acute hypoxia (decreasing PO_2 from 150 mmHg to 17 mmHg) is not energy limiting but can alter the function of a number of ion channels. Acute hypoxia decreases the transient Na^+-current ($I_{Na\text{-}T}$) but increases the persistent Na^+-current ($I_{Na\text{-}P}$) (Ju *et al*. 1996). Acute hypoxia also decreases the basal current through the L-type Ca^{2+} channel ($I_{Ca\text{-}L}$) (Hool, 2000) and decreases the slow component of the delayed rectifier K^+ channel (I_{Ks}) but not the rapid component (I_{Kr}) (Hool, 2004). Coronary occlusion is also associated with an increase in circulating and tissue catecholamines. Acute hypoxia increases the sensitivity of $I_{Ca\text{-}L}$ to β-adrenergic receptor stimulation (Hool, 2000). However, hypoxia also increases the sensitivity of I_{Ks} to β-adrenergic receptor stimulation, without altering I_{Kr} (Hool, 2004) which may counteract the effects of hypoxia on $I_{Ca\text{-}L}$. The net effects of acute hypoxia in the absence and presence of β-adrenergic receptor stimulation on the cardiac action potential are not known.

We incorporated all the published data reporting the effects of acute hypoxia on cardiac ion channels into the Luo-Rudy model. We then compared the results obtained from the model to experimental data obtained from ventricular myocytes isolated from anaesthetised adult guinea pigs using the current clamp configuration of the patch clamp technique. Our modelled data predicts that acute hypoxia has little effect on resting membrane potential (RMP, −88 mV *vs* −88 mV), action potential peak (APP, 47 mV *vs* 45 mV) and action potential duration (APD, 225 mV *vs* 219 mV). Furthermore, hypoxia alone could not trigger EADs. We then kept the values of all other channels at normoxic levels and only modified $I_{Ca\text{-}L}$ to hypoxic conditions. In the presence of the β-adrenergic receptor agonist isoproterenol (Iso) at a concentration of 0.6 nmol/L, (sub-threshold concentration during normoxia), hypoxia significantly prolonged APD to 450 ms and induced EADs. When this was repeated for I_{Ks} alone, there was no substantial change in APD and no EAD generation. Modelling the effects of hypoxia on $I_{Ca\text{-}L}$ and I_{Ks} together also prolonged APD and induced EADs indicating that any anti-arrhythmic effect of I_{Ks} is only small and that the effects of hypoxia and β-adrenergic receptor stimulation on $I_{Ca\text{-}L}$ predominate.

Our experimental data corresponded well with our modelled data. Acute hypoxia (PO_2 of 17mmHg) had little effect on RMP (−67 ± 3 mV *vs* −68 ± 2 mV), APP (56 ± 2 mV *vs* 56 ± 2mV), APD (227 ± 19 ms *vs* 221 ± 18 ms, n = 9). In the presence of 1 or 3 nmol/L Iso and hypoxia there was no change in RMP or APP but a significant increase in APD by 10% (n = 5, p = 0.036) and 20% (n = 7, p = 0.022) respectively were recorded. Three of the seven cells exposed to 3nmol/L Iso and hypoxia developed EADs and spontaneous tachycardia. This is in contrast to normoxic conditions where 3nmol/L Iso did not alter any action potential parameters (n = 4, all p > 0.5). We conclude that during acute hypoxia, EADs are induced predominantly as a result of an increase in the sensitivity of the L-type Ca^{2+} channel to β-adrenergic receptor stimulation.

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Ageing related change in store-operated $Ca2^{+}$ influx and TRPCs expression of sinoatrial node

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The sinoatrial node (SAN) is the primary pacemaker of the mammalian heart. Dysfunction of the SAN increases exponentially with age. The age-related remodelling of pacemaker currents and pacemaker action potential may contribute to age-related slowing of the heart rate and reduction in conduction velocity which result in arrhythmias (Kistler *et al*., 2004). We recently discovered that store-operated Ca^{2+} channel (SOCC) activity is present in the mouse sinoatrial node leading to Ca^{2+} entry (Ju *et al.*, 2007). In the present study we tested the hypothesis that changes in SOCC activity might play a significant role in ageing related SAN dysfunction.

The experiments were carried out in intact mouse SAN preparations from either adult (2 months) or old (> 18 months) mice. Intracellular Ca^{2+} was detected with the fluorescent Ca^{2+} indicator indo-1. The SOCC activity was determined from the influx of Ca^{2+} following depletion of the sarcoplasmic reticulum Ca^{2+} store. We found that the Ca^{2+} influx was reduced in old mice compare to adult mice (2 month) ($p < 0.05$, $n = 4$).

It is widely believed that the transient receptor potential cannonical (TRPC) gene family are candidates for encoding SOCC. Recently we have developed one-step quantitative PCR to study gene expression in mouse SAN. This method allows us to study expression levels of up to 8 genes from one mouse SAN. Central or peripheral SAN samples were isolated from mouse hearts and stored in RNAlater solution (Ambion Inc.). One-step real-time PCRs were performed using the Cells Direct kit (Invitrogen, Australia). We tested 4 housekeeping genes including 18s rRNA, β-actin, HPRT1, B2m as reference genes, to normalise the changes mRNA expression of TRPCs or other genes of interest. We also used TRPC1 DNA as sample input reference. HCN4 used as a positive control for the central SAN region. We quantified the mRNA expression of TRPCs (TRPC1, 3, and 4) and stromal interacting molecule 1 (STIM1), an endoplasmic reticulum-Ca^{2+} sensor protein thought to associate with TRPCs. We found a reduction of the TRPC3 mRNA expression in the SANs from old mice (20-24 months) compared to young mice (1 month). Our preliminary data suggests that changes in SOCC activity and expression of TRPC genes might play a role in SAN dysfunction, specifically in the ageing heart.

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This work was supported by NH&MRC, Australia.

Delineating the gating pathway in the α1 glycine receptor ligand-binding domain

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Glycine receptor chloride channels (GlyRs) mediate inhibitory neurotransmission in the central nervous system. They are members of the pentameric Cys-loop ligand-gated ion channel family. Individual subunits are each composed of a large N-terminal extracellular ligand-binding domain and 4 transmembrane α-helices (M1-M4). The ligand-binding domain of Cys-loop receptors is comprised of an inner and an outer β-sheet. Although glycine induces global changes in the structure of this domain, little is known about which conformational changes are crucial for the gating of these receptors. Recent studies on other Cys-loop family members have demonstrated an important role of the inner β-sheet during channel activation. Here, we used voltage-clamp fluorometry to address the question of whether the outer β-sheet and the domain immediately preceding the M1 domain (the pre-M1 domain) also form an essential element of the α1 GlyR activation pathway. The voltage-clamp fluorometry technique involves introducing a cystine residue into a domain of interest, specifically labelling it with a sulfhydryl-reactive fluorophore and simultaneously measuring current and fluorescence changes. This technique allows us to monitor movements in domains distant from the channel gate. *Xenopus laevis* frogs were anaesthetized in 1g/l ethyl-m-aminobenzoate according to procedures approved by the University of Queensland Animal Ethics Committee. Stage VI oocytes were then removed and injected with 10 ng of wildtype or mutant α1 GlyR mRNA into the cytosol and incubated for 3-10 days at 18°C. For labelling, oocytes were placed into ice-cold ND96 saline solution containing 10μM sulforhodamine methanethiosulfonate for 25s. Oocytes were then washed and stored in ND96 for up to 6 h before recording. For recording, oocytes were placed on the stage of an inverted fluorescence microscope. Fluorescence signals were recorded by a photodiode and membrane currents were recorded using conventional two-electrode voltage-clamp. We labelled multiple residues in three agonist-binding domains (loop E, loop F and loop C) and in the pre-M1 domain with environmentally sensitive fluorophores to follow structural rearrangements during activation. Our results confirmed that current and fluorescence changes in loop E in the inner β-sheet are closely correlated, implying a role for this domain in channel gating. In contrast, labelled residues in the outer β-sheet (loop F and loop C) and the pre-M1 domain exhibited fluorescence responses only at high glycine concentrations. Additionally, fluorescence responses in loop F and loop C did not discriminate between agonist (glycine) and antagonist (strychnine) binding. These results imply a gating pathway in the α1 GlyR ligand-binding domain that involves loop E in the inner β-sheet. We propose that structural rearrangements in the outer β-sheet are not involved with gating but maybe associated with locking the ligand into the binding site at high concentrations. The results presented here and elsewhere strongly suggest that loop E in the inner sheet is one of the main structural elements triggering channel opening. It is thus tempting to speculate that the local agonist-mediated conformational change at the binding site is forwarded to the transmembrane domains *via* the conserved Cys-loop (or the β6−β7 loop) which is directly connected to loop E. As the Cys-loop lies in close physical proximity to the M2-M3 linker, a gating pathway connecting binding site and channel gate *via* loop E, Cys-loop, M2-M3 linker and finally the M2 helix containing the channel gate, appears a likely scenario.

External divalent ions increase anion-cation permeability in glycine receptor channels – consideration of ion activities, surface charge and conductance measurements

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The ligand-gated glycine receptor ion channels (GlyRs) mediate fast inhibitory synaptic transmission in the central nervous system and their functional role depends on whether they are predominantly permeable to cations or anions. Measurements of relative anion-cation permeability of such channels are often made with different concentrations of external divalent ions. We have already reported (Sugiharto *et al*., 2007) that dilution potential measurements of P_{Cl}/P_{Na} in wild-type (WT) homomeric α1 GlyR have been found to give larger values, implying a greater relative anion-cation selectivity, in the presence of external divalent ions rather than in their absence. We have now further evaluated the effects of divalent ions: (1) by examining the use of activities for liquid junction potential (LJP) corrections, (2) by considering surface charge effects and (3) by directly measuring the effect of divalent ions on single channel conductances.

A recent paper of ours, on the relationship between anion-cation permeability and equivalent hydrated counter-ion size (Sugiharto *et al*., 2008), highlighted the importance of ensuring that ion activities, rather than concentrations, are used in calculating liquid junction potential (LJP) corrections in all dilution potential measurements. We used the MS Windows version of *JPCalc* for such calculations (Barry, 1994). In the case of our divalent ion experiments, using ion activities for LJP corrections reduced the magnitude of P_{Cl}/P_{Na} to 20 ± 1 (compared to the previous value of 25 ± 2, using concentrations) in the presence of 4 mM external [Ca^{2+}], and to 10.9 ± 0.3 (compared to the previous value of 12.4 ± 0.4, using concentrations) in the absence of external [Ca^{2+}]. This means that many of the previous measurements of P_{Cl}/P_{Na} that used concentration for LJP corrections for anion-selective channels in the presence of external [Ca^{2+}], in particular, have overestimated this permeability ratio.

We have also explored the effect of ionic strength and divalent ions in the presence of surface charge near ion channels on relative anion-cation permeability. We have showed in a theoretical analysis that any change in local concentration of ions due to surface charge (with a resultant surface potential) near the channel is completely compensated for by the boundary potential from that channel to within the channel vestibule, in the absence of divalent ions, and almost completely compensated for if the divalent ion concentration is only a few mM. This point and other considerations suggest that the effect of external [Ca^{2+}] on P_{Cl}/P_{Na} cannot be primarily explained by surface charge effects.

The question may be asked whether the effect of external divalent ions was to increase Cl^- permeability or to decrease Na^+ permeability or both. Single channel conductance measurements on GlyR channels in NaCl solutions indicate that while external [Ca^{2+}] did increase Cl^- permeability to some extent, the major effect was to decrease counter-ion Na^+ permeability.

Our results still suggest that the presence of impermeant Ca^{2+} ions in the external vestibule affects anion-cation permeation by altering the electrostatic energy profile in that region.

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FRET study of C-terminal movements of the cytoplasmic tail of human skeletal muscle chloride channel, hClC-1, during gating

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Like other members of the CLC channel and transporter family, ClC-1 is a homodimeric protein with individual chloride permeation pathways through each subunit. It exhibits quite complex transport/gating characteristics with two distinct gating processes being identified, so far. One is called protopore gating (or fast gating), which regulates each individual pore independently and the other is common gating (or slow gating), which closes both pores simultaneously. An understanding of these gating processes is still far from complete, especially the common gating, which, because of its high temperature coefficient, is presumed to involve large conformational rearrangements of the protein. Some basis for this has recently been found using fluorescence resonance energy transfer (FRET) by Bykova *et al*. (2006) in the related chloride channel, ClC-0, from electroplaque cells of *Torpedo* where large displacements of the C-termini were shown to accompany common gating. Encouraged by this work, by known effects of site-directed mutations in the cytoplasmic tail on gating and by knowledge of the characteristics of naturally-occurring myotonic mutants in this domain of human ClC-1 (hClC-1), we decided to analyse its common gating, also using FRET. We first prepared a variety of full-length, truncated and split hClC-1 constructs with and without fluorescent tags (Cerulean and Enhanced Yellow Fluorescent Protein, eYFP) to determine whether these would affect channel function as determined by patch-clamp when heterologously expressed in HEK293 cells. As for ClC-0, using both full-length hClC-1 (M_1-L_{988}) and a shorter version (M_1-F_{887}, truncated just after the second CBS domain of the carboxyl tail), we then observed changes in FRET suggesting a significant movement of the C-termini that was functionally associated with common gating. Closure of the common gate was accompanied by a physical separation of the C-termini of the two subunits, whereas, on opening the two C-termini moved closer to each other. These movements and, presumably, associated conformational changes in hClC-1 during common gating are considerably smaller than in ClC-0, and this might then underlie the differing properties of common gating in these two channels, such as, their different temperature dependence of relaxation and their very different time scales. Interestingly, although the voltage dependence of common gating is opposite in the two channels (apparent open probability of the common gates increasing on depolarization for ClC-1), C-terminal movement is congruent. A comparison of single mutants E232Q and C277S, and double mutants E232QA272E and E232QC277S in our FRET study strongly suggests that fast gating and common gating are closely linked in hClC-1. It seems that in the absence of the carboxyl side chain of E232, neither fast nor common gating can occur and the channel remains open. On the other hand, FRET shows that the conformational rearrangements of the C-terminals, normally associated with common gating, are retained in E232Q even though chloride current is unimpeded by any gating. Positive FRET between split channel fragments Cerulean-M_1-H_{451} and Y_{380}-L_{988}-eYFP provides answers to several further questions. It shows that these two channel components, split within the membrane resident domain of the protein, but with overlap including helices K and L, must, indeed, combine appropriately to form the functional channel. Secondly, the putative crystal structure for CLC membrane resident domains is supported because the Cerulean and eYFP must be located sufficiently close to each other across the membrane for FRET to occur. Thirdly, and perhaps more importantly, this construct forms the basis for future studies of relative movements of different regions of the channel vertically through the membrane during gating.

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Neonatal hypoxia increases hippocampal excitability in adulthood: Gender differences and prevention by neurosteroid treatment

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Perinatal events can profoundly influence health and wellbeing throughout life. Perinatal hypoxia is a major adverse effect of pregnancy and birth. Epidemiological evidence suggests that obstetric complications are correlated with impaired neural function postnatally that includes cognitive disability, schizophrenia, cerebral palsy and epilepsy.

Rat pups were placed in a chamber containing 3% oxygen in N_2 (hypoxia) or normal air (control) for 15-20 min on postnatal day 10. Some treated pups were injected subcutaneously with the stable allopregnanolone analogue, alfaxalone 4 mg/kg, 30 min before hypoxia. Brain activity was tested when the rats were 10-12 weeks of age. Rats were decapitated under isoflurane anaesthesia and the hippocampus cut into 300 μm slices. A stimulating electrode was placed on the Schaffer collateral nerve tract and activity was recorded from CA1 and CA3 neurons. Slices were studied electrophysiologically: (1) network activity was tested by exposing slices to Mg^{2+}-free solution until electrical activity appeared; (2) pre-synaptic events were tested using paired pulse facilitation (PPF), with pulses delivered 70 ms apart; (3) post-synaptic events were tested in terms of potentiation of synaptic responses for 2 h following tetanic stimulation (4 episodes of 100 Hz stimulation for 1 s).

Following hypoxia, the time for network bursts to commence in Mg^{2+}-free solution was halved (Table), suggesting increased excitability. PPF was increased by 50% indicating facilitation of transmitter release. Tetanus-induced increases in excitability were immediately increased by 116% in hippocampal CA1 neurons and this potentiation was sustained for at least 2h in females, indicating immediate and medium-term changes in post-synaptic responsiveness. In males, immediate potentiation of post-synaptic responses did not occur, but medium-term synaptic responses did not fade as they did in control animals, indicating delayed excitability. The changes in excitability, PPF and post-synaptic potentiation observed as a result of brief neonatal hypoxia were all prevented when the pups were treated with alfaxalone before hypoxia.

	Time to burst onset (min)	PPF (%)	Tetanus treatment (% at 2 h)
Control (n=10)	28±4	175±6	117±16
Hypoxia (n=10)	11±2	225±11	233±22
Hypox+alfaxalone (n=6)	21±2	186±6	92±14

In conclusion, a single brief episode of hypoxia on day 10 of life leads to increased excitability in hippocampal neurons at 10-12 weeks of age. This is prevented by pre-treatment with a stable analogue of the endogenous neurosteroid allopregnanolone, an agonist at inhibitory $GABA_A$ receptors. This suggests that brief hypoxia disrupts normal development of pathways in the neonatal brain, and that synthetic neurosteroids may be suitable for treating neonates at risk of hypoxic events.

A quantifiable approach to filtering and analysis of cellular fluorescence imaging

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Introduction:

Cellular bioimaging has become standard practice in one form or another for its ability to attain detailed structural and functional information at the cellular level. As imaging systems and computer capabilities have evolved, image resolution, imaging speed and volume of collected data has increased dramatically. With increased imaging data, especially in the case of functional data, comes the need for timely, more consistent quantitative measurement and analysis techniques applicable to multiple cellular imaging methods and cell types. Integrity of data is essential, and some image and video filtering and analysis techniques not only have the potential to hide important quantitative information, but to actually distort the information that has been obtained. This paper presents consistent and reproducible image and video filtering techniques for use in the analysis of cellular bioimaging data, concentrating specifically on fluorescence imaging techniques.

Methods:

Filtering Techniques: Linear mean filtering algorithms are commonly employed to remove noise from imaging data. Whilst linear and non-linear mean filtering have merit based mainly in short analysis time and strong noise reduction, it has an inherent problem of incorporating noise into the image rather than removing it. Mean filtering also blurs spatial image segment interfaces within a frame, and may distort temporal activity when applied across multiple frames of a video, so reducing the effective sampling rate of the collected data. Non-linear ranked filtering methods such as median filters employ a more complex form of filtering, however have the essential benefit of avoiding both spatial and temporal blurring of data segments by rejecting impulse noise (Sabri *et al.*, 1997), resulting in strong noise rejection whilst maintaining signal and data integrity.

Automatic Image Segmentation and Cell Detection: Manual cell counting in cellular imaging is a common and time consuming process, there are a variety of image segmentation algorithms to remove the majority of the manual labor involved in this process. These techniques may also be applied to functional and structural fluorescence imaging data to identify specific cells of interest in the culture, slice, or whole animal.

Functional Quantification: Much functional imaging data is presented in a qualitative or inconsistent quantitative fashion that varies markedly between research groups. The quantifiable properties of stochastic noise, namely confidence levels of the normal distribution, may be utilized to quantify functional fluorescence signals in terms of cellular activation level above baseline at given confidence intervals.

Correlation of Activity Between Cells: Correlation of activity between cells may be achieved after applying the combined cell detection and functional quantification methods discussed above. Detection of networks and cellular information transmission through investigation of cellular activity correlation allows determination of network formation and quantification of function, critical aspects of true physiological condition of biological tissue. Timing, strength, and reliability of cell-cell interactions may, using the methods above, be quantified without excessive analysis time, allowing the lag between cells activity to be determined. These methods may well allow investigation of neurotransmitter function, synapse formation and efficiency, and the effects of receptor agonists or other pharmacological interventions on cell-cell interactions.

Conclusion:

Cellular bioimaging techniques currently employed have the potential to limit accurate quantification of data and hide vital information from researchers. Using non linear median filtering along with the known properties of stochastic noise allow a more robust, consistent and highly quantitative methodology for cellular imaging. This has the potential to improve both structural and functional imaging procedures and assist in the testing and development of pharmacological interventions at the intra- and inter-cellular level. These techniques may be applied equally well to traditional two dimensional and three dimensional confocal microscopic techniques.

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