IMAGING INTERCELLULAR CALCIUM WAVES IN THE DEVELOPING NERVOUS SYSTEM OF ZEBRAFISH

by

GANESH SRINIVASAMOORTHY

(under the direction of Andrew Sornborger and James Lauderdale)

ABSTRACT

Several critical developmental events in the brain are believed to be regulated by spontaneous calcium waves. Commonly used calcium imaging methodologies have not been successful in exploring calcium waves beyond the embryonic stages of zebrafish, due to their invasivity, high noise, poor spatial resolution and other problems. However, using zebrafish transgenic for *Cameleon* - a FRET based calcium indicator and SOARS - a statistical optimization technique for data analysis, we were able to overcome these limitations and were able to image spontaneous calcium waves *in vivo* in a live zebrafish brain. The zebrafish that we imaged stably expressed *cameleon* in all the neurons. Changes in the *cameleon* FRET signal were detected using SOARS, which is capable of detecting signals from noisy ratiometric datasets. Our results represent the first successful instance of imaging calcium waves with well preserved spatio-temporal information in a live larval zebrafish brain *in vivo.*

INDEX WORDS: Intercellular calcium waves, Zebrafish, Cameleon, Neuronal calcium waves, Developing brain, Zebrafish imaging

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# CHAPTER 1

# INTRODUCTION

Propagating waves of calcium play several important roles throughout embryonic development. Starting with regular calcium spikes that sweep through the egg during fertilization [Miyazaki et al., 1993], Calcium waves are functionally implicated in mitosis, progression through cell cycle [Kono et al., 1996], cell differentiation, proliferation, gastrulation, formation of somites and neural induction [Gilland et al., 1999, Webb et al., 2000, Creton et al., 1998]. As brain development initiates, calcium waves perform some of the major pivotal functions in the establishment of the central nervous system, which include the regulation of neuronal migration, differentiation, survival and the development of the multi-cellular architecture of the nervous system [Fulton et al., 1995; Katz et al., 1993; Jeffreys et al. 1995].

Changes in cytosolic free calcium are known to have several important functions in a cell's physiology. Several transcription factors that mediate changes in gene expression are known to be regulated by changes in cytosolic calcium levels. Calcium sensitive transcription factors have been known to regulate DNA synthesis, cell differentiation [Ferrari et al., 1998], cell proliferation [Wang et al., 1996] and trigger apoptosis [Kass et al., 1999]. In addition to being important in normal cells, deregulation of calcium has been associated with cancers such as melanomas [Benzaquen et al., 1995]. Neuronal development is also known to be regulated by spikes in calcium levels,

which include the control of expression of specific neurotransmitters and ion channels [Gu et al., 1997, Carey et at., 1999], growth cone behavior [Gomez et al., 1999] and the formation of specific connections in neural circuits [Wong, 1999]

The zebrafish, a popular model organism for studying development, has been very useful in studying calcium dynamics during the early developmental stages [Webb et al., 2003]. Transparent embryonic and larval stages make zebrafish uniquely accessible for *in vivo* imaging and the availability of transgenic lines specifically for studying calcium dynamics [Higashijima et al., 2003; Ashworth, 2004] makes the zebrafish a very promising candidate for exploring the roles of intercellular neuronal calcium waves in the development of the nervous system.

Single wavelength calcium sensors have been popular in biological imaging, but have there own set of problems. Signal-to-Noise Ratio (SNR) is often significantly reduced due to motion and other artifacts [Broder et al., 2007], which are particularly pervasive in imaging *in vivo*.

FRET microscopy, which reads out the signal as a ratio of two wavelengths has been a good alternative, as a majority of the artifacts cancel out while ratioing [Grynkiewicz et al., 1985; Takahashi et al., 1999; Bright et al., 1987]. Several transgenic lines of zebrafish have been specifically engineered to take advantage of FRET microscopy [Miyawaki et al., 1997].

Cameleon, a transgenic line of zebrafish that expresses the protein yellow cameleon (YC2.1) pan-neuronally (using the neuron specific promoter HuC) has been developed in the laboratory of Dr. Joe Fetcho at Cornell University. Cameleons are chimerical fusion molecules built around the calcium sensitive protein Calmodulin, with

CFP and YFP tagged at either end. Conformational changes in calmodulin during calcium binding, bring CFP and YFP close enough to effect changes in fluorescence resonance energy transfer between the molecules, which serves as a measurable signal of calcium activity [Miyawaki et al., 1997; Higashijima et al., 2003].

Cameleon gives a good FRET signal *in vitro* and in transiently transfected cell samples, but the Signal-to-Noise Ratio of the fluorescence emission is poor when expressed in the nervous systems of vertebrates [Miyawaki, 2005; Miyawaki et al., 2003; Nagai et al., 2004; Reiff et al., 2005]. Although spatial and temporal averaging have been effective in producing measurable signal [Higashijima et al., 2003], spatial averaging calls for predefined masks, which cannot be determined for waves propagating spontaneously *in vivo* (as the spatial distribution is unknown) and temporal averaging blurs out important small timescale events which could be of significance [Broder et al., 2007].

SOARS, a statistical optimization technique, has been demonstrated to be capable of detecting calcium signals from a noisy dataset, without compromising the detection of important spatial and temporal information. Using masks that are weighted dynamically, in a user-independent way and by retaining sharp spatial and temporal intensity changes, SOARS overcomes the above mentioned limitations and is a promising technique [Broder et al., 2007] for imaging calcium waves *in vivo*, where retaining important spatial and temporal features is of essence.

# CHAPTER 2

# CALCIUM WAVES IN THE DEVELOPING NERVOUS SYSTEM - LITERATURE REVIEW

## CALCIUM: AN IMPORTANT SIGNALING MOLECULE

Calcium ($Ca^{2+}$) plays an important role as a signaling molecule throughout the life cycle of an organism. During fertilization, mammalian eggs generate regular $Ca^{2+}$ spikes that persist for a few hours and initiate development [Miyazaki et al., 1993]. As the zygote proliferates rapidly into the next stages of development, the $Ca^{2+}$ signaling system controls cell differentiation and the specification events that are responsible for pattern formation [Miyazaki et al., 1993; Gilland et al., 1999; Webb et al., 2003; Creton et al., 1998]. Post differentiation, $Ca^{2+}$ signaling in conjunction with other signaling pathways is responsible for the regulation of cell proliferation in response to growth factors [Lu et al., 1993; Berridge et al., 1995]. $Ca^{2+}$ is also responsible for controlling gene expression and the activation of several transcription factors, particularly those involved in cell proliferation [Crabtree et al., 1999]. $Ca^{2+}$ signaling has also been implicated in the control of the expression of apoptotic signaling components, in addition to being capable of directly inducing apoptosis under certain pathological conditions [Kass et al., 1999].

## CALCIUM WAVES AND THEIR SIGNIFICANCE

Propagating waves of calcium represent an important signaling mechanism during development. Starting with a global calcium wave that sweeps through the egg during fertilization [Miyazaki et al., 1993], they play important roles throughout development. Intracellular calcium oscillations and spikes have been demonstrated to be associated with initiation of mitosis and cell differentiation [Buonanno et al., 1999; Ferrari et al., 1998]. Intercellular calcium waves in zebrafish have been observed during early synchronous cleavages [Creton et al., 1998], specification of the dorso-ventral axis [Reinhard et al., 1995] and during segmentation [Creton et al., 1998]. They have been well characterized during the entire gastrulation phase [Gilland et al. 1999].

## INTERCELLULAR CALCIUM WAVES IN THE DEVELOPING BRAIN

Calcium waves have several crucial functions in the developing nervous system. They have been proposed to be involved in regulation of neuronal migration, differentiation, survival and in the development of the multi-cellular architecture of the nervous system [Fulton et al., 1995; Katz et al., 1993; Jeffreys et al. 1995]. Synchronized spontaneous discharges are believed to contribute significantly in establishing functional properties of neural circuits by influencing the development of excitatory and inhibitory synapses [Sanes et al., 1988]. They also appear to play an important role in the developing spinal cord, particularly in the spatial organization and specificity of motor neuron and skeletal muscle connections [Walton et al., 1991]. In mammals, brain waves and oscillations involving calcium [Kotak et al., 2007] have been shown to exist during development, aging, cognitive performance and sleep cycles

[Buzsáki, 2006; Ren et al., 2006]. Oscillations in calcium levels have also been observed in small neuronal ensembles [Yuste et al., 1992], and large-scale networks in the developing cortex of rat and mouse [Garaschuk et al., 2000; Adelsberger et al., 2005]. Waves in the developing hippocampus have been proposed to play a significant role in the formation of hippocampal circuits [Palva et al., 2000].

Calcium waves in the developing cortex have been reported in resting mouse pups that are not detected when the pup is in motion, indicating that the calcium wave dependent network development might occur during intermittent sleep-like resting phases [Adelsberger et al., 2005].

Although a good number of important developmental functions have been associated with calcium waves, many of the studies have been restricted to calcium waves in glial cells (astrocytes) and not much is known about intercellular calcium waves in neurons even though these have been well characterized in *in vitro* studies. Intercellular neuronal calcium waves have been characterized in culture preparation of mouse cortical neurons and GT1-1 neurons [Charles et al., 2006]. Endogenously generated oscillations in calcium levels have also been reported in the developing thalamorecipient auditory cortex. These endogenously generated waves in the auditory cortex ceased with onset of early hearing activity [Kotak et al., 2007].

## ZEBRAFISH AND CALCIUM WAVES

The zebrafish has become a model system for studying large scale calcium dynamics. Its transparent embryo makes it optically well-suited for imaging calcium waves. The zebrafish has served as a useful model for studying calcium signals that are

associated with ooplasmic segregation [Leung et al., 1998], early blastomere cleavage [Webb et al., 1997], and many later morphogenetic events [Gilland et al., 1999]. Early development through mid-blastula stages has also been extensively studied using fluorescent calcium reporters [Chang et al., 1995; Reinhard et al. 1995]. Developmental stages from 30% epiboly to 16 somites, covering primary and much of secondary gastrulation [Kimmel et al., 1995; Kanki et al., 1997] show large scale rhythmic calcium waves, that have been well characterized [Gilland et al., 1999].

Although calcium waves during early embryonic stages of development in zebrafish have been well studied, their role and characteristics in the developing nervous system remain unexplored [Ashworth, 2004]. Particularly intercellular calcium waves in neurons have remained largely unexplored even in other model organisms, even though they are believed to have significant roles during development. With extensively documented developmental stages and several inherent advantages that are discussed in detail in the following sections, the zebrafish serves as an excellent system to explore the function of calcium waves in a developing nervous system *in vivo*.

# CHAPTER 3

# ZEBRAFISH AND CALCIUM IMAGING – OVERVIEW OF LITERATURE

Over the past decade the zebrafish has emerged as an important model organism for studying development. Genetic abnormalities, cancer, brain development and function, diseases of sensory systems, pigmentation defects and pollution detection are just a few of the broad ranging disciplines in which the zebrafish has served as a very useful and successful model organism.

## ADVANTAGES OF ZEBRAFISH - AN IMAGING PERSPECTIVE

One of the most distinguishing aspects of zebrafish is that it is transparent through much of its early development. This, combined with the fact that it is a vertebrate, makes it a particularly favorable model organism for optical methods of investigation. Other important advantages of using the zebrafish as a model system are that, fertilization and development is external with offspring available in large numbers. Zebrafish also develop rapidly, with hatching beginning at about two to three days from fertilization, and the larvae becoming free-swimming at about five days from fertilization [Kimmel et al., 1995].

The transparency of zebrafish during embryonic and larval stages not only allows one to observe development but also to image cells, tissues and activity in an intact animal. This feature alone serves as a strong motivating factor for choosing zebrafish

for imaging experiments, as, there are only a few other groups of vertebrates (some larval fishes and some amphibians) that are accessible for imaging an intact live animal [Fetcho et al., 2004]. Further, this feature also makes it possible to image neurons throughout the nervous system, which is essential for studying large scale intercellular calcium waves.

## CALCIUM IMAGING IN ZEBRAFISH - APPROACHES

Intracellular calcium concentration can be estimated using several techniques. Bulk loading of cells in culture with synthetic calcium indicator dyes [Takahashi et al., 1999] has been promising and has been effective in the measurement of cellular calcium levels [Fan et al., 2007]. This technique can be extended to *in vivo* imaging of calcium waves by injecting a suitable calcium indicator in the blastomere stage during the development of the fish [Cox et al., 1996]; however, this method produces a weak labeling and not all cells are identifiable. Backfilling of neurons with calcium indicators also serves as a poor option for imaging, since this method is invasive and has insufficient spatial resolution [Fetcho et al., 2004] for measuring large scale intercellular calcium waves.

Transgenic zebrafish have ushered in a new wave of neurobiological studies and provide an elegant solution to the above mentioned problems [Higashijima et al., 1997; Lin, 2000; Long et al., 1997]. Genetically encoded GFP-based indicators, not only allow visualization of individual cell types, but can also act as sensors of important biochemical variables [Miyawaki et al., 1997; Nagai et al. 2000; Yu et al. 2003]. Genetically encoded fluorescent indicators, which show a change in the magnitude of

emitted signal based on changes in cellular calcium concentration [Nagai et al. 2000; Yu et al. 2003] have facilitated the study of calcium dynamics in cells, tissues and in intact live animals. Transgenic lines of zebrafish, expressing GFP based proteins, whose signal output changes with cellular calcium concentration, have been successfully used in producing detectable calcium signals [Higashijima et al., 2003]. Pan-neuronal expression of GFP and GFP based GECIs (Genetically Encoded Calcium Indicators) has been successful and unique genetic lines have been developed that have a pan-neuronal expression of calcium indicators [Miyawaki et al., 1997].

Pan-neuronal expression of a calcium indicator provides the essential nervous system-wide distribution of the calcium indicator that is needed for studying large scale multicellular neuronal events. (Refer Figure 1 for a visual aid)

This unique combination of optical accessibility and availability of suitable genetic lines makes zebrafish an ideal candidate for exploring intercellular neuronal calcium waves *in vivo*.

## ARTIFACTS, PROBLEMS AND SOLUTIONS

Although, genetically encoded calcium indicators are immensely useful, a single indicator has several shortcomings from an imaging perspective. In laser scanning confocal microscopy, chromophores (such as GFP, YFP, CFP) tend to induce artifacts in the output due to bleaching, variable illumination, etc. Further, changes in focus and movement of the object also pose problems in image analysis. These issues can be significantly reduced if the output may be measured as a ratio of emissions, both of which are subject to the same artifacts. FRET, (Fluorescence Resonance Energy

Transfer) is a technique that offers a solution to this problem, as the output consists of two wavelength emission measurements, which can be ratioed to eliminate the above mentioned artifacts [Miyawaki et al., 1997]. It has been successfully utilized for imaging and studying calcium dynamics [Higashijima et al., 2003].

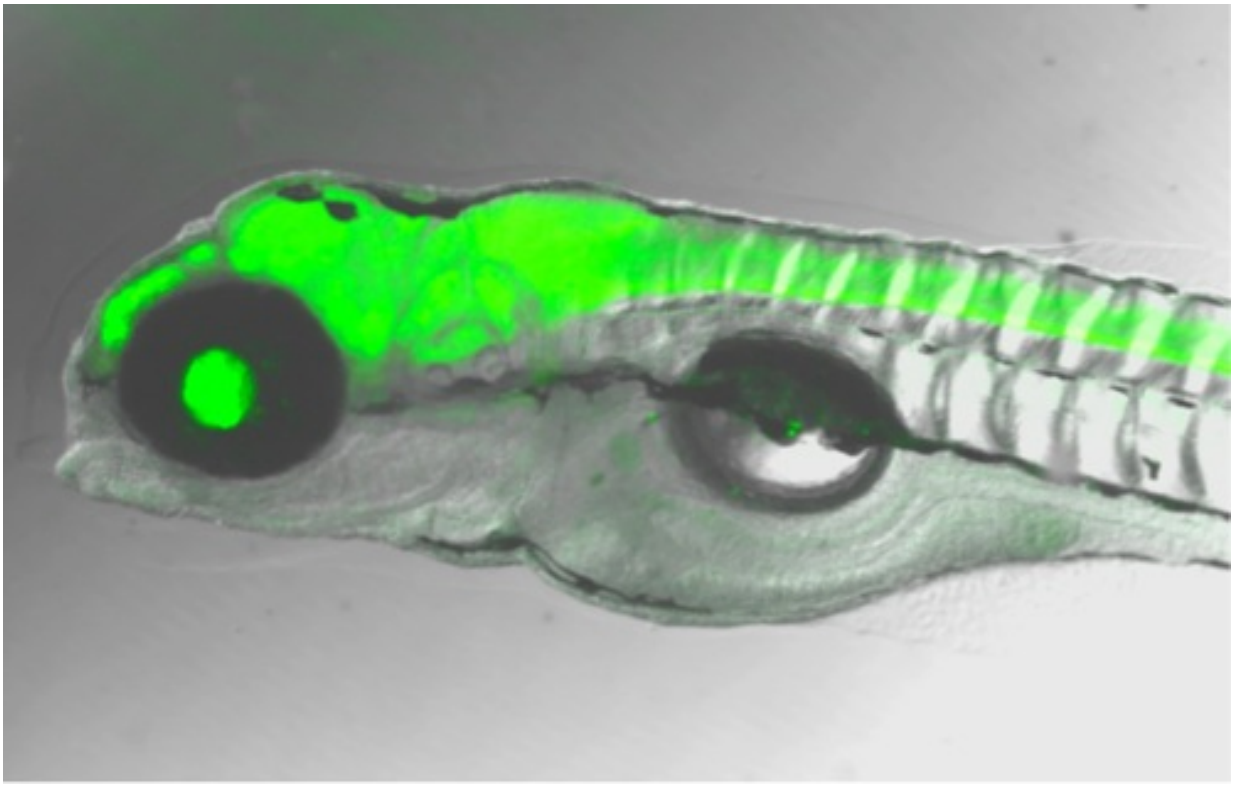

FIGURE 1 Image of a five day old zebrafish larva, expressing the genetically encoded calcium indicator, Cameleon, pan neuronally, under the neuron specific promoter HuC (Photo: Anirban Majumdar)

# CHAPTER 4

# CAMELEON AND FRET – OVERVIEW OF LITERATURE AND DISCUSSION

## FRET

Fluorescence Resonance Energy Transfer is a mechanism of energy transfer between two chromophores. When a donor chromophore and an acceptor chromophore are in close proximity (< 10 nm), the donor, in its excited state can transfer long-range, non-radiative energy, to the acceptor through dipole-dipole coupling. A special case of this phenomenon is when the donor and acceptor molecules are fluorescent. The donor molecule decays from its excited state by transferring its energy to the acceptor molecule. The acceptor molecule then decays to a lower energy state by emitting light at a higher wavelength [Lacowicz J, 1999].

FRET has become a very useful tool for studying and quantifying molecular dynamics in biological systems. CFP and YFP, color variants and derivatives of GFP have become the most useful FRET donor and acceptor pair for studying biological systems. These proteins can be genetically engineered to attach to a host protein and offer an elegant alternative option to synthetic dyes [Zhang et al., 2002; Miyawaki, 2005; Miyawaki et al. 2003; Miesenbock et al., 2005; Miesenbock, 2004].

CAMELEON

Sensing calcium in cells has been an area of intense interest. Calmodulin, a protein that changes its conformation upon binding with calcium, along with FRET, has allowed sensing and quantifying calcium dynamics at the cellular level. Cameleon, a chimerical protein, is made from the fusion of CFP, YFP, calmodulin and a binding domain (M13) for calmodulin [Nagai et al., 2004]. Calmodulin undergoes conformational changes upon binding to calcium and interacts with M13. This conformational change brings the CFP and YFP close enough to cause measurable changes in the FRET between the two molecules. Thus, changes in FRET signal can be used as a read out for rise and fall in cellular calcium levels, using the chimerical protein cameleon [Nagai et al., 2004]. (Refer Figure 2)

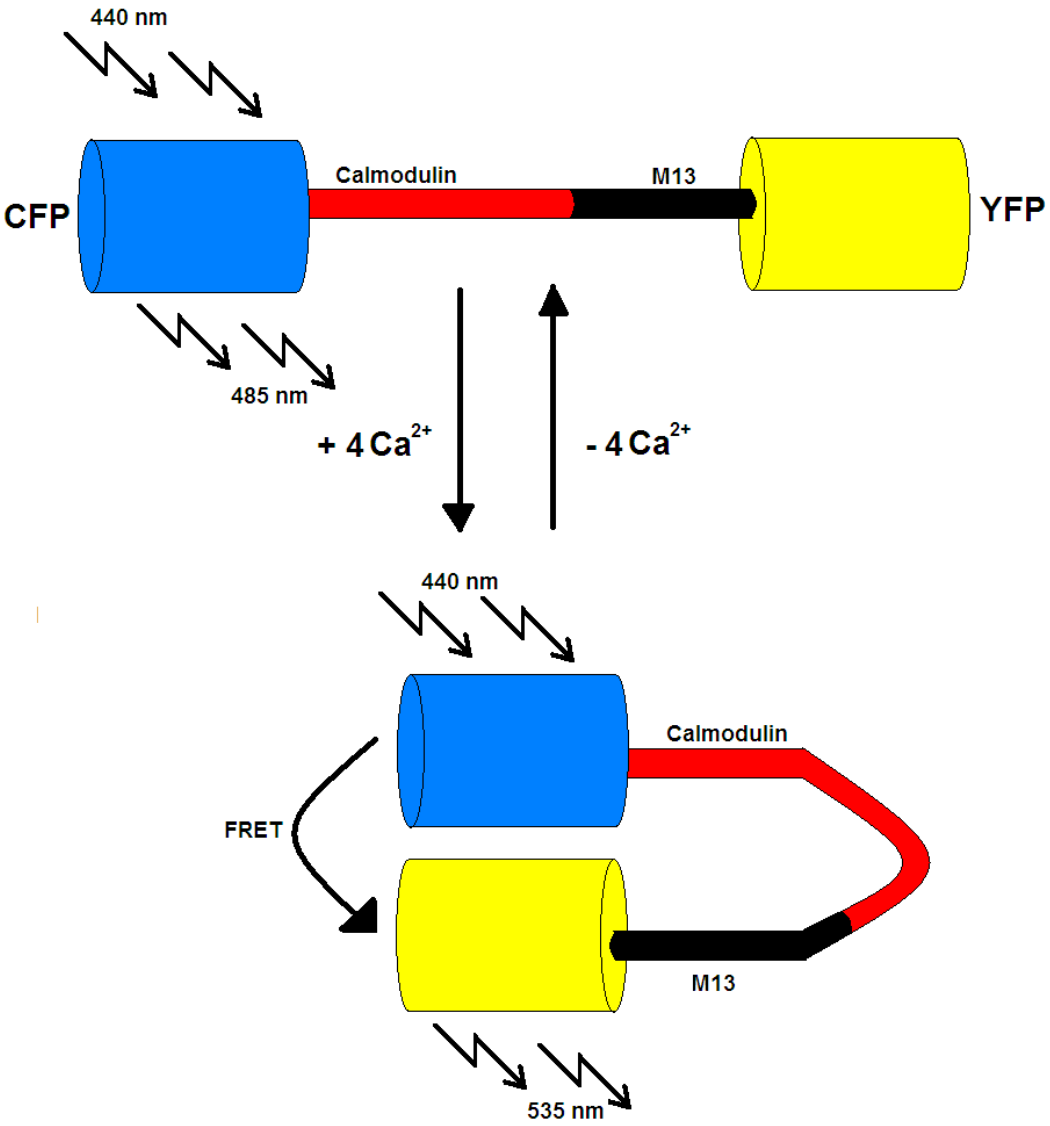


FIGURE 2 A schematic depicting the change in FRET between CFP and YFP in cameleon. CFP and YFP are attached to calmodulin via an M13 binding domain to form a fusion protein called cameleon, Calmodulin changes its conformation upon binding with calcium and interacts with M13. This conformational change brings the CFP and YFP closer and hence causes a change in the FRET between them.

# CHAPTER 5

## ZEBRAFISH GENETICALLY ENCODED WITH CAMELEON

YC2.1, a version of Yellow Cameleon has been expressed at high levels in sensory neurons and spinal cord neurons in zebrafish, using the neuron specific promoter HuC, which normally directs the expression of an RNA-binding protein in all neurons. This represents a successful and stable transgenic line of zebrafish expressing cameleon (YC 2.1) in all neurons. The transgenic line thus generated is healthy; the buffering of calcium by the indicators does not affect the viability of zebrafish [Higashijima et al., 2003]. Cameleons are well suited for *in vivo* studies and have been used to study the activity of Rohon-Beard neurons *in vivo*, in response to electrical shock [Higashijima et al., 2003].

With single wavelength indicators, movement of the imaging sample or a change in focal plane can result in artifacts. Since not all regions in the imaging region have the same amount of indicator, a change in brightness can either be due to change in calcium level, movement of the imaging sample or a change in illumination. Cameleons are inherently ratiometric, and are less sensitive to artifacts induced by movements, tissue thickness and focal plane variations [Miyawaki, 2005].

Being built with calmodulin as a component, cameleons have a Dissociation Constant ($K_d$) for calcium binding between 0.1–1 μm, and buffer calcium relatively less when compared with many synthetic calcium indicators [Takahashi et al., 1999].

Synthetic dyes such as Fura-Red have been shown to completely damp the calcium response due to excessive calcium buffering [Fan et al., 2007]. These properties make cameleon expressing zebrafish an ideal choice for FRET based calcium imaging *in vivo*.

# CHAPTER 6

# CAMELEON AND CURRENT RATIOMETRIC DATA ANALYSIS METHODS

## PROBLEMS WITH IMAGING CAMELEON *IN VIVO*

Although cameleon has been successfully expressed *in vivo* in zebrafish, there are still several limitations. Cameleon displays a good calcium response *in vitro* and in transiently transfected cell samples, but the SNR of the fluorescence emission is very poor when expressed *in vivo*, suggesting that the protein may be modified *in vivo* by its environment [Higashijima et al., 2003]. The dynamic range is significantly reduced in the nervous systems of vertebrates [Miyawaki et al., 1997; Nagai et al., 2000] and in primary neurons from vertebrates [Truong et al., 2001]. While YC2.1 shows almost 120% change in the ratio of CFP to YFP upon calcium binding in solution, interactions between CaM/CaM associated proteins and the sensing domains of YCs in neurons attenuates this dynamic range. Particularly so in neuronal cell types that have a large amount of CaM and CaM associated proteins [Palmer et al., 2004; Heim et al., 2004; Hasan et al., 2004].

## IMAGING CALCIUM WAVES *IN VIVO*: DRAWBACKS OF SPATIAL AND TEMPORAL AVERAGING

Although, YC2.1 in transgenic zebrafish was successful in showing measurable stimulus-dependent response in Rohon Beard neurons, the signal detection required

extensive spatial averaging of the fluorescence output before subjecting it to ratiometric analysis [Higashijima et al., 2003]. Whilst spatial averaging is a popular technique for signal detection, it calls for the use of ROI's (regions of interest). This procedure involves retaining pixels in a predefined region and masking the remaining pixels, and then averaging the retained pixels. This method has several limitations. Firstly, in data with low SNR, as is typically the case with ratiometric indicators, important information might be lost in averaging. Secondly, although the method may detect a response, important spatial information is lost, as the mask defined has to be static. Spatial and temporal information is of significant importance in studying propagating calcium waves in neurons and sacrificing this information impairs one's ability to interpret data for detecting waves. Further, a mask, if chosen, cannot be static, as we do not know the spatial distribution of a propagating wave.

Averaging temporally is another method for detecting signal, wherein the pixel time courses are averaged [Russ et al., 2006]. This is typically accomplished by using a low pass filter, however, too much averaging can over-smooth the data and blur out fast dynamics that might be of interest, particularly in the context of propagating waves. This limitation thus restricts the amount of temporal averaging that can be done, and hence cannot be effective in datasets with high noise content (effective only when $\Delta F/F_0 = 0.4$ or more) [Cheng et al., 1999].

From the above discussion, it is evident that to detect calcium waves in neurons *in vivo*, from a noisy dataset, without compromising on important spatial and temporal information: [Broder et al., 2007]

- We need masks that are weighted dynamically, in a user independent way, based on their variance and co-variance information (as opposed to static predefined masks).
- We must avoid pre-smoothing of data, to retain sharp spatial and temporal features.

# CHAPTER 7

# SOARS

## A SOLUTION TO THE DRAWBACKS

Statistical Optimization Analysis for Ratiometric Signals [SOARS] is a technique for statistically optimized analysis of ratiometric data, which overcomes the above mentioned limitations, and offers other significant advantages. This method has been demonstrated to be capable of reading out signals from very noisy data (with SNR at 1/16 or poorer) without sacrificing any spatial and temporal information [Broder et al., 2007].

This method takes advantage of the fact that in the physiological context, meaningful signals are usually spatially correlated. It detects spatially and temporally correlated signals by applying multivariate statistical optimization methods to the full covariance matrix of the data [Sirovich et al., 2002; Yokoo et al., 2001; Sornborger et al., 2005].

## SOARS: AN OVERVIEW OF THE TECHNIQUE

Calcium signals in ratiometric data are often represented as anti-correlations in the data from the two channels (donor and acceptor). Binding of calcium to calmodulin, causes a conformation change and brings CFP and YFP closer. The resulting increase in FRET between YFP and CFP, causes a reduction in emission of YFP (as the energy

is transferred to CFP instead of radiation) and an increase in the emission of CFP (additional increase in emission due to increase in FRET). Thus, a rise in the level of intercellular calcium causes a rise in CFP emission and a decrease in YFP emission, which results in a characteristic anti-correlation in the dual channel data [Miyawaki et al., 2003].

The problem of signal detection in our context can be thus be stated as that of finding a subspace of the datasets from each of the channels that has maximum temporally anti-correlated and spatially correlated information. SOARS accomplishes this objective by using multivariate statistical optimization. The maximally anti-correlated subspace of the data is first estimated and then the dual-channel measurement is *de-noised* by projecting the dataset into the subspace. The technique thus filters the data in a space of anti-correlated eigenimages, by choosing the statistically significant eigenvectors from the data. Filtering pixels (ROI) or filtering temporally (Boxcar filters) and their associated limitations are thus overcome and high resolution spatial and temporal information is retained in the estimated ratio by using multiple significant eigen images [Broder et al., 2007].

## DETECTING CALCIUM WAVES AND THE NECESSITY OF SOARS

The important advantageous feature of SOARS is that the masking in the dataset is user-independent, i.e., the anti-correlated subspace is estimated objectively without any user bias. This is particularly important in studying propagating calcium waves because a user defined mask might mask out regions of importance that could be participating in wave propagation and secondly in noisy data, regions with lower

background intensity but significant response, might go undetected. By determining anti-correlations based on weighted masks, instead of user defined ROIs, SOARS retains a high degree of spatial information, even in very noisy datasets.

## SOARS – DEMONSTRATED EFFECTIVENESS

SOARS has been demonstrated to reliably detect small changes in the ratio of YFP/CFP in YC2.1 expressing cultured zebrafish spinal neurons in response to potassium stimulation. In PC12 cells bulk loaded with ratiometric pair dyes, Fluo-4 and Fura-Red, SOARS was effective in reading out the anti-correlated calcium response signal (upon stimulation with KCl), even when traditional fluorescence averaging was unable to detect the signal [Broder et al., 2007]. It has also been shown that while the first eigenimage often displays the dominant response, subtle statistically significant information in the FRET response is captured by a few other eigenimages, which can correspond to hard-to-detect physiological changes which might otherwise be undetectable.

# CHAPTER 8

## LARVAL DEVELOPMENTAL STAGE FOR IMAGING

In zebrafish, large scale calcium waves sweep across the early stage embryos up to around 24 hours [Gilland et al., 1999]. In the embryonic nervous system, particularly at and before 24 hours, developmental activity is dominated by primary neurogenesis [Mueller et al., 2005], which is believed to involve the generation of transitory neurons such as spinal motor neurons, that are involved in a reflex circuit with Rohon-Beard neurons [Chitnis, 1999], and the early axonal scaffold formed by the first differentiated brain neurons [Chitnis et al., 1990; Wilson et al., 1990; Park et al., 2000].

In contrast, larval stages are characterized by secondary neurogenesis, which involves the generation of neurons that are not formed during primary neurogenesis [Mueller et al., 2005]. Secondary neurogenesis includes much of the primary and all of secondary neurogenesis described in amniotes [Hatten, 1999]. This includes events such as non-radial glia guided neuronal migration in the cerebellar EGL, hippocampal dentate gyrus and neuronal generation from the subventricular zone of the telencephalic lateral ventricle, which results in the rostral migratory stream to the olfactory bulb [Bayer et al., 1995].

The zebrafish larva at five days post fertilization undergoes significant development in the central nervous system. Intense proliferation of neuronal cells is observed in the pallial and subpallial cells in the telencephalon, and in the medial

proliferative zone of the olfactory bulb. Neuronal proliferation is also significant in the ventricular lining of the preoptic region, parts of eminetia thalami, basal part of habenula, dorsal and ventral thalamus, and medial zones in the anterior optic tectum [Mueller et al., 2005].

The five-day-old larva is also characterized by the separation of the preoptic region from the emerging rostral hypothalamus caudally by the post-optic commissure. Proliferation of neurons is observed in the posterior tuberculum, pretectum and the formation of a tubercular region, which is destined to form the preglomerular complex. Intermediate and caudal hypothalamus also show neuronal proliferation. Proliferative zones are also observed in the medial and ventral zones of the cerebellum, eminentia granularis and the rhombic lip [Mueller et al., 2005].

Intercellular neuronal calcium waves have been proposed to be involved in neuronal migration, differentiation and survival [Charles et al., 1996; Komura et al., 2005] and among other roles, in the development of the architecture of the nervous system. The fifth day post-fertilization in zebrafish serves as a good time point in the nervous system development in which spontaneous calcium waves can be expected to propagate. This stage is thus a suitable point for imaging calcium waves during the zebrafish larval development.

# CHAPTER 9

# HYPOTHESIS

From the previous discussions, we can state that it is a testable hypothesis, that imaging larval zebrafish expressing cameleon around the 5$^{th}$ day post fertilization and utilizing the SOARS technique for analysis of the ratiometric imaging data would reveal spontaneously propagating calcium waves in the developing nervous system.

From the literature review presented above, it can be concluded that significant variation is observed in the frequency, the exact timing and the factors governing the propagation of these waves [see for example Adelsberger et al., 2005] during development. It would hence be difficult to frame an expected frequency and the onset and ceasing stages of the calcium waves in the developing larva. The SOARS technique however has been demonstrated to be successful in detecting anti-correleted signals in ratiometric data, and other conditions remaining the same, spontaneously propagating waves of calcium in the developing zebrafish nervous system offers a testable hypothesis to be verified experimentally.

# CHAPTER 10

# MATERIALS, METHODS AND EXPERIMENTAL SET UP

## ZEBRAFISH STRAINS AND MAINTENANCE:

Transgenic zebrafish expressing YC2.1 cameleon [Higashijima et al., 2003] were obtained from Dr. Joe Fetcho (Cornell University, NY). Embryos were staged by hours at 28$^{o}$C and by standard staging criteria [Kimmel et al., 1995] and reared as described previously [Westerfield, 2000]. Heterozygous cameleon embryos were generated by crosses between wild-type adults and cameleon heterozygous adults.

## FISH SAMPLE PREPARATION:

Zebrafish larvae at 5$^{th}$ day post fertilization were sorted to collect larvae expressing cameleon. The larva to be imaged was then anesthetized using 40 µg/ml of Tricaine (3-amino benzoic acid ethyl ester, Sigma Cat.# A – 5040). The larva was then embedded in 3% low melting agarose, containing 40 µg/ml of Tricaine, over a coverglass. The coverglass was then incorporated into a Dvorak-Stottler chamber and the chamber was sealed.

## CONFOCAL MICROSCOPE:

LSM 510 Meta (Carl Zeiss Inc.) was the upright confocal microscope to be used for imaging larval zebrafish expressing cameleon. The 458 nm line of a 50 mW argon

laser served as the excitation laser, with emission bands centered on 485 (cyan) and 535 (yellow) nm, respectively. The transmitted image was captured with a separate Photo Multiplier Tube.

IMAGING:

Egg Water [Westerfield, 2000] was perfused into the chamber at a rate of 8.3 µl/sec. Images were taken with excitation set to deliver 9-10% of the laser intensity. This excitation level was set to be generally able to excite approximately 10% of the pixels in the cyan image, and a few pixels in the yellow image, to saturation (grey level 255 in an 8-bit image). The gain of the photomultiplier for each channel was then adjusted downward to eliminate saturated pixels.

Images for ratiometric analyses were collected at a rate of 1/second, at a resolution of 256X256 pixels. The fish samples were imaged in continuous slots of 30 minutes with a short break in between to monitor the health of the fish and to address for motion if observed.

IMAGE PROCESSING AND ANALYSIS:

The dual channel images along with the images from transmitted channel were collected from the Zeiss 510 Meta confocal microscope. The images were then binned down to 128 X 128 pixels due to computational memory constraints. The ratiometric imaging data were then analyzed with the multivariate SOARS method [Broder et al., 2007]. This method makes use of the anti-correlated nature of ratiometric signals. SOARS is capable of finding orthogonal weighted masks (eigenimages) that are

statistically significant estimators of anti-correlated activity in the two-channel data. These eigenimages and the temporal eigenvectors were observed and analyzed to observe calcium waves in the nervous system. The data in the two channels were then denoised using the eigenimages. Finally, the ratio of the two channels was taken to give an estimate of $Ca^{2+}$ activity [Broder et al., 2007].

## EXPERIMENTAL PLAN

### Test Experiments:

#### Experiment Set I

#### IMAGING ZEBRAFISH TRANSGENIC FOR CAMELEON AT 5 DPF – LATERAL ORIENTATION

Zebrafish transgenic for Cameleon were imaged at 5 days post fertilization to detect spontaneous calcium waves.

In order to determine the regions of the brain participating in wave propagation, a laterally mounted larval fish provided a large region of the brain that could be directly captured for imaging. As the whole brain was represented as individual planes for confocal microscopy, a laterally mounted fish, in other words was used to provide the maximum number of Z – planes that were accessible for confocal imaging. This was however at the cost of detailed information that was available in each of the individual layer. Despite this disadvantage, a laterally mounted fish served as the most advantageous starting point for initially probing the distribution of the spontaneous waves.

Experiment Set II

IMAGING ZEBRAFISH TRANSGENIC FOR CAMELEON AT 5 DPF – DORSAL ORIENTATION

Once the broad ranges of the brain where the calicum waves propagate were determined, the next set of experiments was be to elucidate the detailed spatial features of the wave and the fine details of different layers of brain that actively participated in wave propagation. To accomplish this objective zebrafish transgenic for Cameleon were be imaged at 5 days post fertilization, in a dorsal orientation.

Dorsal orientation provided the detailed spatial information for each of planar region that would be determined to be of importance in wave propagation after imaging in the lateral orientation, and was the choice orientation for the experimental objectives.

Control Experiment:

Experiment Set I

IMAGING WILD TYPE ZEBRAFISH INJECTED WITH OTHER CALCIUM SENSITIVE DYES

Whilst cameleon in itself was a reliable indicator of calcium activity in neurons [Higashijima et al., 2003], it was important to set up control experiment where we can compare the activity reported by one calcium indicator to another calcium indicator, so that we could observe any indicator induced artifact, which might otherwise be confounded as calcium activity.

To achieve this objective, an imaging experiment with Fluo4 and Fura Red, a pair of fluorescent calcium indicator dyes was performed. Fluo4's fluorescent emission

decreases upon binding with calcium and Fura Red has an increase in fluorescent emission upon binding with calcium. Together, this pair of dye, serve as a reliable calcium indicator pair that will report calcium changes as an anti-correlation and do away with several artifacts that results from single wavelength indicators [Fan et al., 2007].

Wildtype zebrafish (Swik) at 5 days post fertilization were used for the imaging experiment. Fluo4 and Fura Red were injected into the optic-tectum region of the brain using a pico-spritzer injection rig. The fish were then imaged with identical conditions as the Cameleon transgenic fish.

# CHAPTER 11

## A STEP FORWARD: IMAGING INTERCELLULAR CALCIUM WAVES IN THE NERVOUS SYSTEM OF LARVAL ZEBRAFISH *IN VIVO* [1]

[1] Ganesh Srinivasamoorthy, James Lauderdale and Andrew Sornborger. To be submitted to *Nature Neurotechniques*.

SUMMARY

Several critical developmental events in the brain are believed to be regulated by spontaneous calcium waves. Commonly used calcium imaging methodologies have not been successful in exploring calcium waves beyond the embryonic stages of zebrafish, due to their invasivity, high noise, poor spatial resolution and other problems. However, using zebrafish transgenic for *Cameleon,* a FRET based calcium indicator and SOARS a statistical optimization technique for data analysis, we were able to overcome these limitations and were able to image spontaneous calcium waves *in vivo* in a live zebrafish brain. We report here the successful imaging of wide-spread calcium changes *in vivo*, suggestive of spontaneous intercellular calcium waves in the larval zebrafish hindbrain. The zebrafish that we imaged stably expressed *cameleon* in all the neurons. Changes in the *cameleon* FRET signal were detected using SOARS, which is capable of detecting signals from noisy ratiometric datasets. SOARS detects signals using masks that are weighted based on the co-variation in the data, in a user independent way and retains sharp spatial and temporal intensity changes. Our results represent the first successful instance of imaging calcium waves with well preserved spatio-temporal information in a live larval zebrafish brain *in vivo.*

INTRODUCTION

Propagating waves of calcium play several important roles throughout embryonic development. Starting with regular calcium spikes that sweep through the egg during fertilization [Miyazaki et al., 1993], Calcium waves have been observed in mitotic events in cell cycle [Kono et al., 1996], cell differentiation, proliferation, gastrulation, formation

of somites and neural induction [Gilland et al., 1999, Webb et al., 2000, Creton et al., 1998]. As the brain development initiates, calcium waves perform some of the major pivotal functions in the establishment of the central nervous system, which include the regulation of neuronal migration, differentiation, survival and the development of the multi-cellular architecture of the nervous system [Fulton et al., 1995; Katz et al., 1993; Jeffreys et al. 1995].

Changes in cytosolic free calcium are known to have several important functions. Many transcription factors are known to be regulated by changes in cytosolic calcium levels, including those that regulate DNA synthesis, control cell differentiation [Ferrari et al., 1998], regulate cell proliferation [Wang et al., 1996] and trigger apoptosis [Kass et al., 1999]. Neuronal development is also known to be regulated by spikes in calcium levels, which include the control of expression of specific neurotransmitters and ion channels [Gu et al., 1997, Carey et at., 1999], growth cone behavior [Gomez et al., 1999] and the formation of specific connections in neural circuits [Wong, 1999].

The Zebrafish has lately become a good model system for studying large-scale calcium dynamics. The transparency of zebrafish during embryonic and larval stages not only allows one to observe development but also image cells, tissues and activity in an intact animal. This feature alone serves as a strong motivating factor for choosing zebrafish for imaging experiments, as there are only few other groups of vertebrates (other larval fishes and some amphibians) that are so accessible for imaging an intact live animal [Fetcho et al., 2004]. Further, this feature also makes it possible to image neurons throughout the nervous system, which makes it a choice candidate for studying large-scale intercellular calcium waves in a system-wide context.

Calcium imaging techniques have been very effectively used in early zebrafish. Some important studies include the elucidation of signals that are associated with ooplasmic segregation [Leung et al., 1998], early blastomere cleavage [Webb et al., 1997], and many later morphogenetic events [Gilland et al., 1999]. Early development through mid-blastula stages has also been extensively studied using fluorescent calcium reporters [Chang et al., 1995; Reinhard et al., 1995].

Although current techniques have been useful in studying calcium events, imaging large scale waves of calcium *in vivo* has been restricted to early embryonic stages of development in zebrafish. Intercellular calcium waves have remained inaccessible for imaging beyond the first few hours of neuronal development. Despite zebrafish being a very promising model organism for studying calcium dynamics, the function and characteristics of these waves in the post-embryonic developing nervous system have remained unexplored due to several limitations discussed in detail in later sections.

Here, we report the successful imaging of large-scale calcium events suggestive of spontaneous intercellular calcium waves in the developing brain of larval zebrafish. Using SOARS, a statistical optimization technique, we were able to overcome most of the limitations and detect calcium waves *in-vivo* in a live larval zebrafish. SOARS is capable of detecting signals from noisy ratiometric datasets [Broder et al., 2007]. The zebrafish imaged were transgenic and genetically encoded with Cameleon, a FRET based calcium indicator [Higashijima et al., 2003].

Although, calcium signals from individual neurons have been successfully measured in larval zebrafish earlier [Higashijima et al., 2003], intercellular calcium

waves in the brain have remained unexplored. Here we show that using SOARS, we were able to measure endogenous wide-spread changes in neuronal calcium in the hindbrain of larval zebrafish at 5 dpf, suggesting that intercellular calcium waves are important for later stages of brain development.

## RESULTS

Larval zebrafish were imaged at five days post fertilization and the images were analyzed using SOARS. The zebrafish were transgenic and genetically encoded with Cameleon. The Cameleon transgenic line of zebrafish expresses the protein yellow cameleon (YC2.1) pan neuronally (using the neuron specific promoter HuC). Cameleons are chimerical fusion molecules built around the calcium sensitive protein Calmodulin, with CFP and YFP tagged at either end. Conformational changes in calmodulin during calcium binding brings CFP and YFP close enough to effect changes in fluorescence resonance energy transfer (FRET) between the molecules. This change in FRET serves as a measurable signal of calcium activity [Miyawaki et al., 1997; Higashijima et al., 2003].

The SOARS analysis detected large scale calcium events across the zebrafish hindbrain, that were suggestive of calcium waves in the rostro-caudal direction. These wave-like events seem to be generated spontaneously; they have a characteristic separation of about 300-400 seconds and a characteristic temporal evolution over 90 - 120 seconds.

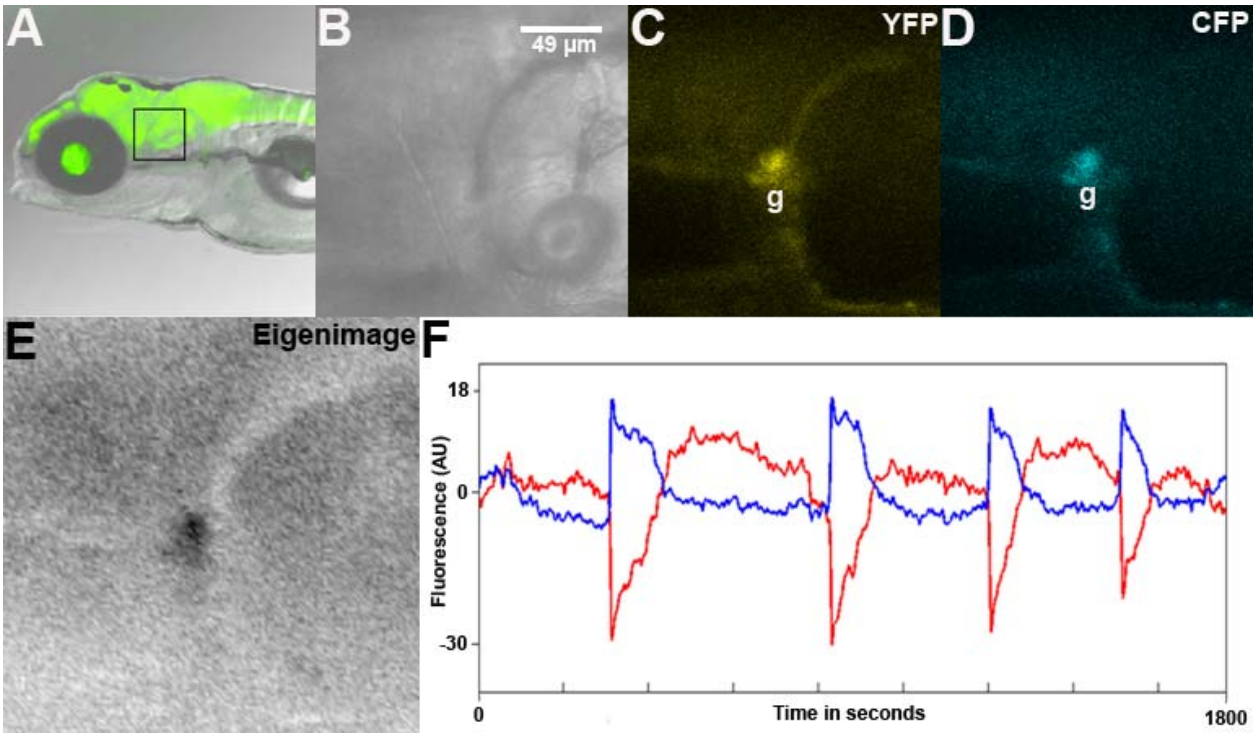


Fig.3. Spontaneous calcium waves in the hindbrain. g: Posterior lateral line ganglion. A) A view of the lateral mount of the fish with the region of the hindbrain that was imaged shown in a box. B-D) The transmitted, CFP and YFP channel images of the hindbrain acquired during confocal imaging showing the hind brain and the posterior lateral line ganglion E) Eigenimage of the region of the hind brain that was imaged. The eigenimage is an eigenvector that shows the relative activity (calcium signal here) in each pixel. Dark pixels indicate high calcium levels and white pixels low calcium levels. The intensity of each pixel corresponds to the activity in that spatial region of the brain (This is a representation of the rich spatial information that is provided using weighted masks, by SOARS). Here we can see that the eigenimage shows the hindbrain to be distinctly darker than the rest of the image F) The time-course of the CFP and YFP after performing SOARS analysis on the imaging data. The time-course over 1800 seconds shows four spontaneous waves, each represented by a sharp anti-correlation between

the CFP and the YFP channels. A typical wave rises swiftly and reaches peak fluorescence intensity within about 10 seconds and then decays slowly, with the entire wave lasting between 90 -120 seconds. A boxcar temporal averaging of 10 seconds was used in the time-course shown above. The time-course also shows the characteristic separation of 300 – 400 seconds between the waves.

Out of 10 fish, each imaged for one hour at 5 dpf, 4 fish showed these spontaneous waves. Given the spontaneous and endogenous nature of these waves, it is technically difficult to pinpoint reasons for not observing these waves in the remaining 6 fishes. One possible hypothesis is that these neuronal calcium waves might have a functional significance that causes them to be stochastic and have variation in the time frame in which they occur. Variation in the observed frequency and in the exact timing of these waves could also be due to a relationship with behavior. For example calcium waves in the developing cortex have been reported in resting mouse pups that are not detected when the pup is in motion, indicating that calcium wave dependent development might occur during intermittent sleep-like resting phases [Adelsberger et al., 2005].

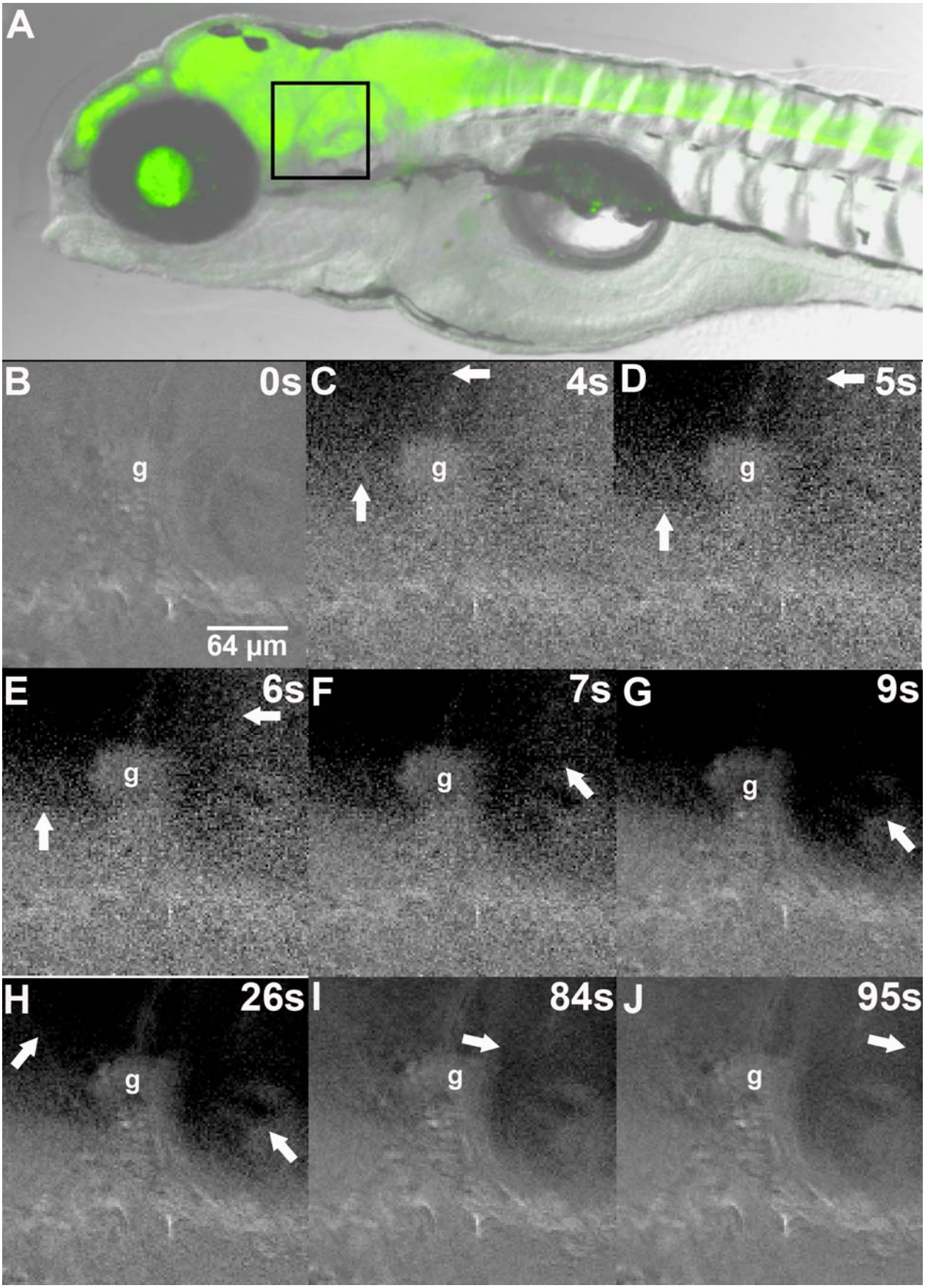
A
B
0s
g
64 µm
C
4s
g
D
5s
g
E
6s
g
F
7s
g
G
9s
g
H
26s
g
I
84s
g
J
95s
g

Fig.4. Rostro-caudal propagation of a spontaneous wave. g: Posterior lateral line ganglion A) A view of the lateral mount of the fish with the region of the hindbrain that was imaged shown in a box. B-J) Snapshots at various time points showing the calcium wave propagating in the rostro-caudal direction. The wave rises to peak fluorescence intensity in about 10 seconds and slowly decays over 100 seconds. The images were obtained using the improved ratio estimated from SOARS. From the ratio obtained by SOARS the background has been subtracted from the images to highlight the signal.

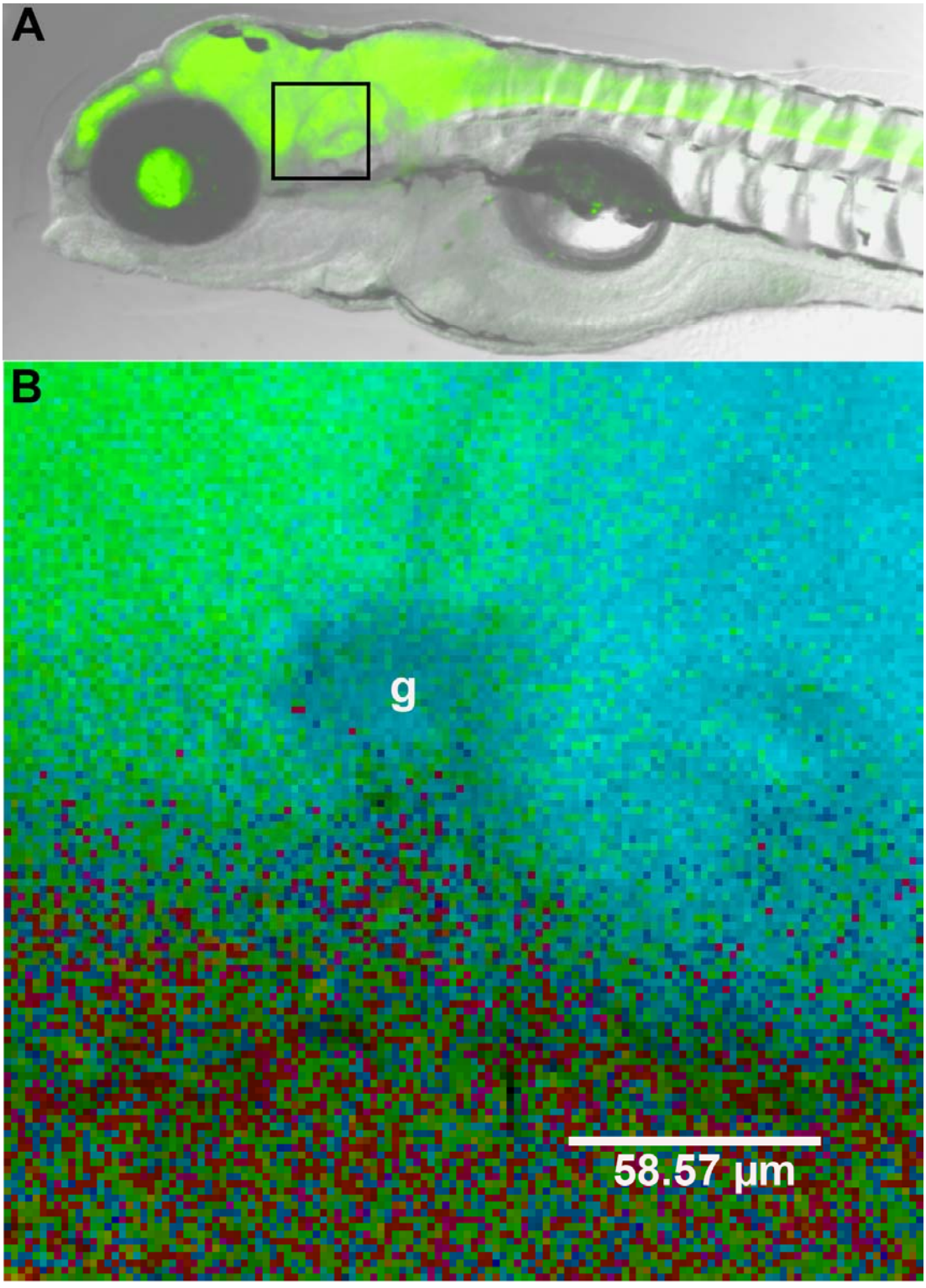
A
B
g
58.57 µm

Fig.5. Time evolution of a spontaneous $Ca^{2+}$ wave in zebrafish hindbrain. g. Posterior lateral line ganglion A) View of the lateral mount of the fish with the region of the hind brain that was imaged shown in a box. B) The hindbrain region that was imaged showing temporal evolution of the spontaneous calcium wave. Colors encode time of maximal calcium change (maximum YFP / CFP ratio). Early times are denoted by Green and later times are denoted by Blue. This analysis also confirms the rostro-caudal propagation of the spontaneous wave. A key aspect is that the image shows both the spatial *and* temporal information about a calcium wave in the brain. The detailed spatial and temporal information used to generate this image was obtained from the improved ratio that is provided by the SOARS analysis.

To rule out any transgenic cameleon specific artifacts or dependency, we also attempted to image these waves in a wildtype fish by injecting calcium sensitive dyes, Fluo 4 and Fura red in the brain (optic tectum). We observed the same characteristic wave (duration of ~120 seconds, separation of ~300 seconds).

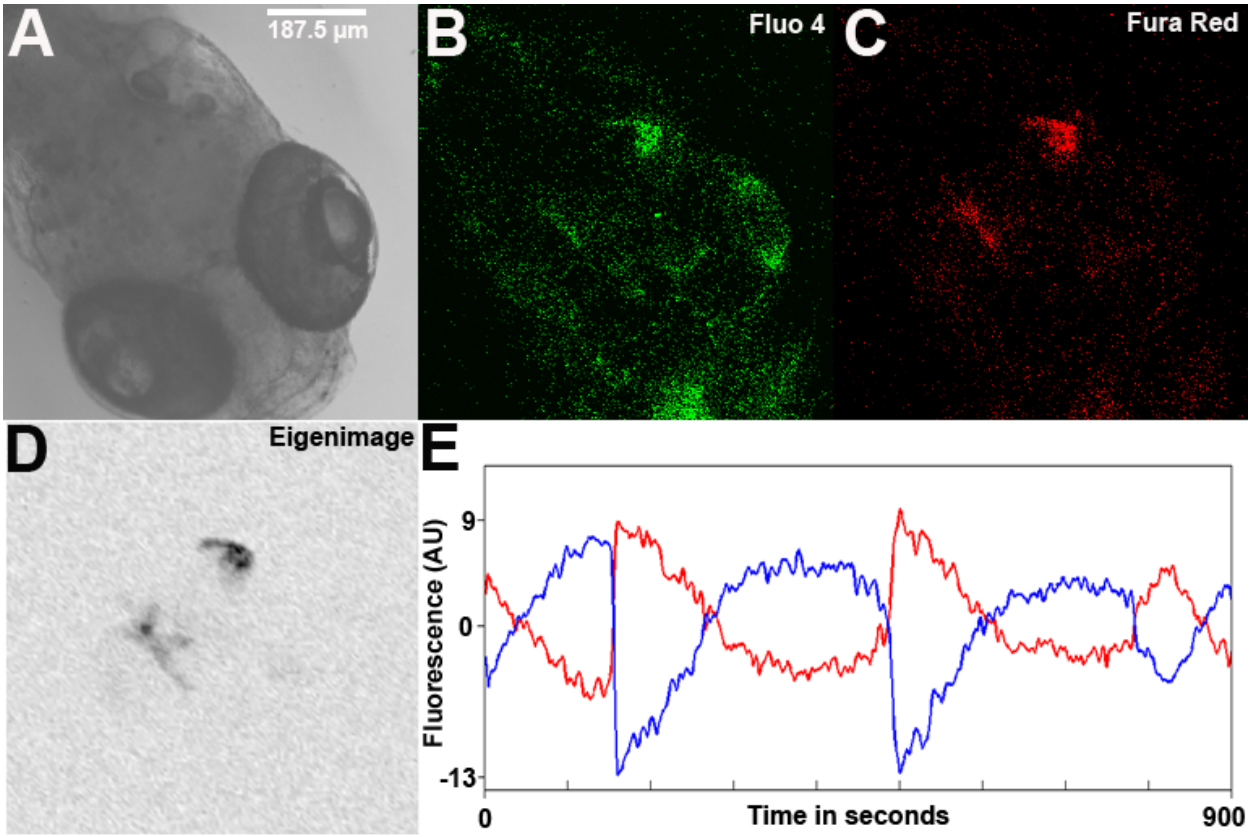


Fig.6. Spontaneous calcium waves in wild type fish A-C) The transmitted, Fluo 4 and Fura Red channel images of a brain in dorsal view, acquired during confocal imaging. D) The eigenimage obtained from SOARS analysis distinctly highlights only the optic tectum, where the dyes were injected. Here, only the optic tectum has fluorescent calcium sensitive reporters and is hence the only region that reports the propagating waves as opposed to the whole brain in the case of transgenic cameleon zebrafish. This also shows the disadvantages of not having a pan-neuronal expression of an indicator, as spatial information on wave propagation is restricted to selected regions. E) The time-course of the Fluo4 and Fura-Red after performing SOARS analysis on the imaging data. The time-course has similar features to the spontaneous waves observed in transgenic cameleon zebrafish. The time-course over 900 seconds shows two spontaneous waves, each represented by a sharp anti-correlation between the Fluo4

and the Fura Red channels, which last for about 120 seconds. A boxcar temporal averaging of 10 seconds was used.

Often, motion of the specimen during the imaging experiment can pose serious difficulties in analysis of data. Since not all regions in the imaging region have the same amount of indicator, movements of the sample can often cause significant noise. Although, the ratiometric nature of the data gets rid of most of the problems due to motion, changes in (CFP / YFP) ratio can still be influenced by movements of the imaging sample.

In order to observe the influence of motion on the improved ratio obtained from SOARS, we performed simulations of the movement of the imaging sample and then analyzed the resulting data. An imaginary plane corresponding to the imaging sample was subjected to simulated motion inside a three dimensional stack of brain images. The three dimensional stack of images for the YFP and CFP channels was obtained from real time imaging of the zebrafish brain, with successive layers one micron apart from each other. The data values on the imaginary plane inside the stack were obtained by interpolating the stack data onto a set of discrete points (here 128 X 128) on the imaginary plane meant to represent an image of the response to motion.

For simulating motion, we divided the observed imaging sample's movements into two categories. First was the fast twitch like movement (over 2 seconds), that is sometimes observed while imaging the fish, and second was slow movement (over 20 seconds) which is rarely observed and causes a gradual drift of the focal plane. We also simulated two types of displacement: small displacement, that usually happens over 10 microns in all three directions (X, Y and Z) and a relatively larger displacement that

happens across 40 microns in the X and Y directions and about 60 microns in the Z direction. (Displacement beyond this range was never observed)

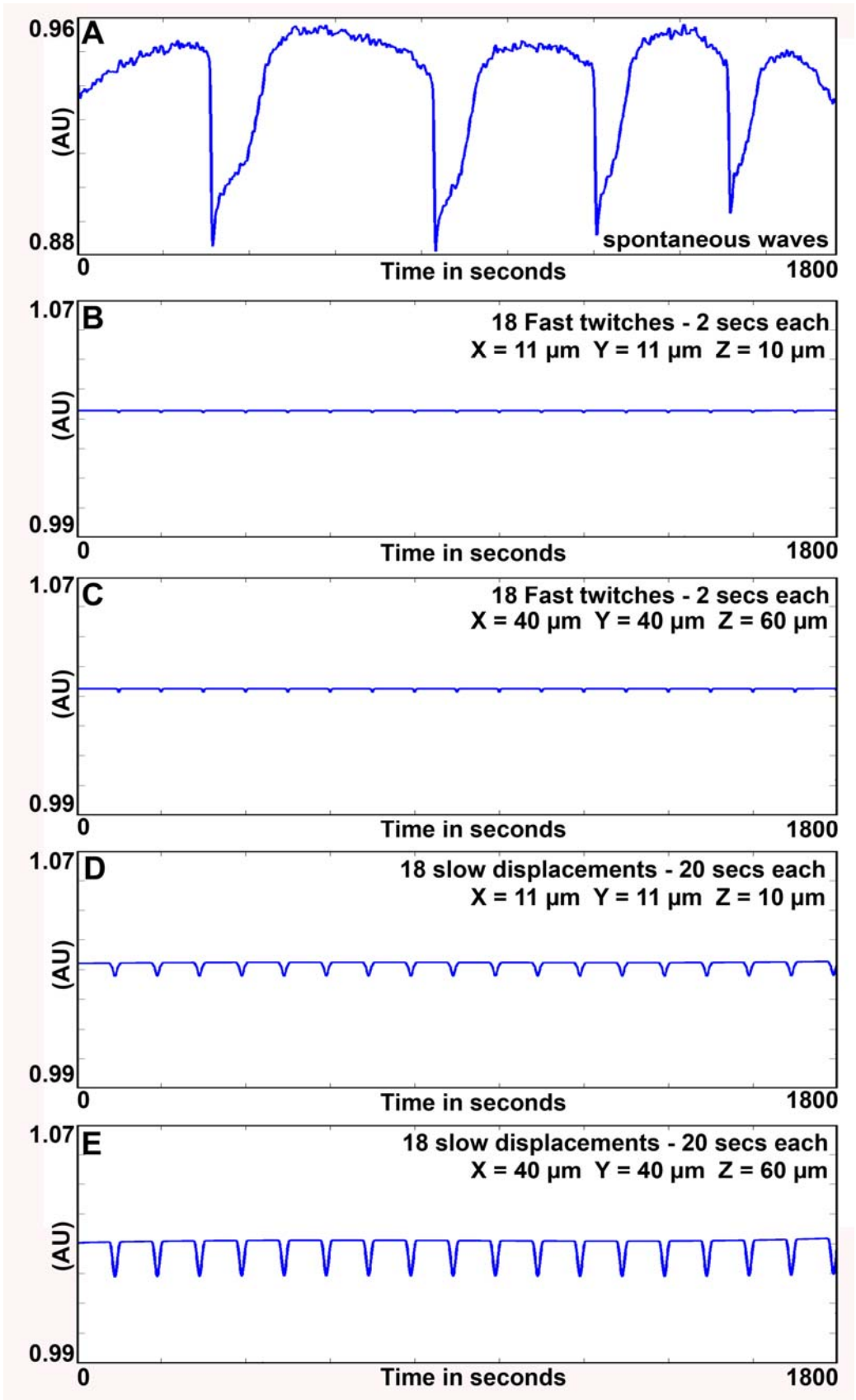
A
0.96
0.88
(AU)
spontaneous waves
0
Time in seconds
1800
B
1.07
0.99
(AU)
18 Fast twitches - 2 secs each
X = 11 µm Y = 11 µm Z = 10 µm
0
Time in seconds
1800
C
1.07
0.99
(AU)
18 Fast twitches - 2 secs each
X = 40 µm Y = 40 µm Z = 60 µm
0
Time in seconds
1800
D
1.07
0.99
(AU)
18 slow displacements - 20 secs each
X = 11 µm Y = 11 µm Z = 10 µm
0
Time in seconds
1800
E
1.07
0.99
(AU)
18 slow displacements - 20 secs each
X = 40 µm Y = 40 µm Z = 60 µm
0
Time in seconds
1800

Fig.7. Comparison of the changes in YFP/CFP ratio obtained from simulation of motion with the change in ratio from actual calcium changes. Note that the range of the Y – axis in all the plots are comparable to each other A) Changes in YFP/CFP ratio over 1800 seconds due to spontaneous calcium waves, obtained after SOARS analysis. B) Changes in YFP/CFP ratio over 1800 seconds due to a quick twitch like (simulated) motion (2 seconds) with a displacement of about 10 microns in X, Y and Z directions every 100 seconds C) Changes in YFP/CFP ratio over 1800 seconds due to a quick twitch like (simulated) motion (2 seconds) with a displacement of about 35 microns in X, Y direction and 60 microns in the Z direction every 100 seconds D) Changes in YFP/CFP ratio over 1800 seconds due to a slow (simulated) displacement (20 seconds) of about 10 microns in X, Y and Z directions every 100 seconds E) Changes in YFP/CFP ratio over 1800 seconds due to a slow (simulated) displacement (20 seconds) of about 35 microns in X, Y directions and 60 microns in the Z direction every 100 seconds

From the simulation results, it is clear that even with severe motion of the imaging sample the change in YFP/CFP ratio is not comparable to the large change that happens during a spontaneous calcium wave. We can confidently interpret the calcium events that we observe to be true signals.

## DISCUSSION:

### SOARS AND ITS IMPORTANCE IN IMAGING CALCIUM WAVES

Statistical Optimization Analysis for Ratiometric Signals [SOARS] is a technique for statistically optimized analysis of ratiometric data, which offers significant

advantages and improvements over prevailing imaging and analysis techniques. This method has been demonstrated to be capable of reading out signals from very noisy data (with SNR at 1/16 or poorer) while retaining much of the spatial and temporal information [Broder et al., 2007]. SOARS takes advantage of the fact that in the physiological context, meaningful signals are usually spatially correlated. It detects spatially correlated and temporally anti-correlated signals by applying multivariate statistical optimization methods to the full covariance matrix of the data [Sirovich et al., 2002; Yokoo et al., 2001; Sornborger et al., 2005].

Calcium signals in ratiometric data are represented as anti-correlations in the data from the two channels (donor and acceptor). Binding of calcium to calmodulin, causes a conformation change and brings CFP and YFP closer. The resulting increase in FRET between YFP and CFP, causes a reduction in emission of CFP (as the energy is transferred to YFP instead of radiation) and an increase in the emission of YFP (additional increase in emission due to increase in FRET). Thus a rise in the level of intercellular calcium causes a rise in YFP emission and a fall in CFP emission, which results in a characteristic anti-correlation in the dual channel data [Miyawaki et al., 2003].

The problem of signal detection in this context can thus be stated as that of finding a subspace of the datasets from each channel that has maximum temporally anti-correlated and spatially correlated information. SOARS accomplishes this objective by using multivariate statistical optimization. The maximally anti-correlated subspace of the data is first estimated and then the dual-channel measurement is *de-noised* by projecting the dataset into the subspace. The technique thus filters the data in a space

of anti-correlated eigenimages, by choosing the statistically significant eigenimages from the data. Averaging pixels (ROI) or filtering temporally (Boxcar filters) and their associated limitations are thus overcome and high resolution spatial and temporal information is retained in the estimated ratio by using multiple statistically significant eigenimages [Broder et al., 2007].

The important advantageous feature of SOARS that facilitates detecting calcium waves is that the masking in the dataset is user-independent, i.e. the anti-correlated subspace is estimated objectively without any user bias. This is particularly important in studying propagating calcium waves because a user defined mask might mask out regions of importance that could be participating in wave propagation and in noisy data, regions with a poor signal might go undetected by the user. By determining anti-correlation based weighted masks, instead of user defined ROIs, SOARS retains detailed spatial information, even in very noisy datasets.

## OTHER APPROACHES TO IMAGING CALCIUM WAVES IN ZEBRAFISH

One of the most distinguishing aspects of the zebrafish is that it is transparent through much of its early development. This combined with the fact that it is a vertebrate, makes it a particularly favorable model organism for optical methods of investigation. Several techniques have been employed for imaging intracellular calcium and each has its own pros and cons. Bulk loading of cells in culture with synthetic calcium indicator dyes [Takahashi et al., 1999] has been promising and has been effective in the measurement of cellular calcium levels [Broder et al., 2007]. This technique has been extended for *in vivo* imaging of calcium by backfilling specific

neurons with a suitable calcium dye [Fetcho et al., 2004] or by injecting indicators during the blastomere stage of development of the fish [Cox et al., 1996];

Backfilling of neurons with calcium indicators serves as a poor option because this method is invasive and has poor spatial resolution for measuring large scale intercellular calcium waves. Injecting suitable calcium indicators in the blastomere stage during the development of the fish, has been a successful technique in reporting calcium waves in the embryonic stages [Gilland et al., 1999] but is not very effective in the later stages of development, as the labeling is weak and not all cells are identifiable [Fetcho et al., 2004]. This has limited the imaging of calcium waves to the early embryonic stages of development.

Transgenic lines of zebrafish, expressing GFP based proteins, whose signal output changes with cellular calcium concentration, provides an elegant alternative to injecting calcium sensitive dyes. Unique genetic lines have been developed that have a pan-neuronal expression of calcium indicators [Miyawaki et al., 1997] and have been successfully used in producing detectable calcium signals [Higashijima et al., 2003]. Another important and distinguishing advantage of using transgenic lines of zebrafish is that pan-neuronal expression of a calcium indicator provides the essential nervous system wide distribution of the calcium indicator that is needed for studying large scale multicellular neuronal events.

While single wavelength calcium indicators have been popular, changes in laser intensity or bleaching can result in artifacts. Since not all regions in the imaging region have the same amount of indicator, a change in brightness can either be due to change in calcium level or intensity or bleaching artifact. FRET, is a technique that offers a

solution to this problem, as the output consists of two emissions, which can be ratioed to eliminate the above mentioned artifacts [Miyawaki et al., 1997]. It has been successfully utilized for imaging and studying calcium dynamics [Higashijima et al., 2003]. Cameleon, which serves as a FRET indicator is inherently ratiometric, and is less sensitive to these artifacts [Miyawaki, 2005]. These properties make cameleon expressing zebrafish an ideal choice for FRET based calcium imaging *in vivo*.

Although, YC2.1 in transgenic zebrafish was successful in showing measurable stimulus-dependent response in Rohon-Beard neurons, the signal detection required extensive spatial averaging of the fluorescence output before subjecting it to ratiometric analysis [Higashijima et al., 2003]. Whilst spatial averaging is a popular technique for signal detection, it calls for the use of ROI's (regions of interest). Although the use of ROIs may detect a response, important spatial information is lost, as the mask defined has to be static. Spatial and temporal information is of significant importance in studying propagating calcium waves in neurons and sacrificing this information would impair our ability to interpret data for detecting waves. Further, a predefined mask, if chosen would be a limitation, as we do not know beforehand which regions of brains participate in wave propagation. SOARS on the other hand determines the spatial features of the wave and provides an elegant solution to the limitation of using predefined masks.

Averaging temporally is another method for detecting signal [Russ et al., 2006]. This is accomplished by typically using a low-pass filter, however too much averaging can over-smooth the data and blur out fast dynamics that might of interest, particularly in the context of propagating waves. This limitation thus restricts the amount of temporal

averaging that can be done, and hence cannot be effective in datasets with high noise content (effective only when $\Delta F/FB_{0B}$= 0.4 or more) [Cheng et al., 1999].

Thus, choosing injection methods for studying calcium waves have problems with distribution of the dye and invasiveness whereas choosing transgenic lines of zebrafish expressing FRET based indicators suffer from insufficient analysis techniques. SOARS, as an analysis technique coupled with the availability of transgenic zebrafish expressing FRET based calcium indicators overcomes all the problems and limitations mentioned above and successfully detects propagating waves of calcium in the developing brain and also retains high spatial and temporal information.

## MATERIALS AND METHODS

### FISH SAMPLE PREPARATION:

Zebrafish larvae were imaged at 5 days post fertilization. The cameleon expressing larva to be imaged was first anesthetized using 40 µg/ml of Tricaine (3-amino benzoic acid ethyl ester, Sigma Cat.# A – 5040). The larva was then embedded in 3% low melting agarose, containing 40 µg/ml of Tricaine, over a coverglass. The coverglass was finally incorporated into a Dvorak-Stottler perfusion chamber.

### CONFOCAL MICROSCOPE:

An upright LSM 510 Meta (Carl Zeiss Inc.) was the confocal microscope used for imaging. The 458 nm line of a 50 mW argon laser was used for excitation, with emission bands centered on 485 (cyan) and 535 (yellow) nm, respectively.

IMAGING:

Egg Water was perfused into the Dvorak-Stottler chamber at a rate of 8.3 µl/sec. Images were collected at a rate of 1/second. The zebrafish was imaged for 30 minutes.

SOARS ANALYSIS:

The dual channel images (CFP and YFP) were first binned down to 128 X 128 pixels and converted to raw images. The raw files were then subject to SOARS analysis. SOARS first standardizes the data from the two channels and estimates their difference. It then utilizes the full covariance matrix to find orthogonal weighted masks (eigenimages) that are statistically significant (and are estimators of anti-correlated activity in the two-channel data). The eigen mages thus found, were then used to denoise the data in both channels. A ratio was then obtained from the denoised data, which gives an estimate of calcium activity. The output from SOARS provided the statistically significant eigenimages, the ratio estimated from the denoised datasets and the time-course of CFP and YFP from their respective denoised channels. Calcium activity was also represented as characteristic anti-correlations in these time-courses (see Fig.3. and Fig.6.).

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