TRACKING of INTRACELLULAR CALCIUM SIGNALS for SEGREGATION of SINGLE-CELLS

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and have found that it is complete and satisfactory in all respects,
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ABSTRACT

The modulation of calcium concentration and rate of elimination in the cytoplasma is a crucial intracellular mechanism that control different cellular process, including gene expression, proliferation, differentiation and energy metabolism. Spikes, waves and continuous oscillation deliver information throughout the cell by frequency and amplitude changes or may adversely affect other functions if modes are altered by external stimuli. Despite the detailed studies and an insight into the mechanism of calcium elevation, elucidating temporal patterning of signals, small number of dynamic imaging and intrinsic noise remain elusive and limits the development of reliable models. Measuring the metabolic changes at single-cell resolution plays a key role to understand the heterogeneous signal propagation in tissues. Hovewer, ensemble measurements mask the activity of single-cells; fast tracking methods are needed to resolve the dynamics at spatiotemporal resolution. Single-cell studies of calcium signaling and tracing can also decipher its association with downstream events. In this thesis, we have used calcium tracking methods to monitor real-time changes, and used statistical methods to elucidate the heterogenous signal formation. Genetically encoded reporters derived from permutated GFP were expressed in different cells and used as a probe to measure calcium waves mediated by various hydroxytrytamine compunds. To determine rare cells, calcium traces from single-cells was analyzed by a linear transformation. The coefficients of principal components with largest eigenvalues were used to replot single-cell with new orthonormal coordinates. We have determined that cells were segregated differently based on initial rate and magnitude of calcium changes, therefore isolated rare and unresponsive cells. After preprocessing, single-cells were selectively sorted by various clustering methods. We found that calcium signal modulated by serotonin and histamine can be divided into two states depending on falling and raising patterns and the length of oscillation. The coefficients were also tested to determine the state of signals that had various signal-to-noise ratios. Our study suggests that dynamic measurements of calcim signaling and fast segregation can be used to cluster calcium patterns at single-cell level. We envisioned that quantitative imaging can reveal spatiotemporal dynamics of metabolic states, therefore establish an alternative method to study calcium signal in single-cells.

ÖZET

Metabolik değişimlerin tek hücre düzeyindeki çözünürlüğün ölçülmesi, hücreler arasındaki sinyal oluşumu çeşitliliğinin anlaşılmasında büyük rol oynamaktadır. Kalsiyum sinyallerinin, çekirdekli hücrelerde oluşan bir çok esas metabolik olayda yer alması yaygın bir mekanizmadır. Bu sinyallerin salınımı ve dalgalanması epithel hücrelerdeki ve sinir hücrelerindeki bilgi aktarımı için temel bir olaydır ya da eğer dışarıdan uygulanan zehirli maddeler bahsedilen kalsiyum sinyalinin salınımını ve dalgalanmanın frekansını ya da büyüklüğünü değiştirirlerse ciddi şekilde hücre işlevini etkiler. Zaman odaklı çözünürlüğün tek bir hücrede gözlenmesi, anlık değişimlerin gözlemlenebildiği çözünürlükte sinyalleri sınıflandırmak icin kullanılan bilgisayar programlarının eksikliği ve bu tür değişimlerin takibindeki eksiklikler yüzünden çalışılması zor konular arasındadır. Hücrelerin metabolik aktivitesindeki gözlenmesi zor değişimleri ve hücrelerin mikro-çevrelerindeki değişimler sebebiyle oluşan gürültüleri bir takım anormal sinyal oluşumlarına sebep olmaktadır. Artık hücrelerin farklı metabolik aktiviteleri analiz teknolojisindeki gelişmelerle birbirinden ayırt edilebilir hala gelmiştir. Hücre dışındaki kalsiyum sinyallerinin iletimi detaylı olarak çalışılmasına rağmen,tek hücre düzeyindeki iletişim hala açıklanamamış bir konudur. Toplu ölçümlerin tek hücre düzeyindeki aktiviteleri gösteren sinyalleri örtmesinden ötürü, hücre dinamiklerinin anlık değişimlerin gözlemlenebildiği çözünürlüğü sağlayacak olan yeni tekniklere ihityac duyulmaktadır. Bu tezde kalsiyum sinyallerinin tek hücre düzeyinde gözlemlenmesi için geliştirilmiş bilgisayar ve deneysel tekniklere odaklanıldı. Aynı zamanda heterojen sinyal oluşumlarını statistiksel olarak açıklayan bir teknik geliştirildi. Bu tezde, kalsiyum sinyallerinin kullanımı açıklandı ve kalsiyum sıralamasına karar vermek için temel bileşenler analizi kullanıldı. Hücre durumunu tespit etmek için hücre morfolojisi ve boyama teknikleri geçerli olsa da, metabolik sinyallerin tek hücre düzeyinde ölçülmesi hücresel değişimleri daha doğru betimler, sonuç olarak sinir hücreleri gibi tek hücre düzeyde bir çok farklı işlev yürüten çok önemli yerlerde hızlı ve güvenilir bir metod geliştirilir

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NOMENCLATURE

GCamP	Green fluorescence-Calcium modulated protein
GECI	Genetically encoded calcium indicator
GFP	Green fluorescence protein
FRET	Fluorescence resonance energy transfer
PCA	Principal compenent analysis
SOC	Store operated channel

Greek Symbols

ν	orthonormal eigenvectors
λ	eigenvalues

Chapter 1

INTRODUCTION

The first study that revealed the physical shape of cells was conducted in the late 1880s. Simply staining of golgi organelle made the cellular connections visible by labelling the individual cells. Development of microscope techniques help researchers to study cells in detail and even control their functions by small molecules, electrophysiological methods and recently lasers targeting light sensitive proteins. In 1971, Richard Fork at Bell laboratories used an intense beam of light to activate the action potential in the ganglion cell of *Aplysia* (moolusca). Fork failed because the application disrupted the cell membrane as well as the integrity of the cell [1]. The dream of controlling cellular function by external methods has continued in the last two decades until less invasive methods have invented by expressing light sensitive proteins called rhodopsin in neurons that conduct small ions upon activated by low intensity laser beam. Another example is photo labile calcium chelates which come from the idea of using light sensitive groups such as photo uncaging of ATP. Therefore, recent studies provide such techniques for photo stimulation of cells with these kinds of molecules.

Techniques that mostly used for photo stimulation can be categorized as Feng Zhang proposed. They have suggested three approaches: photo stimulating of chemically modified signaling molecules (with help of light, uncage the signaling molecule); chemical modification of ion channels or receptors to make them responsive towards light (i.e. retinal in Rhodopsin); introduce light-sensitive proteins into light insensitive cells (i.e. using E.coli which has channel rhodopsin protein) [2]. As a third approach, mammalian expressed rhodopsin protein is a good candidate for monitoring electrical signal through cell membrane because while activating and inhibiting the cells, their electrical signals can be monitored by such genetically encoded fluorescent reporters [3]. Recently, achievement of manipulating neural activity by optically switchable manner with fluorescent protein reporters was awarded the Brain Prize in 2013 by the European Neuroscience Society [4]. This achievement shows rapid development of the technique that used light sensitive protein reporters for controlling cells within last few

years. As well as controlling cellular function of cell population is achieved; parameters of controlling single-cell among the population still remain elusive. Earlier studies demonstrated that single-cell play a key role for affecting various functions in tissues. Therefore, mechanisms that regulates cellular activity can give a clue to understand function of single-cell. Moreover, the failure of current investigations to control the cell invasion in tissues results from the limitation of single-cell analysis approaches to reveal the heterogeous population of cells. Therefore, the specific aims in this project are to be demonstrated the overall goal which were to develop sensitive probes to control membrane potential and revealed the heterogenity in cells using statictical approaches.

In Chapter 2, we described the current issues on studying the single-cell functions. The significance of single-cell analysis methods and its advantegous was also given in detail which includes the brief overview of biophysical techniques used previously to achieve a high spatio-temporal resolution. These methods have been used to study various cellular functions including calcium imaging, controlling action potential of cell and tracking fluorescence reports in living cells. Developing sensitive probes to control membrane potential and revealing the heterogenous calcium signaling in cells using statictical approaches are described as ultimate goals of project.

In Chapter 3, experimental procedures of single cell calcium tracking experiments were described. Statistical analysis for single cell tracking which is main motivation of this thesis was depicted. We presented experimental results of calcium signaling changes upon addition several important chemical situmulant including histamine, acetycholine and serotonine. Then, time-lapse videos were described which were used to measure the calcium signal changes in individual cells. As a result, cells, which are responsive to these chemicals in the same population, are shown by using statistical methods to reveal unknown connection between them.

In Chapter 4, this thesis is concluded with summary of work and overview for future work that will be conducted to answer single cell heterogeneity in response to calcium signaling.

Chapter 2

LITERATURE REVIEW

2.1. Single Cell Studies

In living systems, variation of cellular response can be associated with non-genetic factors, where the cells are stimulated with numerous small molecules, hormones and peptides. The interaction between extracellualar cues and membrane proteins induce dramatic physiological changes in cells that includes differentiation, tissue formation or immune response. Most of the drugs and theraupautic reagents tigger signaling pathways in population of cells, hovewer heterogeneous singal formation was usually observed. Most of the bulk measurements provide an average measurement of response and do not provide single cell information (Fig.2.1). Ensemble and bulk measurments of cells inherently mask the individual cells and cell-to-cell variations, therefore signalling cannot be distinguished by convetional methods. Current methods are specifically focused on monitoring the spatiotemporal imaging of single-cells in the presence of exogenous factors, therefore manipulating them and measure the singal changes. Development of imaging technologies or genetic tools provides a precise spatia and temporal control of signaling and monitoring changes at living cells. Additionally, content of cellular environment is the major factor that effects response of a cell. Therefore, population of cell shows different response due to minor difference in their microenvironments. These responses can be listed as changes in membrane potential and ion channel activities so numbers of ions and signaling molecules act as a messenger to direct cellular activities. For that reason regulation of messengers in the environment is a method to control single cell activity within a population of cell. On the other hand, one of the most common experimental methods, western blot, fails to detect protein localization within the cell or possess any individual cell variability for subjected biomolecules in genetically homogenous population [5]. Additionally, technologies such as flow cytometry detecting single cell at a time can be also insufficient to observe single cell response for continuous activity within many experimental repeats so variability of single cell within a clonal population in the same conditions remains as a challenge to report [6]. Flow method has strong capability of

separating individual cells but a limited use for monitoring of cells due to its low temporal resolution where cells are usually followed at a single time point. Therefore there is limited monitoring of single-cell results in poor characterization of gene expression variations in literature. Such bulk measurements provide average value for a population of cell so these kinds of molecular biology methods only deliver information for mixture of proteins or average response of population. Moreover, conventional bulk measurements provide a single mean value but not statistically meaningful data for expression of protein at single cell level [5]. On the other hand, microscopy imaging techniques can be used for recording of many changes in cells. For example, different fluorescence intensity of individual cell shows variability within the same population of cell. As a result, researchers can detect single cell and also have more reliable quantitative data for protein expression, signaling changes, ions levels at single-cell level [5].

2.2.1. Source of Cellular Heterogenity

Source of non-genetic heterogeneity can be listed as intrinsic and extrinsic factors which effect gene expression and cellular metabolism, respectively. Cells result from identical parents may have different response to external factors. Stochastic noise and cell variation has been studied previously to understand the role of these changes. Cellular heterogeneity is a crucial factor to increase the survival rate of cells. However, the noise in signaling pathways would have an adverse affects in cell populations. Since it is an essential process and increases the resistance of cells, it is crucial to study and characterize the mechanism at a single-cell levels where the inherent noise can be detected. Bacterial resistance, failure of cancer drugs are examples of existing of cell heterogeneity. Intrinsic factors related with the gene expression level of regulatory proteins such as polymerases and their concentration creates stochasticity for the activity of the protein. Therefore, metabolism of the cell varies depending on the amount of the protein. There is a loop between intrinsic and extrincis factors that feed each other while regulating cell function [6].

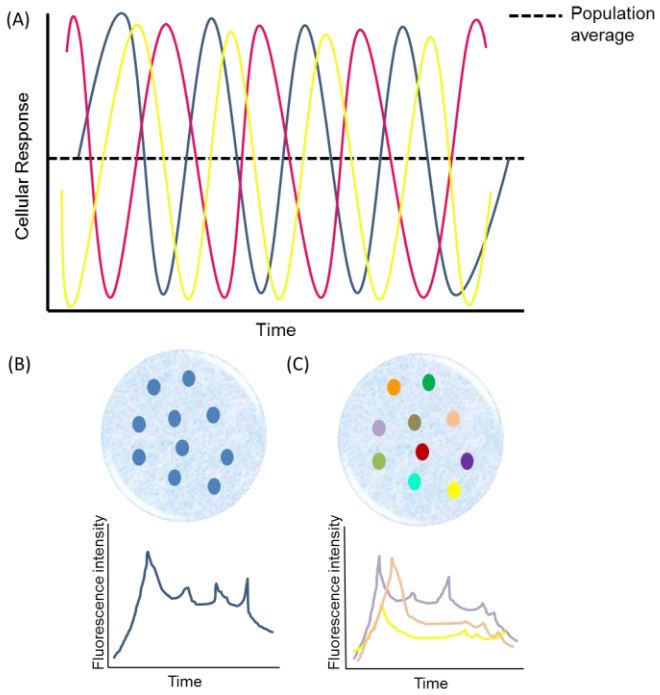

Figure 2-1 Schematic representations of single cell variabilities. (A) Each color represents a different cell from the population. Each cell has characteristic frequency and response but average value is not able to provide information about single cell difference. (B) At first, each cell is supposed to be the same and fluorescently tagged signals show the same response to their physical microenviroment but (C) shows how each cell differs from each other. (Adapted from Spiller D.,G. 2011[7])

There are many studies to address the importance of single cell analysis and data collection. For example, calcium mediated ATP efflux is not homogenous in cytosol of both excitable and non excitable cells. ATP released from a point, finite source and dissapiated through cell and not suprizingly, each cell has different fluorescence intensity value during dissapiation of ATP [8]. This observation only revealed by single cell based imaging. Another example is epithelial cell population which is subjected to agonist or scratching wound in order to observe calcium signal variability and to find a source of this variability in seemingly homogenous cell population. Agonist factor was used as an intrinsic source of variation and scratching wound was considered as an extrinsic factor. According to algorithm to understand variation in calcium transients, cells locating nearby the wound give common response as sub-population, which can be the reason for the source of variation [9]. Therefore, suitable algorithms for single cell analysis provide valuable information about the variation within a population. Although

source of variation can caused by interaction of cells with their physical environment and with each other, there are some evidence that shows it is not the case all the time. For example, difference in calcium response of astrocytes is a known fact. To test whether some downstream reagents have an influence on that variation; they use different Ca^{2+} initiating factors such as ATP, glutamate and histamine. However, they concluded that the type or concentration of these reagents did not effect this variation [10]. Additionally, in 1990s, it was reported that varied calcium response of human mesangial cells did not depend on the confluency and cell cycle stage of them [11]. As a result, there should be other parameters to cause this variation.

2.2. Ca^{2+} signalling changes in cancer cells

Cancer is a complex disease that effected by interconnected network between molecules and their receptors. Most of the cases, cancer cells are affected by their microenviroment which includes ions and signaling proteins. Additionally, some tumorigenic behaviors such as altered expression of calcium channels and pumps are obvious in some of the cancer types [12, 13]. Therefore both components of microenviroment and their effect on cells should be considered during tumorigenesis. Cells maintains an approxiamtely 100 nM cytoplasmic calcium levels while extracellular levels are 10^5 orders of magnitude higher with a range of 1-3 mM. It was observed that amplitude and rate of calcium flux remaind unchanged, however the decay rate was slow for some cancer cell lines. Modeling of calcium pathway demostrated that the cells had a different levels of membrane proteins such as channels, pumps expression that may alter the calcium signalling. For example, SOC protein levels were higher in Human glioblastoma cells, it may promote cell proliferation nad invasion.

It was reported that serotonin has an effect of tumor growth via serotonin receptors [14, 15]. Additionally, it was proposed that cell proliferation of agressive cell types is also affected by this biogenic amine [16]. Since HeLa cell line is a aggresive cervix cancer type, serotonin may also have an affect on proliferation of other agressive cancer types.

Another stimuli for calcium regulation is overexpression of proteins which cause leakeage of ER calcium stores and resulted in resistance to apoptosis. When ER calcium stores are exhausted, it couldn't provide sufficient calcium overload for mitochondria which mainly involve in induction of apoptosis[17]. Normally, calcium has a role in a way to activate apoptotic enzymes ie.caspase in mitochondria.Moreover, it plays a role in drastic increase of receptor activation which caused by high intracellular calcium concentration[17].

2.3. Calcium Regulation in Cell

Calcium dynamics of cells play a key role for regulating much physiological function in tissues. Signals encoded in calcium waves mediate various cellular reponses, including neural communication, muscle contraction, and cell differention. Understanding the spatio-temporal dynamics of calcium waves may elucidate the coordinated response of cells. Despite the detailed studied of intracellular studies of calcium signaling, intercellular communication remains elusive. Wave signals were found in variety of different cells. Abnormal signal formation may activate important regulatory pathways that lead to cells misregulate differentiation, proliferation and carcinogenesis. Calcium regulates intercellular communication through gap junction. Cell surface receptors activated by exposure to numerous neurotramissters and factors are essential for amplifying signals thought the membrane regulated by dynamic changes in activation rate of receptors on the cell membrane. It has been challenging to directly monitor the calcium level rise at a single-cell resolution and associate it for a given physiological function in living tissues [11]. Calcium concentration is tightly regulated within the cell. Ca^{2+} pumps, channels and exchangers are main components to regulate the concentration. Ca^{2+} pumps use ATP to work against gradient whereas channels facilitate the diffusion through high to low concentration. Ca^{2+} exchangers, use additional ions such as Na^{+} to take calcium inside the intracellular matrix. Intracellular concentration of calcium is nearly 100nm; however, extracellular concentration is around 1.2mM scales [18]. This gradient makes the movement of calcium faster than other molecules and, it plays a main role in signal transduction by altering shape and charge of functional proteins. Why calcium ion is so special? First reason is that Ca^{2+} is

fairly reactive with water compared to other cations, Mg^{2+}. As it is known that cytosol has high content of water, cells keep its concentration low in order to avoid hazardous interactions. Secondly, Ca^{2+} is not chemically altered; therefore, when cells control cellular activity over Ca^{2+}, adaptations such as compartmentalization of organelles or chelating molecules contribute signaling process. Moreover, large calcium affinity ranges over a million-fold (nM to mM) causes adaptation for various cellular proteins.

Calcium regulation is facilitated by various proteins. These are calcium channels in plasma membrane and endo (sarco) plasmic reticulum; accessory proteins such as CaM; pumps (ATPases, SERCA) in plasma membrane and ER; and Na^{+} /Ca^{2+} exchangers (NCX) (Fig.2-5) [19].

Interestingly, oscillations during calcium signaling provide an advantageous to protect cell from deleterious effects of sudden calcium influx [20].Moreover, some of the transcription factors (NFAT) are increase in activity from the extent and duration of these oscillations rather than constant open form of calcium channels in plasma membrane.

Calcium regulation in cell can also be manipulated by some biogenic amines or neurotransmitters. Some of these chemicals are acetycholine, histamine and serotonin. For example, histamine has an important role in inflammatory response. Especially, histamine release of mast cell cause immune activation in cell through histamine receptors on membrane. And it was known that every cell type express at least one type of histamine receptor. Therefore, increase in cyctosolic calcium is the main effect of histamine binding on histamine receptor 1(H1R), 3(H3R) and 4(H4R) by inhibiting cAMP. Other mechanism such as IP3 affects endoplasmic reticulum which is calcium store of cells to release calcium into cytosol from 100nm to 1 μM $[Ca^{2+}]_i$.

In order to maintain low $[Ca^{2+}]_i$ in cytosol, $[Ca^{2+}]_i$ s are either actively pumped from cytosol to extracellular matrix or stored at endoplamic reticulum of the cell.

Calcium release from ER is associated with mitochondrial uptake [21] of ions and this process is facilitated by ER-mitochondria joints [19] in nm scales.

Excitable cells regulates voltage dependent calcium channel; non excitable cells depend on voltage independent calcium channel while regulating calcium entry to the cell. Store operated channels (SOC) are used while depletion of calcium occur in the

cell. In depletion of calcium, cell opens SOC to take calcium from extracellular space. G coupled or kinase receptor on cell surface effects calcium influx by the release of calcium with IP3 .Production of IP3 resulted in increase in intracellular calcium.It is understood that IP3 acting on receptor/calcium channel in the ER, so make them open to release calcium in its store. Depletion of ER store effects store operated channels (SOC) with an unknown mechanisms, than the SOC opens to take up calcium from extracellular space (Fig.2-3) [21]. According to "capacitative calcium entry "hypothesis, ER as a capacitor was depleted its store and then sends a signal to the channels to take up calcium in order to refill its store [22].

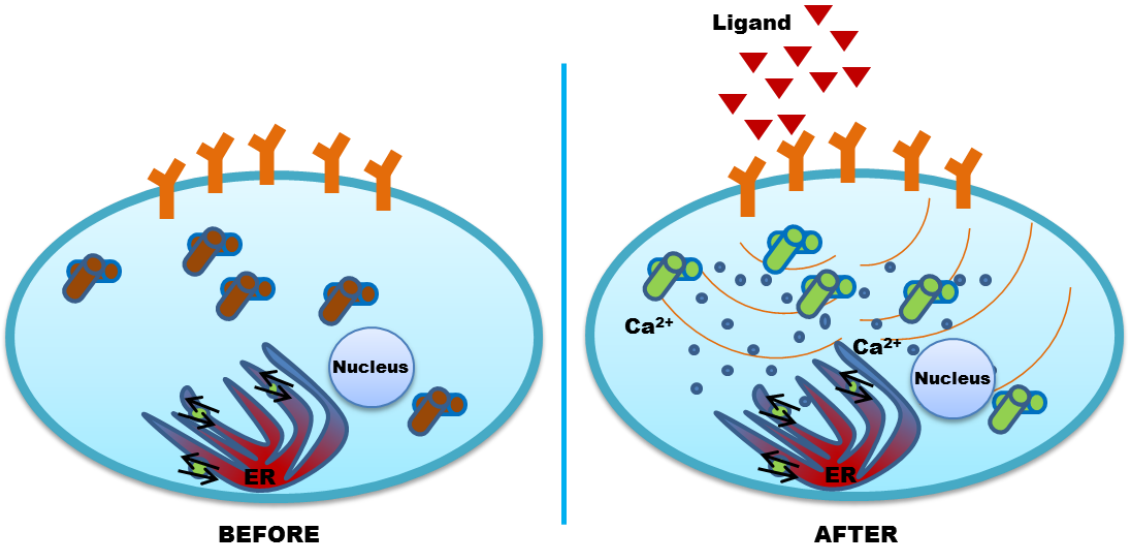

Figure 2-2 Schematic depiction of calcium indicator activity. GCamP (orange) has a low fluorescence intensity at small calcium levels (50-100 nM). External stimuli activates GPCR receptors on cell membrane (yellow). The dissociation of messenger ligand from GPCR receptor occurs quickly and diffuses in the cytoplasm. The binding of messenger to receptors (Ex, IP_3R) on ER opens the channel. As calcium levels increase, the fluorescence intensity of GCamP becomes brighter. Since the calcium and CaM has a strong affinity (Kd, ~0.5-1.0 μM), small increase of calcium results in strong shift in equilibrium. After CaM con formation changes binds to M13 that induces a strong change in GFP emission.

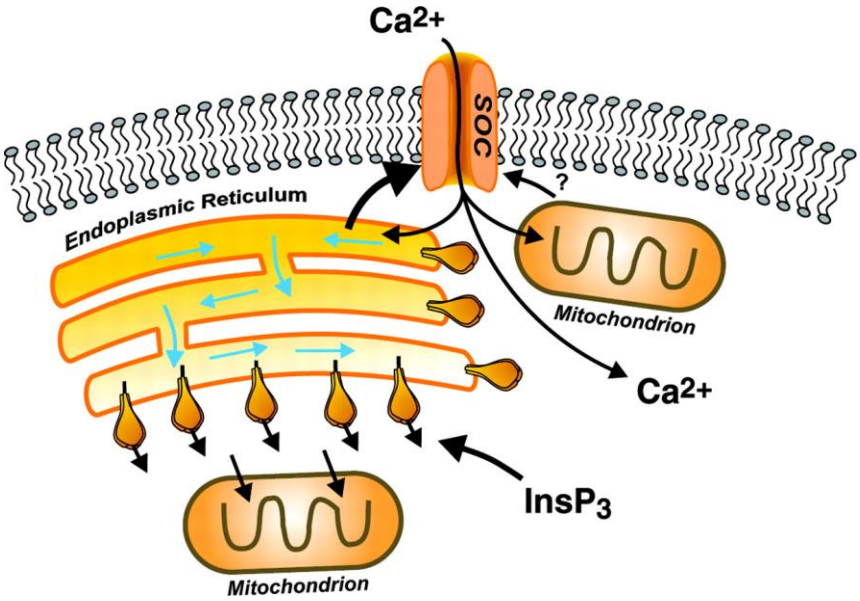

Figure 2-3 Store operated channel model for calcium uptake. (Retrieved from Anant, B. 2005[23])

External stimulants evokes in rapid raise of cytoplasmic calcium in cells. The binding of activator to G protein couple receptor result in increased levels of inositol 1,4,5-triphophate (IP_3) or ryanodine messenger activation that is produced by the cleavage of PIP_2 into IP_3 and DAG. IP_3 then binds to IP_3 receptor (IP_3R) that is a ligand gated calcium channel. It mediated the rapid increase of calcium in cells. Calcium induced calcium release meachanism was also described in previous studies [17]. However, cells cannot maintain high levels of calcium due to its toxicity. Thereore, If calcium levels exceed the threshold values in cells, calcium ions are either separated in cellular organnels or secreted to the extracellular space.

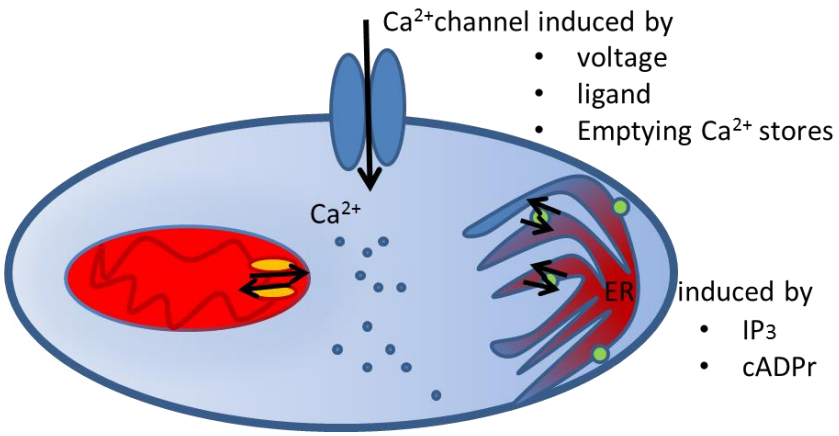

Figure 2-4 Schematic of calcium regulation in cell. Calcium uptake of cell is facilitated by pumps and channels. In plasma membrane, Ca^{2+} channels are gated by intra or extracellular stimuli: emptying calcium ion stores, voltage or ligands. Endoplasmic reticulum are activated by second messanger IP_3 or cyclic ADP ribose[24]. Mitochondria is close contact with calcium regulation and apoptosis with Na/Ca exchanger.

2.4. Mechanism of GCAMP

The emergence of new optical and genetic methods is important for enabling calcium measuremtns in living cells. The development of calcium sensitive indicators such as fura2 and genetically encoded indicators provide sensitivity for measuring cytoplasmic calcium levels. By suing these tools, the changes in calcium levels was measured in different cellsGreen fluorescent protein- Calmodulin (CaM- calcium modulated protein) fusion protein (GCaMP) sensor has circularly permuted enhanced GFP attached to calmodulin and calmodulin peptide binding site M13pep (Fig.2-1) [25]. First GCaMP sensor was dim and has poor folding properties at 37 ^{0}C which is disadvantages for using it in neurons. After multiple and randomized mutation at domain interface of GCAMP, resultant protein was GCAMP2. Eventhough GCAMP2 was extensively characterized in mouse brain cell, poor signal to noise ratio still not make GCAMP2 desirable for reproducible detection of single action potential in those cells [26].

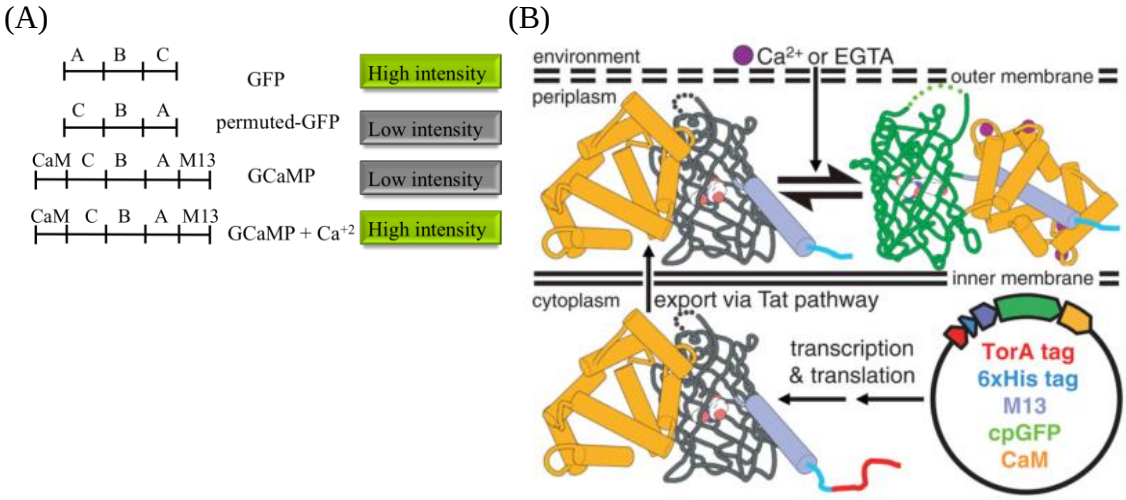

Figure 2-5 Genetically encoded fluorescent sensors. (A) Permutated GFP, low fluorescence intensity, can be prepared by recombining the domains of GFP. Calmodulin and M13 was enoded to the N and C terminus of the permutated GFP. Intensity of GCaMP can be modulated by calcium levels in cells. (B) Schematic depiction of GcamP expresein and translocation in *E.coli*. Strong binding of calcium ions to Calmodulin recruits M13, therefore induces a conformation change that evokes increased fluorescence intensity. (Retrieved from Zhao, Y, 2011 [27])

There are two kinds of GECI according to number of fluorescence protein they have. These two calcium based reporter emmit fluorescence in a different way. First type includes two fluorescent proteins which are donor and acceptor. In this class, signals are facilitated between pairs by FRET and provide emission depending on the orientation of two fluorophores. Other type has one fluorophore whose emission or intensity varies depending on intracellular calcium ion concentration. Therefore, these kinds of GECI such as cameleon emit light in response to change in fluorescence intensity of chromophore[28].

2.4.1 GCAMP and Calcium measurements

Mechanism of Genetically encoded Calcium Indicators (GECI) bases on change in position of M13 peptide upon binding of calcium through calmodulin (CaM) binding

site of protein, and the resulting structure have green fluorescent signals. Most common drawback of GECI is low signal to noise ratio. Variants of GECI overcome that problem to enhance fluorescence signals by illumination. Until now, there are red-shifted and blue shifted fluorescent indicators rather than green one [27]. Since crystal structure of GCAMP had been described at 2009, many studies use solutions to measure calcium activity in cells by using Gcamp.

Calcium is important secondary messenger molecule due to its role in neural and muscle cells. Intracellular free calcium concentration creates polarization or depolarization of cells during creation of action potential. Voltage gated ion channels and calcium release in neurotransmitter receptors regulates signal transduction between excitable cells. Functions of calcium in those cells excites scientist to investigate mechanisms of firing of action potential in neurons. Calcium sensitive dyes as fluo-3 or 4 and proteins such as GECI are widely used for that purpose.

2.5 Principal Component Analysis

Principal Component Analysis (PCA) is a statistical tool to identify the relation of data components of a given multivariable systems by using al linear tranformation. The method takes n-dimensional data where n is the number of observations at each data set and represents it by one or two-dimensional space. The analysis methods are used extensively in signal processing and applications such as understanding the relation between gene clusters or elucidate the link of networks. In bief, PCA reduce the multidimensions of data and produces a linear communication of the original variables to generate new set of axes also known as the principal components (PC). The variance effect of the PCs, basis set of alternative space, is proportional with the magnitude of the eigenvalue. What is the contribution of the each component to the variance of the data? If eigenvalue is high for the corresponding eigenvector, positions of variables change significantly on the principal axis. For each PC, the eigenvalue of the PC effect on the variance depending on the values of the eigenvalues. They are linearly proportional. In brief, we can shuffle the variables along the PC axes where the distrbition and relation of the variables can be clearly observed; otherwise underlying

complexities of variables cannot be explained by evaluating the data and difficult to elucidate the mechanism of signallling. We see the separation of variables by using increasing the number of PC, for example PC1, PC2 etc., therefore the realtions can be identified propertly. The variables (cells) are separated at maximum strength along the principal axis. Singular value decomposcition method can be effectively used to compute the eigenvalues of the PCs. By minimizing the covariance of the principal components, the magnitude of eigenvalues (along the diagonal of the matrix) can be computed by iteration until the values are maximized in the diagonal matrix. Covariance matrix is divided into three parts. The iteration is being performed until maximize the variance values along the diagonal. After sorting of eigenvalues, PCs can be used to plot the variables. The covariance matrix of an m x n matrix, Y, can be given by,

$$Cy = \frac{1}{n-1} YY^T \qquad (1)$$

where the C is the covariance matrix and Y is the data. Covariance matrix is a symmetric n x n matrix. We can find a matrix P, therefore PX is a diagonal and covariance of its entires are sorted in a descending order. Then new variables of PY represent the linear combination of the original variables whose weigths are given by matrix P.

$$Cy = \frac{1}{n-1} (PY)(PY)^T \qquad (2)$$

$$Cy = \frac{1}{n-1} (PY)(X^T P^T) \qquad (3)$$

$$Cy = \frac{1}{n-1} P(YY^T)P^T \qquad (4)$$

Then, $Cy = \frac{1}{n-1} PAP^T$ (5) $where\ A = YY^T$

A, a symetric n x n matrix has a set of n orthonormal eigenvectors (ν_1, ν_2, ν_3,…, ν_n) and associated eigenvalues (λ_1, λ_2, λ_3,…, λ_n). A represents an orthnormal diagonal matrix

where the entries along the diagonal are the eigenvalues for corresponding principal component. Then A can be written as,

$A\,\nu_I = \lambda_I \nu_i$ where $\langle \nu_i, \nu_j \rangle = 0$ if $I \neq j$ and

If A is a symmetric matrix and U is an n x n matrix whose i^{th} column is the ith eigenvector of A. Eigenvectors of A can be sorted in an decreasing order based on the eigenvalues in matrix, D. Then A can be given by,

$$A = UDU^T \quad \text{or} \quad A = P^TDP \qquad (6)$$

Where D is the diagonal matrix (noted as covariance matrix) and $P = U^T$. SVD are handful properties for the computation of parameters. Then computation of matrix P is initiated by $A=YY^T$. Then, Eigenvectors of A is computed to build the matrix, P. Finally, Eigenvectors from matrix P can be used to compute PX where the rows of P are the principam components. Its robustness and relative simplicity makes it very practical method for correlating single-cells and identify heterogenous singal formation. It can describe how signaling in single-celll varies and very sensitive technique if small perturbations occurs in a given condition. Single-cell analysis studies increase the data acquisition rate, therefore convential analysis and sorting methods are not failing to identify different number of signal where some cells may have high amplitude changes while others may have small or no change at all. Moreover, calcium waves should be recorded for a large number of cells. We can normally represent each calcium waves as a single point, but to identify all calcium waves from single cells, each point should be represented in n-dimensioanl space where n is the total number of data points [5]. As the number of observations for a given data gets larger, dimensional space increases that requires n-dmensinal space to represent all cells. PCA, alternatively, compress the given data and generates a few principal components. It can also be used to determine the noise levels. We can elucidate the number of clusters of calcium singalling in single-cells and determine role of different modulation of signaling on cell functions [29].

Chapter 3

INTRODUCTION

Identification of rare signals in a mixed population is an important goal to study their functional role of single cells in tissues. Single-cell studies of signaling and transcriptional processes have revealed fundamental dynamic regulatory mechanisms that differ between cells in the same population. Many earlier studies confirmed that cells are unequally distributed in tissues; therefore they had different functional role in tissues, their sensitivity to other biochemical functions. Changes in a single-cell can change the state of the surroundings and active signaling pathways of other cells by the release of small molecules and hormones. Many tools have been developed to identify and isolate the single cells. Despite recent developments of using different approaches for analyzing single cells, the quantitate methods remains as a challenge. Current tools are limited by the identification of limited number of cells, due to the unavailability of scanning multiple cells in vivo [30]. To determine the changes or response or cells for external stimuli of their microenvironment, the cells should be monitored at high spatial and temporal resolution. Ultimate goal of current studies is to assess the single-cell basal metabolism and compare the changes upon exposure to external stimuli. To determine the cellular state, cells basal levels for various chemicals has to be distinguished otherwise single cell response can disappear due to the variation and high noise levels in bulk measurements. Monitoring of single-cells at spatiotemporal resolution after perturbation of intracellular signaling by chemical and physical stimuli are important for the evaluation of morphology and functional changes and understand the coordinated changes in tissues.

We hyptosized these cells can be scored based on their sensitivity against small molecules and developed an algorithm to distinquish cell based in their frequency and amplitude modes. Recent studies demonstrated that the exchange and encoding of information was determined by the frequency of calcium oscillations. We then used these scores and and cluster them. We concluded that our method provides a sensitive tool to measure for calcium singalling.

PCA, which is a well-known technique for dimensional reduction, was used to compute the principal components where cells coordinates can be represecented by a few dimensions, therefore used to distinquish them based on their calcium signal changes. Briefly, basis vectors were identified from the variance where they are sorted from largest to smallest. We did use high rank components since the magnitude of variance are high compared to other components. Our results indicated that the amplitude and shape calcium signal changes are highly variable at a single-cell level. Cells can be separated based on their response leves to various chemical stimuli. Furthermore, time-resolved changes of single-cells can be used to classify cells. An exciting application of our method is to determine the cluster structure of IP3 receptors that is one of key regulator of calcoim wave formation in cells. We can also use similar method for in-vivo studies where the pattersn and clusters can be used to role of cells in tissues.

Genetically encoded calcium reporters provide most sensitivity measurements of intracellular changes of calcium after the cells are exposed to various external stimuli. Calcium imaging is the most sensistive indicator of cell state changes and is a routine for the study of neuron function and tissue development. An increase of calcium evokes wide array of cellular process such as cell proliferation, motility and gene expression. Correlation of intracellular signal in individual cells can reveal the coordinated behaviors in tissues. The analysis of time-resolved calcium imaging in single-cells is a challenging task due to poor isolation of single-cells and unavailability of automated tools to classify cells based the shape and amplitude of calcium changes [31, 32]. Thus, the studies of the single-cell coordination remain remains elusive even though the presence of established methods to measure the calcium level at single-cells resolution.

Here we used a PCA method to determine the single cell diversity to determine the sensitivity of cells against small molecules. Computational framework for PCA analysis of calcium signaling and segmentation of cells were also explained in detail. The method integrates the computational techniques with signal tracking in living cells. First, we used single cell signal tracking method to measure the changes in calcium levels, thereby assessing the cells response to various neurotransmitters. Then, the

covariance matrix, indicating the correlation of variables, was computed to identify the principal components.

We further used the confficients from PCA for learning-based calcium signal recognition; therefore classify the cells response after exposed to various stimuli. We used the algorithm to measure the effects of neurotramitters and found that each molecules addition to cell media results in diverse signal in cells, suggesting these signal cannot be determined in bulk measurements. Altough the method presented here was developed to classify cells based on their calcium response, kinase or redox activity iof cells with other genetic reporertes can be used to measure the single-cell respond. Different pathways regulating calcium signal has been studied previously, however understanding driving force for these signals and their biological significance remains elusive. It is difficult to monitor the transmembrane state of cells, therefore difficult to understand the mechanism of calcium oscillation increase in cells and its physiological functions.

MATERIALS AND METHODS

3.1. Reagents

Histamine dihydrochloride and serotonin hydrochloride were obtained from Alfa Aesar (Germany). Acetylcholine chloride was obtained from Santa Cruz Biotechnology (Dallas, TX). 3-hydroxytyramine (dopamine) hydrochloride was purchased from TCI (Tokyo).Trizma® hydrochloride, trizma® base, sodium chloride, calcium chloride dehydrate, dimethyl sulfoxide (DMSO), dulbecco's modified eagle's medium - high glucose, fetal bovine serum, and penicillin-streptomycin, dulbecco's phosphate buffered saline with $MgCl_2$ and $CaCl_2$ (liquid, sterile-filtered, suitable for cell culture) were purchased from Sigma-Aldrich (St. Louis, MO). Opti-MEM® (reduced serum media) and Turbofect transfection reagent were obtained from Life Technologies and glycine was purchased from Fisher Scientific (Carlsbad, CA).

3.2. Preparation of Cells and Transfection

HEK293T and HELAS3 cells were used for imaging experiments. HEK293T and HeLaS3 cells were kindly provided by Tamer Onder and Nurhan Ozlu, respectively

from Koç University, Istanbul. Sigma DMEM with high glucose was supplemented with 10 % FBS and 1 % Penicillin/Streptomycin and used for regular growth of cells at 5 % CO_2 37^0C incubator. Cells were passaging each 2 days to avoid overpassaging and overgrowth. For harvesting cells, aspirate the medium and to wash unattached cell in >60% confluent plate use pre-heated +Ca +Mg PBS. 1ml of SIGMA 0.05% trypsin EDTA solution was used to detach the cells for 3-5 min at incubator and neutralized with 3 ml complete DMEM solution. Cells were collected through centrifugation for 5 min at 1400 rpm. For 6 well plates $0.2x10^6$ and for 12 well plate $0.1x10^6$ cells were counted at dilution factor (DF) 10 with hematocytometer and seeded in 2ml for 6 well plate and 1 ml for 12 well plate at calculated volume according to formula [(number of cells in 4 well)/4] xDFx10.000bcell/ml. For storage purpose, from >80% confluent plate, at least 2 vial were done. All the steps through cell collection is the same described above. After centrifugation cell pellet was resuspended into 1ml of 30%FBS 10%DMSO containing complete DMEM for each cyrotube vial and stored at -80^0C. MatTek, 35mm glass bottom plates (petri dish, 14 mm microwell, 1.5 coverglass 0.16-0.19 mm, Poly-d- lysine coated,ɤ-irradiated) was used for transfection. Each plate was transfected with 1000ng Lck_Gcamp3 and 1000ng Chr2, 14aadel and 10aadel as double transfection each had been labelled as Lck_Gcamp3+ori. ; Lck_Gcamp3+ 14aadel; and Lck_Gcamp3+10 aadel. Passage number 9, HEK293T cell plate which has over 80% confluency was passaged into these 3 plates, a day before the experiment. After 18 hours, 3 plates reached the over 60% confluency.

Transfection protocol:

HEK293T transfection:

Half an hour before transfection protocol, aspirate the medium inside the plates and add 2ml DMEM solution (includes only Dulbecco's modified eagle's medium w/o FBS and pen/strep antibiotics). In protocol, there are two kinds of tubes; first kind includes pre-heated OPTIMEM (Opti-MEM®, life tech.) + 6µl Turbofect (Thermoscientific) and second kind includes pre-heated OPTIMEM + certain amount of plasmid DNA. 6 µl turbofect is added into eppendorf tube including 150 µl Optimem for one plate. And certain amount of DNA is added into another eppendorf tube

including 150 µl optimem, we had double transfect so first add one kind of plasmid DNA and finger hit to eppendorf tube then add second kind of plasmid DNA to the optimem+dna mixture. From OPTIMEM+lipofectamine mixture, take all 156 µl mixture, and add into OPTIMEM+plasmid DNA tube and mix them finger hit then incubate them outside the hood for 5 minute. After incubation, add all the content on top of the plate in dropwise manner. Afterwards, plates were placed in incubator (37^0C, 5 CO2 %). Channel rhodopsin 2 EYPF containing plates were subject to 40uM retinal addition, one hour later transfection.

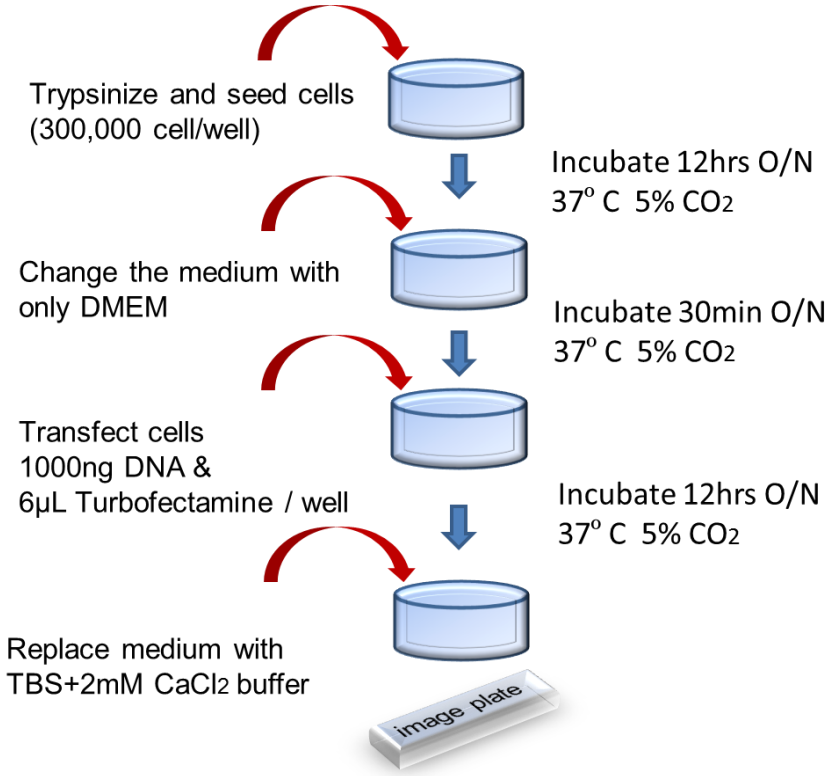

Figure 3-1 Schematic for HEK293T transfection experiments. Cells are seeded onto 35 mm regular 6 well plate and transiently transfected with GCAMP3. Transfected cells are used experimentally 12 hrs after transfection to allow appropriate expression of the introduced genetic material.

HELAS3 transfection:

12 well plates (corning, Multiple Well Cluster Plate, 12 Well, TC-Treated, Sterile) were used. >60% confluent plates were transfected with 1:3 ratios (µg DNA: µl Turbofectamine). In 100 µl pre-heated OPTIMEM Eppendorf tube, first appropriate amount of transfection reagent, than plasmid DNA were added. Mixture of transfection reagent and DNA should be incubated for 30 minutes. Afterwards, mixture was added on top of cells without changing the medium in dropwise manner. Cells were incubated in 37 ^{0}C, 5 CO_2 % for 19 hours. Channel rhodopsin 2 EYPF containing plates were subject to 40uM retinal addition, two hours before the microscopy experiments.

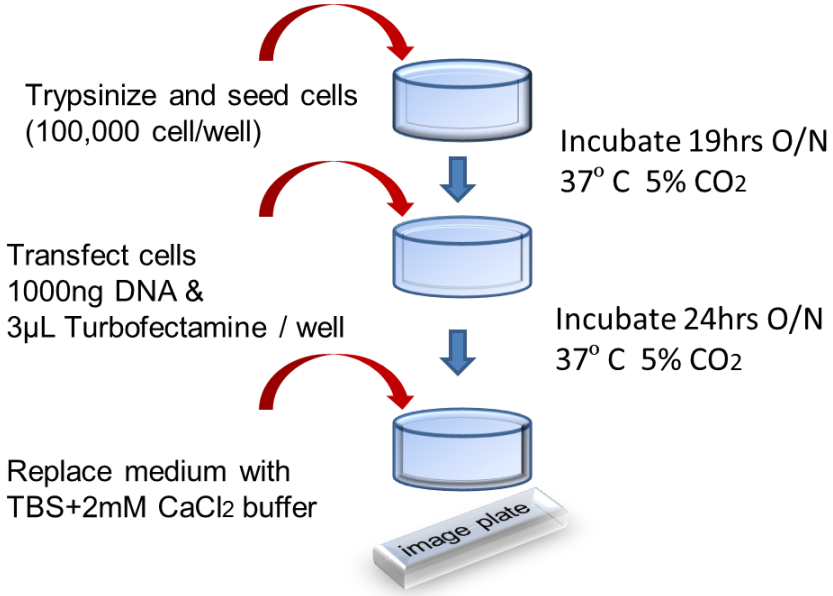

Figure 3-2 Schematic for HELAS3 transfection experiments. Cells are seeded onto regular 12 well plate and transiently transfected with GCAMP3. Transfected cells are used experimentally 24-36 hrs after transfection to allow appropriate expression of the introduced genetic material.

3.3. Fluorescent Microscopy

Nikon eclipse inverted fluorescence microscope with a 10X and 60X objective(Nikon,Tokyo), numerical aperture 0.3 and 1.4, repectively were used for fluorescence measurement.

The fluorescence images of HEK293T and HELAS3 cells were recorded by an Andor Luca S EMCCD. Images were analyzed with ImageJ (1.6.0v, Bethesda, Maryland). All experiments were performed at room temperature. Custom scripts by using MatLab (MathWorks, 2014) were used to analyze the data.

Microscopy experiments:

200 µl acetycholine from 300 µM acetycholine +1x TBS-Tris-Buffered Saline-(diluted from 10x TBS solution: including 0.152 mM TrisHCl, 0.0462mM Tris Base, 1.5mM NaCl,pH:7.6) + 2 mM $CaCl_2$ was directly added into complete DMEM containing medium. Final concentration of acetycholine was 50 µM. For 10 and 25 µM recordings were done with in DMEM HELAS3 Gcamp transfected cells. 100 µM recording was done in TBS2+mM $CaCl_2$ containing plate, complete DMEM replaced with 1xTBS+$CaCl_2$ (due to low fluorescent change in DMEM for 100 µM acetycholine). Calibration curve for histamine was done with 1xTBS2+mM $CaCl_2$ containing medium. Freshly prepeared 300µM histamine in TBS+$CaCl_2$ was used and diluted in 1ml TBS+$CaCl_2$ containing well. Calibration curve for serotonin was prepeared from 1mM serotonine solution containing 1x TBS2+mM $CaCl_2$. Final concentrations were calculated by addition of specific amounts (x µl) of serotonin into 1ml TBS+$CaCl_2$ containing well.

Experimental procedures

Aspirate DMEM and all transfection content and wash 1 times with 0.5mM $CaCl_2$ recording buffer (110mM NaCl, 5.4mM KCl, 0.8mM $MgCl_2$, 10mM HEPES, 10mM D-glucose and varied concentration of (2mM to 50mM) $CaCl_2$ at pH:7,4 w/ HCl). Place 2ml of 0.5mM $CaCl_2$ containing recording buffer and manually add varied concentration of $CaCl_2$ into the plate.

RESULTS AND DISCUSSSION

It has been known that addition of small neurotramistter molecule leads to Ca2+ influx into cells. It has been shown that Ca2+ can increase up to 1-5 uM. Recently, some studies demonstrated the asymmetric Ca2+ accumulation inside the cell. During the activation, Ca2+ rises quickly in cells, presumably through GPCR protein on the membrane. It implies that signaling events recruits essential proteins inside cells and triggers the Calcium channels in ER where the calcium are generally deposited in unstimulated cells. The measurement of calcium in individual cells allows observation of receptor activation since it would be rate-limiting step.

3.4. Calibration curve for histamine, acetycholine and serotonin:

First, we used single cell signal tracking method to measure the changes in calcium levels, thereby assessing the cells response to various neurotransmitters. Then, the covariance matrix, indicating the correlation of variables, was computed to identify the principal components. HELAS3 cells were used for transfection with Turbofect reagent in 12 well plates. 1000 ng GCAMP3 plasmid DNA was diluted into OPTIMEM with 1 μg:3 μl ratio of transfection reagent (3 μl turbofect). Complete DMEM was present during transfection protocol. Plates were incubated with transfection reagent at least 24 hours; Gcamp3 expression and activity were observed within 24-48hours.

There are calibration curve for acetylcholine, histamine and serotonin. Each data point for each chemical was collected from the same experimental batch. 100 μM acetylcholine addition showed bigger change when we compared to histamine and serotonin. On the other hand, serotonin showed relatively small effect for each concentration. This variation can be caused by varied receptor in cancer cells. Functional or unfunctional receptors can affect the ion balance in the cell and thus cancer cell had a response to those chemicals. It was known that serotonin had an effect to inducing growth of prostate cancer (PC) cells which affected by the activation of oncogene[33]. In addition, nicotinic acetylcholine receptor is also known to trigger calcium influx into cell and involving in signaling pathway of cancerous cell growth[34].

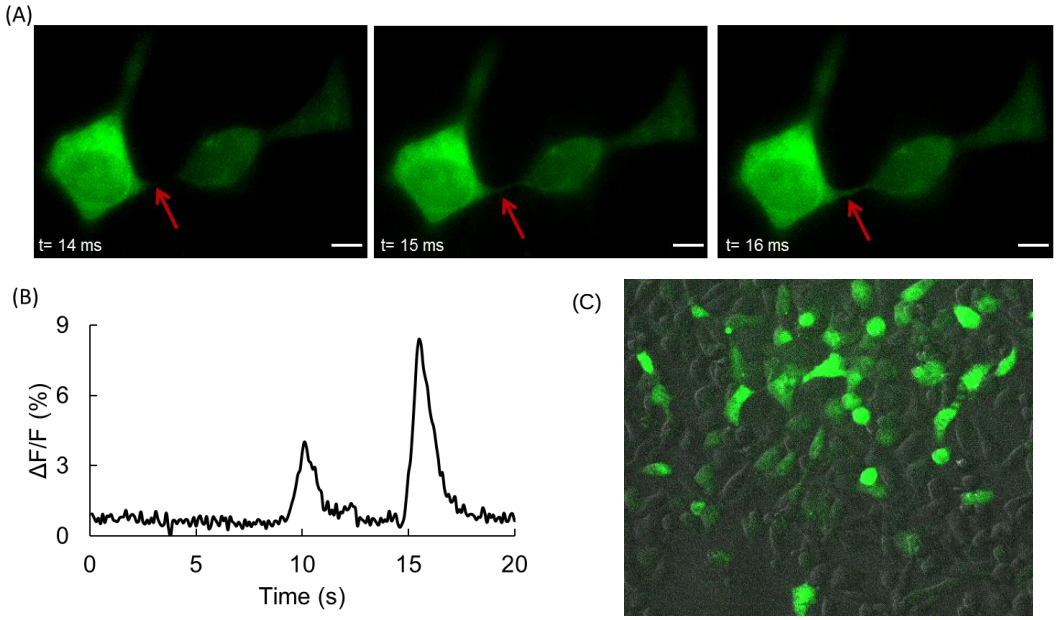

Figure 3-3 Representation of GCAMP3 expression and activity. (A) Expression of Gcamp3 GECI in HEK293T cell line. The rise of action potential was indicated (red arrows). (B) Fluorescence change of HEK293T cells was plotted versus time. Scale bar: 10 μm. (C) Gcamp expression in cells.

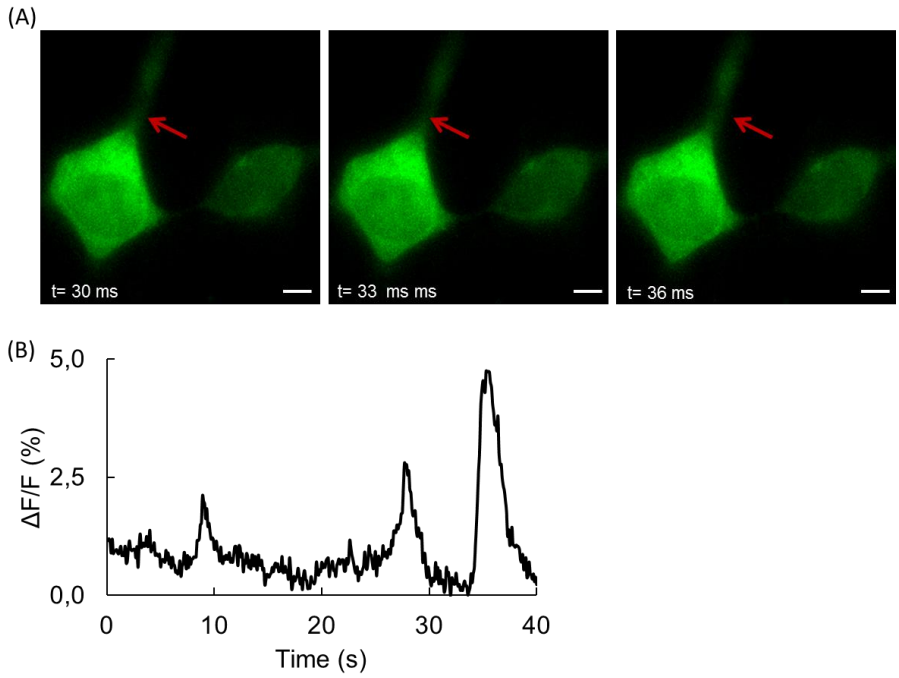

Figure 3-4 Representation of GCAMP3 expression and activity in different part of the same cell. (A) Expression of Gcamp3 GECI in HEK293T cell line. The rise of action potential was indicated (red arrows). (B) Fluorescence change of HEK293T cells was plotted versus time. Scale bar: 10 μm.

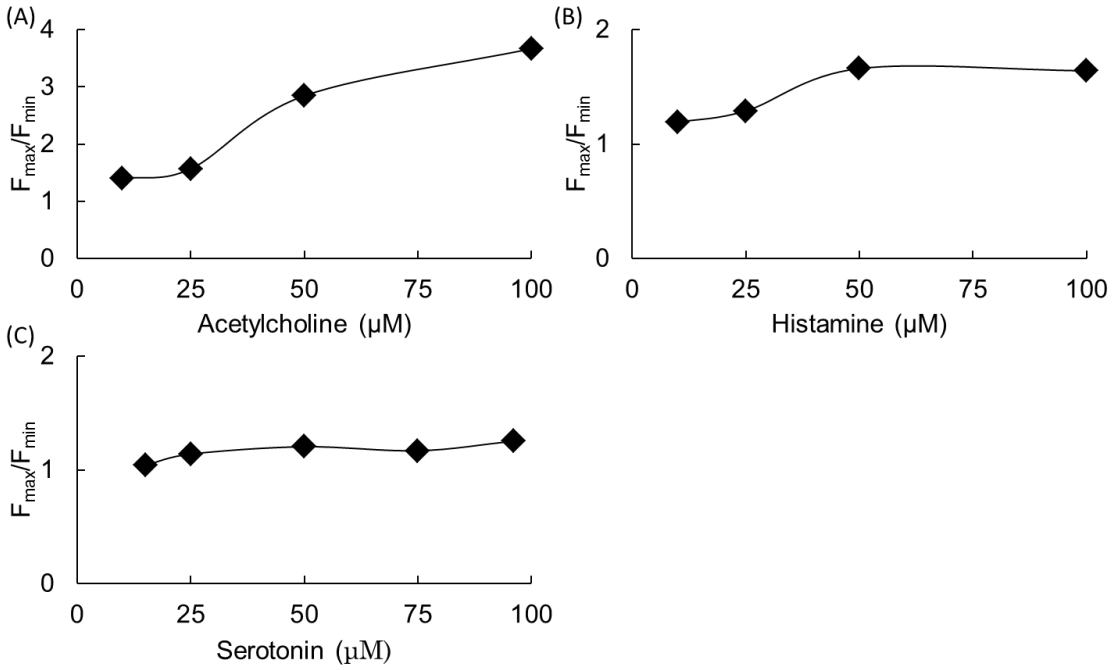

Figure 3-5 Calibration curve for acetylcholine, histamine and serotonin.Fluorescence change (Fmax/Fmin) plotted versus concentration for acetylcholine (A), histamine (B) and (C) serotonin in HELAS3 cells.

3.5. Single-cell response to histamine, acetylcholine and serotonin**:**

HELAS3 cells transfected with Gcamp3 by using Turbofect reagent. 1000ng plasmid DNA was used. Plates were incubated at least 24 hours with transfection reagents. For microscopy experiments, complete DMEM was replaced with 1xTBS+$CaCl_2$ solution and 100µM histamine, 100µM acetylcholine, 96 µl serotonin were added seperately into well during recording from freshly prepared 300 µM and 1mM 1xTBS+$CaCl_2$ solution stocks of histamine, acetylcholine and serotonin, respectively.

For both calibration curve and varied histamine response experiments, Nikon eclipse inverted fluorescence microscope with a 10X objective, numerical aperture 0.3 was used for fluorescence measurement. Blue laser with the wavelength of 475nm was used to visualize GCAMP3 activity. Fluorescence images of HEK293T and HeLaS3 cells were recorded by an Andor Luca S EMCCD. Images were analyzed with ImageJ (1.48v, Bethesda, Maryland). All experiments were performed at room temperature.

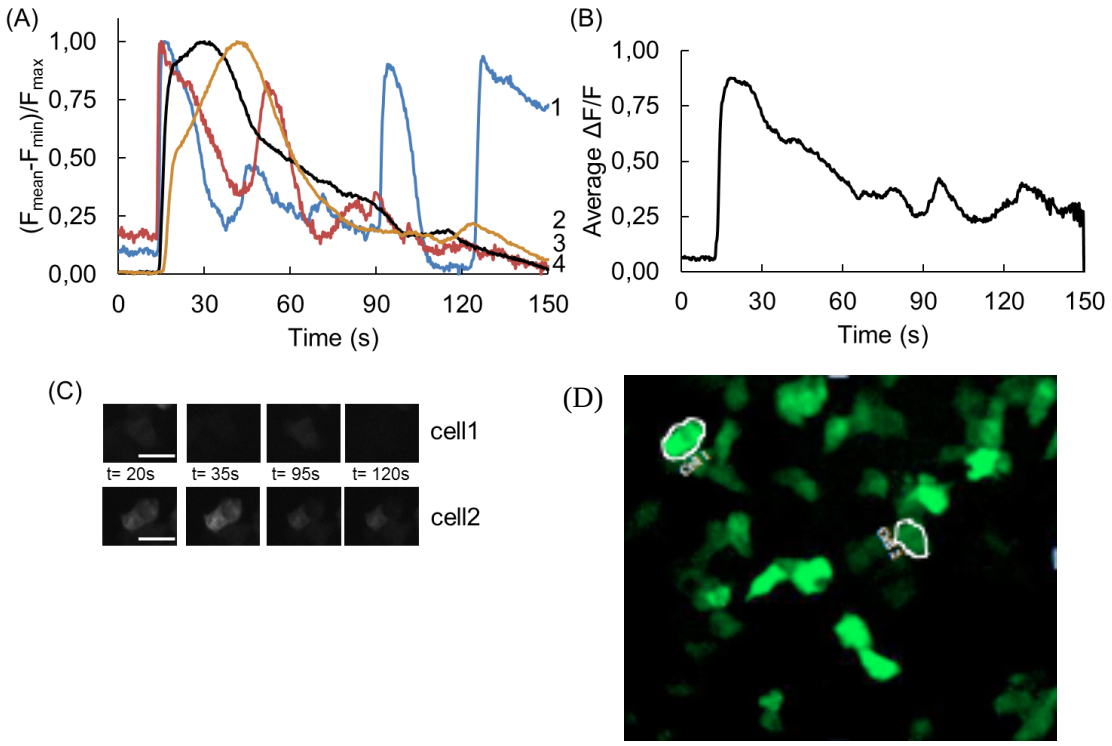

Figure 3-6 Stimulated **(**histamine) calcium concentration changes on single-cells. (A) Normalize fluorescence intensity change of 4 HELAS3 single-cells after addition of 100 µM histamine at the same condition (1xTris-Base Saline, 2mM $CaCl_2$) as a function of time. (B) Average of normalized fluorescence intensity change of 10 HELAS3 cells. Calcium fluctuation of cell 1 was not disappeared due to ensemble average. (C) Image of single-cell 1 (blue line from part A) and single-cell 2 (yellow line from part A) show different brightnein response to time. Scale bar: 40 µm.

Fig3.6. represent the effect of histamine on HeLaS3 cell line. Panel (A) shows that histamine caused fluctuations for cell 1 and cell3.There were sudden peaks at 60s and 100s for cell1 and cell3, respectively. These cells differ in activity of calcium channel on membrane or ER stores. Also, microenviroment contains varied concentrations of calcium ions depending on channel activity. Additionally, some cells were affected from histamine in a manner that fluctuate more than once compared to acetylcholine and serotonin, it may due to the nature of histamine or its role in biological systems. Inflammatory properties of histamine have adverse effect on cell, so

each cell has different sensitivity to histamine in response to their specialized receptors on cell membrane.Panel B indicated the average value of ten cells and histamine shows drastic reduction in fluorescence intensity, it is due to uptake of calcium from cytosol to ER stores and cell become exhausted of calcium ion in cytoplasm. Panel C shows the fluorescence intensity change in cell1 and cell2. At time 20 and 95, cell1 showed up relative increase in fluorescence intensity so fluctuations were visible; however, in the case of cell2 fluorescence intensity decrease even it was subjected to the same conditions.

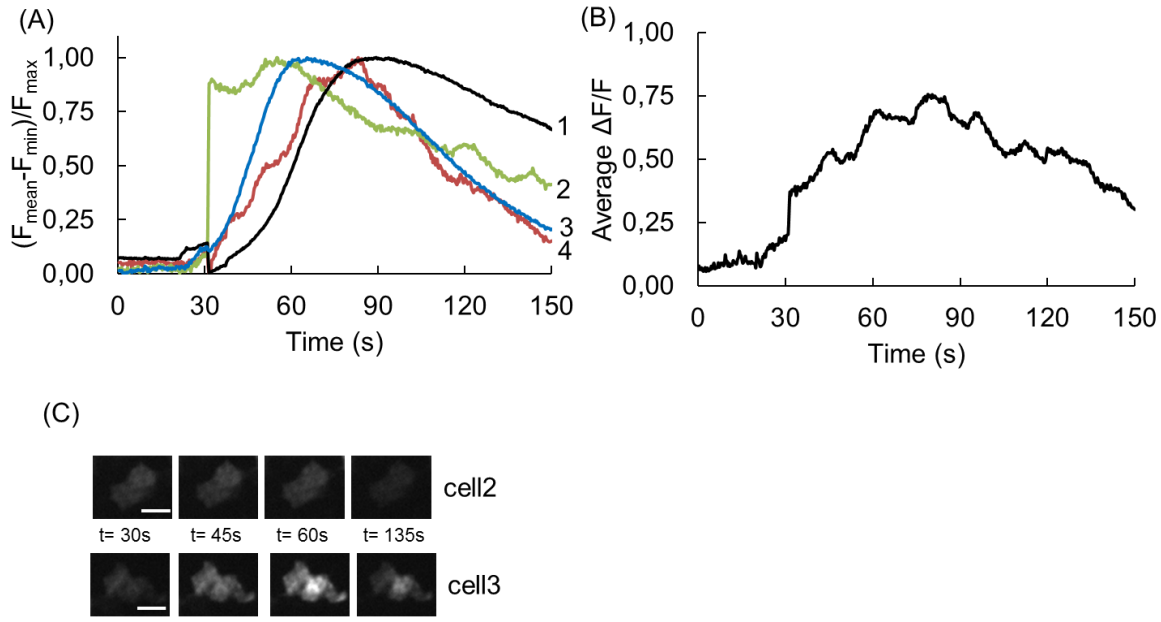

Figure 3-7 Varied single-cell response to acetylcholine. (A) Four different HELAS3 cells response to 100 µM acetycholine in the same condition (1xTris-Base Saline, 2mM $CaCl_2$). (B) Average fluorescence change of ten HELAS3 cells. (C) Representative images of cell-2 (green) and cell-3 (blue) show different brightness in response to time. Scale bar: 40 µm.

Fig 3.7 shows that there is single cell heterogeneity in response to acetycholine. After addition of acetylcholine, fluorescence of calcium indicator was increase. All four cells show the same peak value but the release of calcium from the indicator show different rate for all cells. The average value of ten cells also display fluctuations, which

indicate the continuous change in calcium concentration within cytoplasm.Panel(C) indicated fluorescence intensity change in cell3 and cell2.They both reached the same maximum value at different time and while cell2 had fluctuations, cell3 showed smooth curve and less fluctuations.This difference can show the varied sensitivity or expression of calcium indicator protein GcaMP as well as activity of calcium pump on cellular or ER membrane.

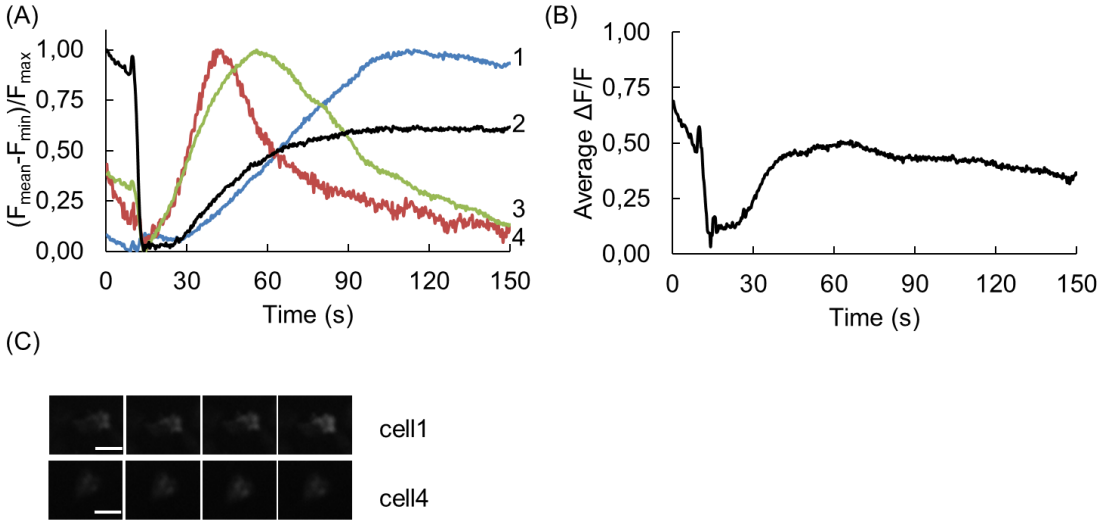

Figure 3-8 Varied single-cell response to serotonin. (A) Four different HELAS3 cells response to 96 μM serotonin in the same condition (1xTris-Base Saline, 2mM $CaCl_2$). (B) Average fluorescence change of twelve HELAS3 cells. (C) Live image of cell 1(blue) and cell4 (red) show different brightness in response to time. Scale bar: 40 μm.

Effect of serotonin was shown in Fig3.8. At panel(A), sharp decrease in cell 2 was observed compared to other cells and the minor decrease was observed in cell 1.Different response of cell at 15 second is caused by serotonine addition. Serotonin effects the cytosolic calcium and after addition ER stores released calcium to cytosol.Therefore, the fluorescence signal was enhance. Additionally, after 30s, intensities show narrower peak for cell4 and broader peaks for other cells that shows calcium restorage at varied rate.After 60s, cell 4 showed drastic decrease which serotonin lost its effect but serotonin can sustain its effect for cell1.At panel(B) there is

an average value of 12 different cell, compared to the effects of acetycholine and histamine, serotonin causes sharp decrease in fluorescence intensity. It was reported that serotonine has an effect to tumor growth via serotonin receptors[14, 15].Additionally,it was proposed that cell proliferation of agressive cell types is also affected by this biogenic amine[16]. Since HeLa cell line is a aggresive cervix cancer type, serotonin can also have an affect on proliferation of the tumor.

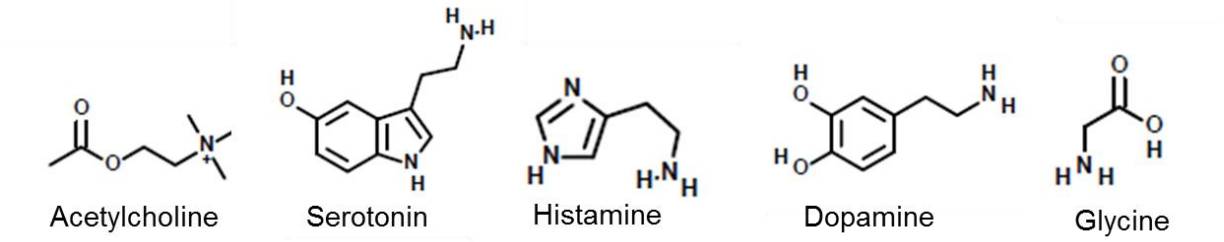

Figure 3-9 Chemical drawings of neurotransmitters used in this study.

Fig 3.9 represents chemical structures of neurotransmitters used throughout the experiments.Histamine molecule has an imidazole ring structure. Depending on ring structure, serotonin and histamine show similarities. For example, five membered ring structures of serotonin and histamine have amine group. Additionally, only serotonin has hydroxyl group.. Amine group in all five chemicals is another common feature of them. Acetycholine and glycine do not have any ring structure rather they have double bonding of oxygen for 4C and 2C molecule, respectively.

Single Cell analysis and propagation of calcium waves to control cell-to-cell communication,

Cytosolic calcium oscullations can be modulated by electric signal or external stimuli which subsequently lead to activatin of proteins/ Spontaneous activation of calcium flux was also observed in different type of cells. Although these waves have been observed in many cells, therir role in cell function remains unclear. We demonstrated that single-cell analysis can be used to determine the cell-cell communication.

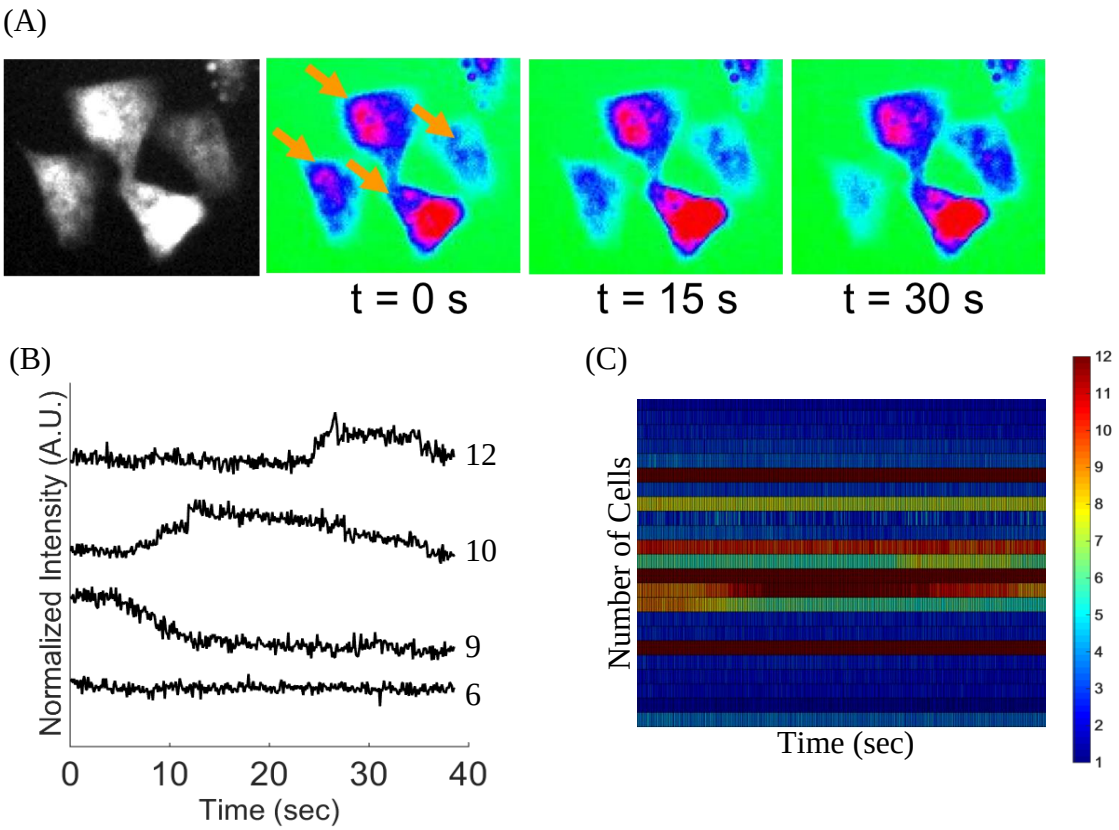

Figure 3-10 Propagation of intercellular calcium waves between hela cells after situmulated by Acethylcholine. (A) Calcium images of Gcamp expressing Hela cells. Cells are labeled as 6, 9, 10 and 12. After the simulation of cells, the calcium wave propagation from cell 9 to 12 was observed. (B) Normalized fluorescence intensity change of selected cells as a function of time. (C) Normalized fluorescence intensity change of all cells in the field of view. The colour scale indicates calcium levels changes in cells.

Wave propagates from cell 6 to cell 12 with a delay of 20 seconds (Fig 3.10). Calcium signals in other cells may be saturated before the stimulation (Fig 3.10). Recent experiments demonstrate that wave propagation between cells can be driven by extracellular mechanism that is driven by the release of secondary messengers. Based on other observations, we proposed that glutamate increase may evoke the calcium release in adjacent cells. The role of ATP release has been also diccused to control the cell-to-cell communication. Glial cells are known to use ATP as a secondary messenger to drive neuronal, ganglion and amacrine cells. We do not know what messenger

initiates wave signals between cells. This studies are underway in our laboratory and will be reported in further studies.

3.6. PCA analysis and clustering of single-cells

Here we used PCA to determine the single cell diversity to determine the sensitivity of cells against small molecules. Later, we cells baed on the results of PCA were clustred into two groups (histamine and serotonin). Computational framework for PCA analysis were described in previous studies. We used PCA method and integrated with the computational techniques of signal tracking to identify the heterogenous single-cells. First, we used single cell signal tracking method to measure the changes in calcium levels, thereby assessing the cells response to various neurotransmitters. Then, the covariance matrix, indicating the correlation of variables, was computed to identify the principal components. The increase magnitude of variance alonf the diagonal raise from the variance of cells. The values at off-diagonal indicated the covariance of single-cells.

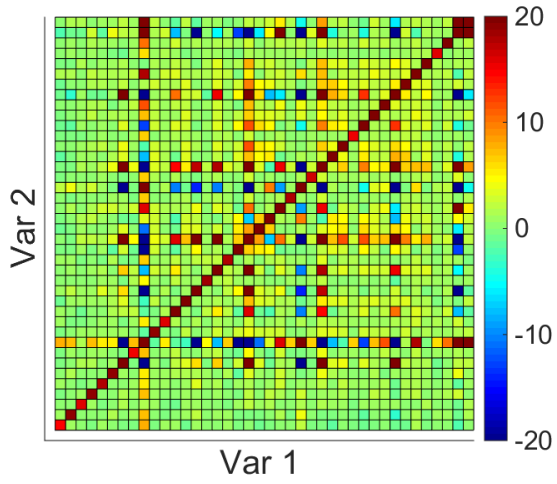

Figure 3-11 Computation of covariance matrix for calcium signaling in single-cells. Diagonal and off-diagonal entries in the matrix indicates the variance and and covariance of calcium signaling in single-cells.

We used the covariance matrix to calculate the singular values of each principal component. Since each cell represents an independent observation and tracking method captures a large number of cells that could enable the accuracy of SVD analysis.

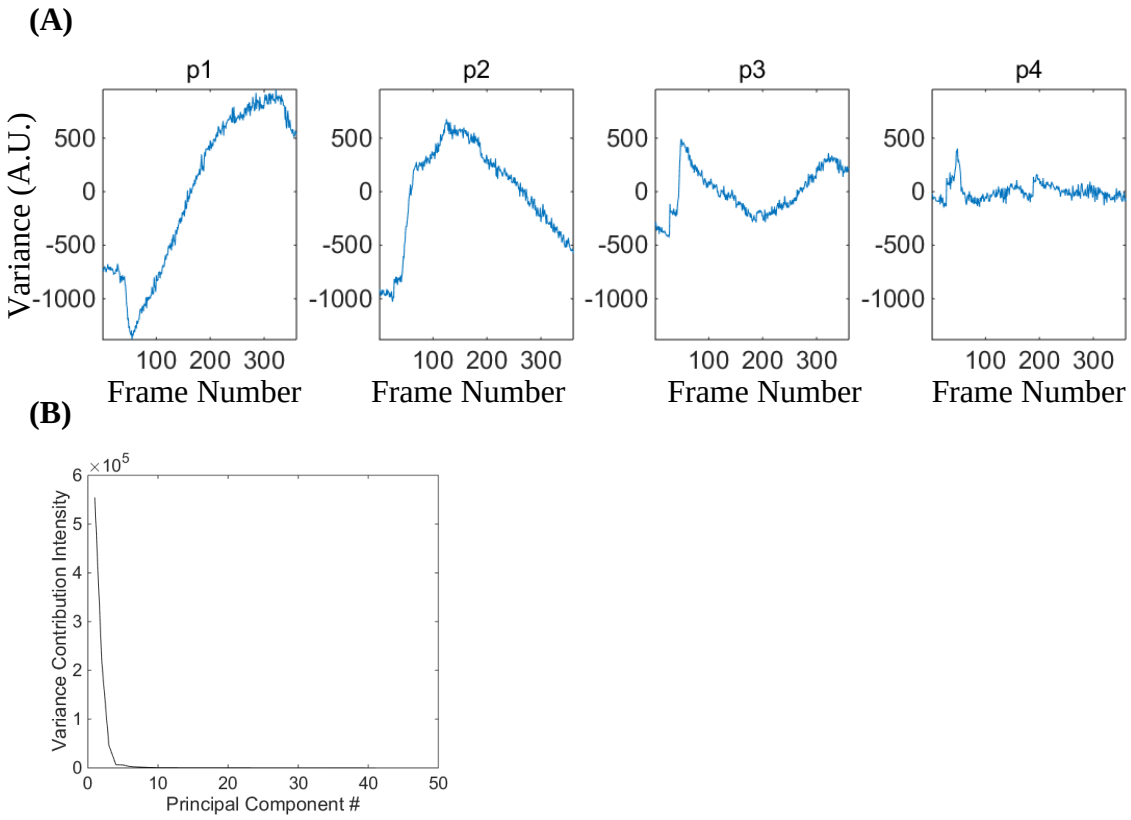

Figure 3-12 Evalation of principal components for single-cell analysis. (A) Time dependent changes of variance for PC1, PC2, PC3 and PC4. (B) Weight of each principal component for single-cells in the new basis set.

Since the first and second eigenvectors had the largest eigenvalues, we used principal components to determine cells coordinates (Fig. 3.13). First principal axis demonstrated the total calcium changes while second axis was the represeantive of calcium rate change. Cells (cluster 2) at the origin had very low calcium levels and did not respond the increased histamine levels. Cell9 (blue cluster) had a high levels of calcium change with a low rate of change. Other cells demonstrate a different rates of change and depicted at different regions. A total of five different clusters were determined.

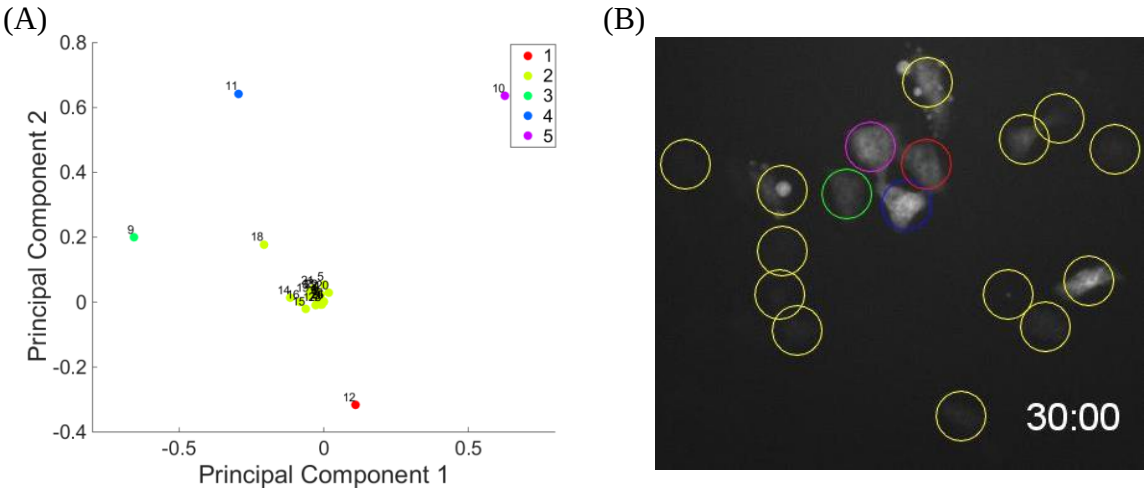

Figure 3-13 Two-dimensional projection of n-dimensional space data. (A) The projection was obtained by computing the covariance matrix that was used to determine singular values and principal components. By using an Euclidean approximation, give different regions were determined. (B) Cells were grouped and colored based on their cluster number that was computed by measuring Euclidean distance in MatLab. These color were automatically assigned to each cell based on magnitude and variance of calcium signals.

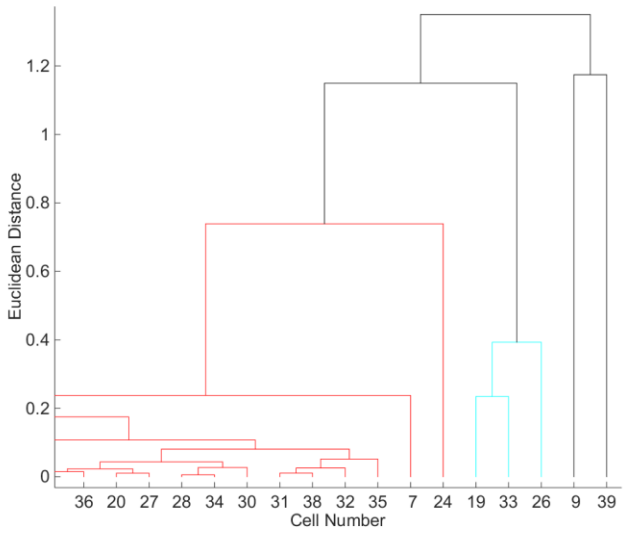

Figure 3-14 Euclidean distance of cells. The relation was elucidated by single-cell analysis of calcium waves.

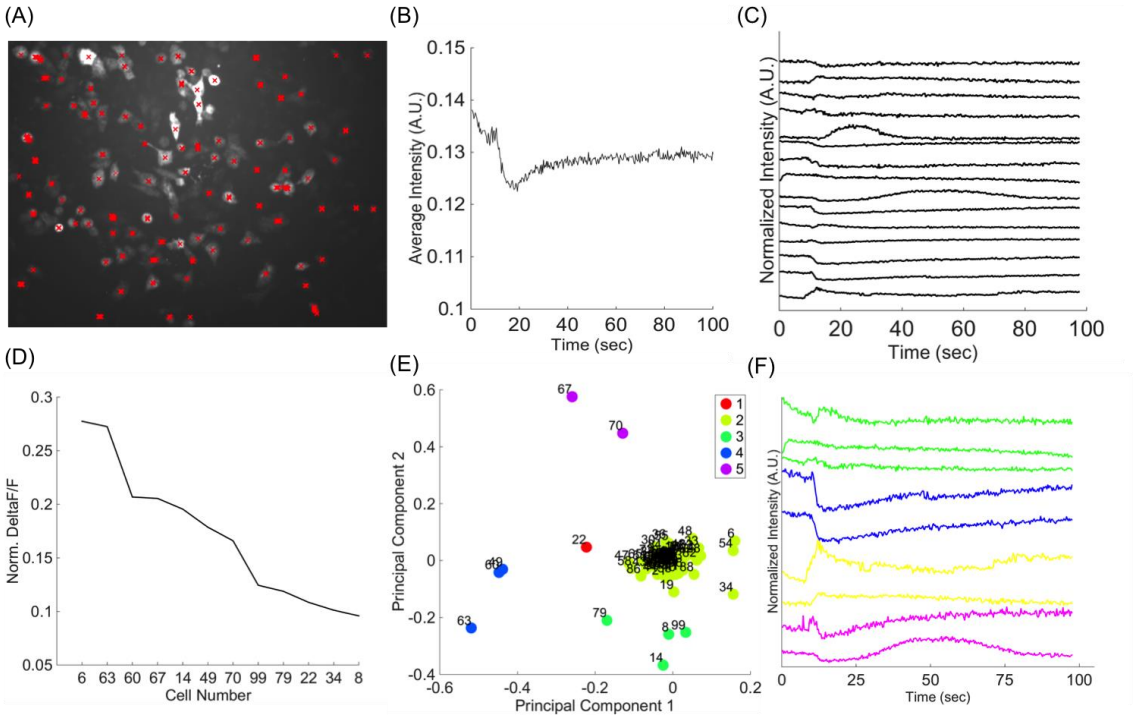

Figure 3-15 Calcium singal changes in single-cells after addition of 50 µM serotonin. (A) Average fluorescen image of single-cell expressing GCaMP calcium indicator. (B) Average calcium level change of cells. Signal decrease exponentially in 1-5 second which follows a long recovery period of cells. (C) Example of calcim signal time courses of 15 randomly selected single-cells. (D) Single-cells were sorted based on magnitude change of intensity. (E) The projection of cells by using principal components and grouping cells by minimizing Euclidean distance between clusters. (F) Cells were automatically sorted based on the cluster mode of cells.

After obtaining all results described above, we finally sought to test the signal-sorting for histamine and serotonin response of cells where the cells average patterns were different. Histamine stimulation induced a signal where the calcium levels initially increases, however serotonin reduced the calcium levels at early stage, later gradually increased calcium levels were observed. The purpose of cell signal sorting is to identify stimulant from calcium time traces, therefore determine the current state of cells and determine the secreted molecules. Cells in tissues are exposed to different stimuli, however identification of these molecules at spatio-temporal resolution is a challenging task. We proposed that the molecules can be determined by using time trace of calcium

signaling and the method characterize the cells in two steps. First, all collected signals from single-cells were pooled that includes time traces from serotonin and histamine. It contains at least 60 cells and time traces.

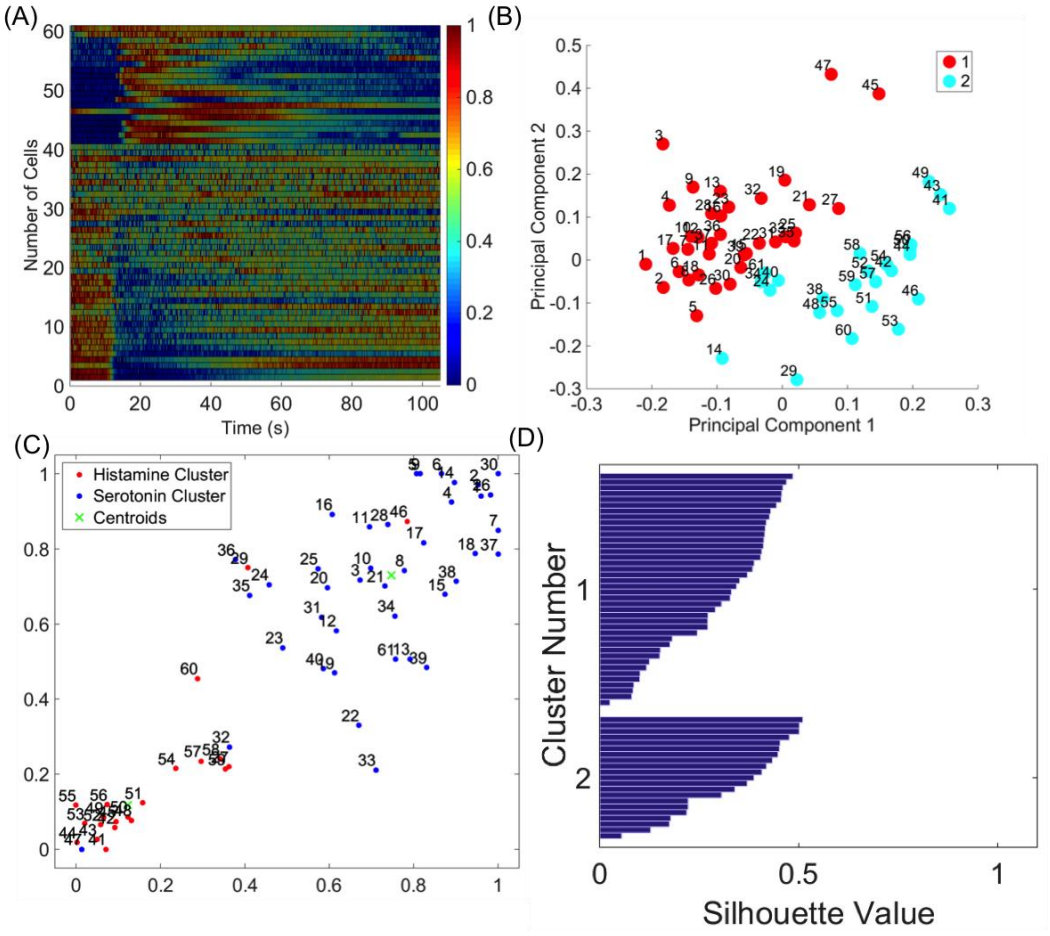

Figure 3-16 The change of calcium signals stimulated by different stimuli and sorting of single-cells. (A) The overlay of single-cells responses for histamine and serotonin. The initial response to both molecules was different. (B) The cells were clustered based on calcium signals after elucidating the principal components. Euclidean distance between cells were measured to cluster cells into two groups. Group 1 (red) and 2 (blue) represents the cells for serotonin and histamine respectively. (C) Clustering of cells by using kmeans method. We used the normalized data to determine the coordinates of cells at a new basis set. Cells were then clustered into two groups after 8 iteration to compute minimum total distance between centes of clusters and cells. (D) Silhouette scores of clustered data were computed to measure the efficiency of kmeans method.

Cells were clustered by using hierarchical method where the cells points are added to each cluster by minimizing the distance between cells and centraind of cluster. Cells cluster number does not change once the group is determined during the analysis. It inially uses a small number of cells for each cluser. Then add each cell to cluster by minimizing the mean square distance of a given cluster. The compute the distance betwwen groupds we used the Euclidean method defined as the square root of the sums of the squares of differences between the coordiantes of the points. It can be written as,

$$d_{ij} = \sqrt[2]{\sum_{m=1}^{n} \left| x_{im} - x_{jm} \right|^2}$$

where n represents the number of rows in the matrix. Xim and Xjm represent the random points in the data set. At each step, we computed the distance between each pair of clusters.

3.7 Single-Cell Clustering by using K-means method

K-means were used to compute the clusters of cell by using the coordinates from pca analysis. The method computes the centroid position for each elements of the cluster and repeats the process until the final position of centroid converges a mean value. K-means method uses known number of clusters, therefore it computes faster than other methods. K represents the number of cluters and was determined before computation. In brief, mean and variance of each cluster is computed for a given data after all cells are assigned to each cluster. The approximate position of clusters can be estimated by using the coordinates of cells. Then, the distance between the each cell point and clusters mean are measured to recalculate the mean of clusters. The process requires the recomputation of centroids at each iteration. However in hierarchical clustering mean values of cluster are given at the beginning and each cell added to a precomputed cluster. If mean value does not change, group receives the cell as an element of the cluster or it stays in the same group. The cells cluster can be changed during the computation of mean cluster values. If a cell point increases the mean value of a given cluster, we reassign the cell to a different cluster and compute the values

again. The process is also repeated for all cells in other clusters until the centroids of clusters remain unchanged. Here, we also used Euclidean distance as a proximity measure to each centroid. Cell position to each centroid was minimized by computing sum of squared distance.

We started with the initial set of data obtained from PCA analysis of calcium signals in single-cells. Our results demonstrated that the sorting efficiency was better that cluster method that was described above. For example, cell 45 and 47 (histamine sensitive) were clustered in the appropriate group (Fig 3.17-a). However cell 27, 29 ad 40 was also grouped in the same cluster even though cell was selected from serotonin group. Calcium signals in these cells were different than other cells in serotonin group. Since the analysis is sensitive to outliers, it is expected to see some assignments moves to other cluster. Our results suggest that we should increase number of cells that would increase the accuracy of results. We have observed few scores in silhouette scores (Fig 3.17-b), however sorting efficiency was similar to the second method where the normalized data was used to group single-cells.

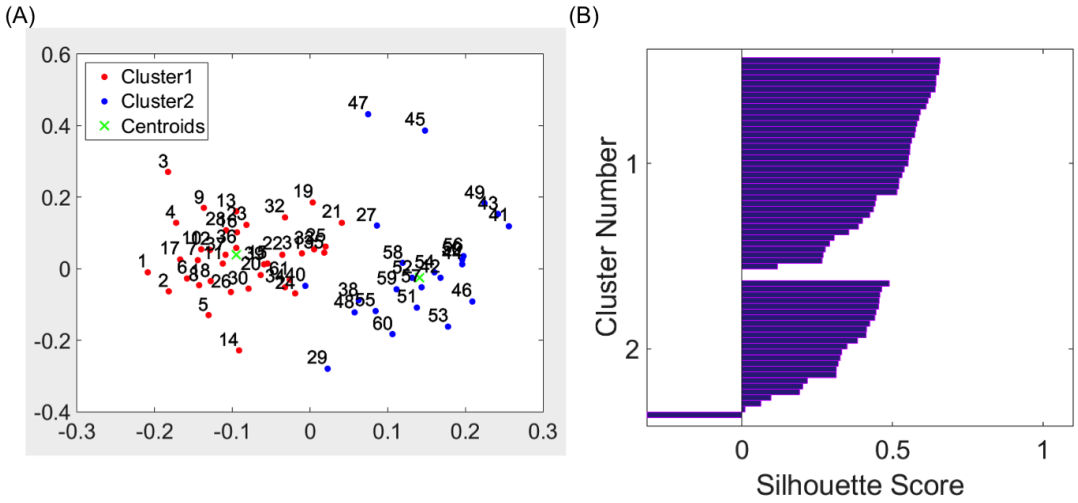

Figure 3-17 Clustering of cells by using kmeans method after computing the principal components of cells. (A) K-means clustering results of cells where cells were divided into two groups. Cluster 1 (red) and cluster 2 (blue) represents the serotonin and histamine respectively. Each number represents the correspoing cell number. (B) Silhouette scores were computed to determine the efficiency of clustering. Low scores and negative values in cluster 2 indicate overlap of two regions where cells can be assigned to both groups. Cells 29, 38 and 27 may affect the scores of cluster 2.

Chapter 4

OUTLOOK AND CONCLUSION

Intracellular signaling in tissues plays a critical role for determining and controlling vital functions. By knowing the signaling condition and external factor at the microenvironment, the dynamics response can be identified, therefore provide a predictable outcome after exposed them to an external cues. Although many factors are previously characterized at bulk measurements, it is now understood that heterogeneous signal formation and single-cell changes demonstrated underlying complexities of signal response that cannot be explained by bulk measurements or considering a single factor. To elucidate the role of single-cell signals, tracking methods and models are required. Calcium signal of cells were trackind in single-cells by using GECI reporters and minimizing mean square displacement methods. The signal derived from experiment data awas further analyzed using principal component analysis.

An important caveat in the use of PCA for the elucidation of calcium signal mechanism in single-cells is that it allows comparing large number of cells and indexes them based on the amplitude and frequency of calcium signal. Given time series data and non-destructive imaging of cells, the method can capture and identify the unique cells. Indeed, present work used a small group if cells, methods can be scaled up and provides a faster and precise identification of single-cell heterogeneity.

The current study was limited with U2OS and HeLa cells. Clinically relevant cell types such as stem cells can be potentially used to classify the cells based on their calcium response. Intracellular signaling at early stages of differentiation plays a critical role for determining the fate of cells. Understanding the dynamics of single stem cells may potentially resolve the issues of having a low yield, efficiency of differentiation and factors driving the fate of stem cells. Low copy number of pluripotent cells reduces the potential of differentiation. Since the decisions of cells are driven by physical and chemical stimuli, it is critical to identify the all factors at single-cell resolution.

Although the thesis describes an early study of single-cell analysis as computationally and experimentally, the methods used here can be applicable in other areas. Single-cell calcium signal tracking and simultaneous measurements of cell states offers a useful approach for rapidly determining the heterogeneous variations of signaling pathways. Extension of single cell studies are currently underway in neuroscience and stem cell biology and whole-cell differ enation of C. elegance where the light-sheet microscopy allows resolving single-cells and identify the migration directions of cells. The methods could allow automated identification of cell variations as well as signal changes after cells are exposed to external cues. Further development of optics and image based analysis of might yield significantly to the understanding of noise in cells. Our present understanding of Ca^{2+} dynamic changes in single cells is based on cultured cells and tissues. The relevance of our obssrvqtion can be studied

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