Confocal microscope is able to detect calcium metabolic in neuronal infection by *toxoplasma gondii*

A D Sensusiati¹*, T K S Priya and Y P Dachlan

¹Medical Faculty of Airlangga University, Dr. Soetomo Hospital, Surabaya

*corresponding email address: radiologidc1@gmail.com

Abstract. Calcium metabolism plays a very important role in neurons infected by Toxoplasma. Detection of change of calcium metabolism of neuron infected by Toxoplasma and Toxoplasma requires the calculation both quantitative and qualitative method. Confocal microscope has the ability to capture the wave of the fluorescent emission of the fluorescent dyes used in the measurement of cell calcium. The purpose of this study was to prove the difference in calcium changes between infected and uninfected neurons using confocal microscopy. Neuronal culture of human-skin-derived neural stem cell were divided into 6 groups, consisting 3 uninfected groups and 3 infected groups. Among the 3 groups were 2 hours, 24 hours and 48 hours. The neuron *Toxoplasma gondii* ratio was 1:5. Observation of intracellular calcium of neuron and tachyzoite, evidence of necrosis, apoptosis and the expression of Hsp 70 of neuron were examined by confocal microscope. The normality of the data was analysed by Kolmogorov-Smirnov Test, differentiation test was checked by t² Test, and ANOVAs, for correlation test was done by Pearson Correlation Test. The calcium intensity of cytosolic neuron and *T. gondii* was significantly different from control groups (p<0.05). There was also significant correlation between calcium intensity with the evidence of necrosis and Hsp70 expression at 2 hours after infection. Apoptosis and necrosis were simultaneously shown with calcium contribution in this study. Confocal microscopy can be used to measure calcium changes in infected and uninfected neurons both in quantitatively and qualitatively.

1. Introduction
Many brain calcifications were found on congenital toxoplasmosis (Couvreur et al, 1962; Dunn et al, 1999). Neurons calcification as the result of *Toxoplasma gondii* infection is associated with important role of calcium for *Toxoplasma gondii* infection. *Toxoplasma gondii* as a part of apicomplexan parasite relies on calcium signaling to review different important functions including protein secretion, motility, cell invasion and differentiation (Negaunee, et al, 2008)[1]. In parasite infection, during invasion and persisting, the host cells need movement and gliding motility, also calcium scavenging (Dobrowolski and Sibey, 1996)[2]. All of this dynamic calcium change can be evaluated by measuring the calcium intra parasite and neuron as a host cells (Lovett and Sibey, 2003)[3]. Confocal microscope has the ability to capture the wave of the fluorescent emission of the fluorescent dyes used in the measurement of cell calcium. The purpose of this study was to prove the difference of calcium changes both in infected and uninfected neurons using confocal microscopy. In order to make the similar condition like infected neuron in neonatal toxoplasmosis, skin-derived neural stem cell was used.
2. Material and methods
A fully synchronized laser scanner in a single compact design for simultaneous laser stimulation and confocal observation (Olympus FV1000) was used to measure calcium intensity of the uninfected and *Toxoplasma gondii* infected neuron.

Two month baby’s preputium was used to grow multi potent neural stem cell. The existence of its growth was confirmed by immunofluorescence of CD 133. After isolation, proliferation, and differentiation of neural stem cell from multi potent, neural stem cells (MNSC) became adult neuron. Beta III tubulin with immunocytochemistry was also confirmed for adult neuron.

*Toxoplasma gondii* isolate from RH lineage taken from Biotechnology Centre of Gajah Mada University Yogyakarta was used in order to increase the number of isolate intra peritoneal mouse injection of $10^3$ in every mouse. After 4 days, the mice were sacrificed to take the intraperitoneal fluid. Parasites inside the peritoneal fluid were counted with haemocytometer, and the number of *Toxoplasma gondii* (tachyzoites stage) in every millimetre was obtained. Adult neurons were divided into 6 groups consisting of 3 uninfected groups and 3 infected groups. Adult neurons then were infected by tachyzoite through incubation with tachyzoite and fixation at 2 hours, 24 hours and 48 hours. The optimal neuron tachyzoites ratio was 1:5 (Rozenfeld, 2005)[4].

Fluorescent calcium indicators fura-2 was used to dye calcium. The excitation was set at 340 and 380 nm. Confocal laser scanning microscopy then used to measure the calcium intensity.

The normality of the data was analysed by Kolmogorov-Smirnov Test, differentiation test was checked by $t^2$ Test, and ANOVAs, and for correlation test was done by Pearson Correlation Test.

3. Result
Laboratory experimental research has been carried out in vitro on the mechanism of deposition of calcium in neurons infected by *Toxoplasma gondii*. It was conducted through observation of neuronal cytosolic calcium, *Toxoplasma gondii* calcium by immunofluorescence using fura-2.

![Figure 1. Neural stem cell at day 7. Blue arrow shows neural stem cell, red arrow shows fully differentiated neuron. Evaluation with 200x](image1)

![Figure 2. Identification and characterization of neural stem cell using fluorescent microscopy, the CD 133$^+$ expression can be detected. Green colour is the expression of neural stem cell.](image2)

As the main ingredient in this laboratory experimental research, neurons were obtained from the process of isolation and culture using discharged skin tissue from prepuce circumcision of a 2-month-old baby. Within 10 days after the process of proliferation and differentiation, fully differentiated adult neurons were formed as seen in Figure 1. Characterization of neuronal stem cells was observed by CD 133 using immunofluorescence. Figure 2 shows that the cells are checked neuron stem cells for expressing the CD 133$^+$. 
Culture examination of neurons with fura-2, which was observed in confocal microscopy, showed the results in a 3-dimensional image based on different interference contrast (DIC) (Figure 5). Intracellular calcium and nucleus was showed in luminescence of green colour of fluorescent fura-2 after incubated in cultured neurons (Figure 6).
Figure 7. Calcium fluorescence intensity on control group was measured using confocal laser microscopy scanning.

The measurement of calcium on experimental group was performed using the method of chemical fluorescent indicator fura-2 and antibodies (double staining, Figure 8 to 10) on *Toxoplasma gondii*. The result then was utilized to distinguish neurons intensity of cytosolic calcium and calcium intensity of *Toxoplasma gondii*. The observations indicated that the intensity of calcium in *Toxoplasma gondii* was gradually increasing in which its second-hour result was equal to the intensity of the control group in 2 hours. It was increasing at 24 hours and 48 hours while the intensity of calcium in neurons was gradually declining (Figures 11 to 17).
Figure 8. Neurons after infected by tachyzoites in two hours. The identification and characterization of tachyzoites used *Toxoplasma gondii* antibody, shown by red fluorescence, and neural intracellular calcium, characterized by green fluorescence.

Figure 9. Twenty four hours after tachyzoites infection, red fluorescence was more prominent than 2-hour tachyzoites infection. The green fluorescence decreased.

Figure 10. Forty eight hours after tachyzoites infection, the neuron population diminished, and tachyzoites intracellular calcium increased.
Figure 11. Calcium intensity measurement on neural cell in 24 hours after tachyzoites infection.

Figure 12. The comparison between the intensity of neuronal calcium of control group and after 2 hours tachyzoites infection.
Figure 13. The comparison between the intensity of neuronal calcium of control group and after 24 hours tachyzoites infection.

Figure 14. The comparison between the intensity of neuronal calcium of control group and after 48 hours tachyzoites infection.
Figure 15. The intracellular calcium measurement on neuron and *Toxoplasma gondii* 2 hours after tachyzoites infection.

Figure 16. The intracellular calcium measurement on neuron and *Toxoplasma gondii* 24 hours after tachyzoites infection.
Figure 17. The intracellular calcium measurement on neuron and *Toxoplasma gondii* 48 hours after tachyzoites infection.

### Table 1. Results of Data Normality Test Using One Sample Kolmogorov-Smirnov Test

| Group                           | Significant value (p) | Description               |
|--------------------------------|-----------------------|---------------------------|
| Control 2 hour (Ca Neuron)      | 1.000                 | Normal Distribution (p > α) |
| Control 24 hour (Ca Neuron)     | 1.000                 | Normal Distribution (p > α) |
| Control 48 hour (Ca Neuron)     | 0.988                 | Normal Distribution (p > α) |
| Infected Neuron 2 hour (Ca Neuron) | 0.764             | Normal Distribution (p > α) |
| Infected Neuron 2 hour (Ca Toxo) | 1.000               | Normal Distribution (p > α) |
| Infected Neuron 24 hour (Ca Neuron) | 0.946            | Normal Distribution (p > α) |
| Infected Neuron 24 hour (Ca Toxo) | 0.984              | Normal Distribution (p > α) |
| Infected Neuron 48 hour (Ca Neuron) | 0.942           | Normal Distribution (p > α) |
| Infected Neuron 48 hour (Ca Toxo) | 0.772              | Normal Distribution (p > α) |

Based on the observation of the control group and infected group, as shown in Table 1, and the analysis using abnormality test data by Kolgomorov-Smirnov’s test, the result showed that the data was in normal distribution.
Table 2. Results of the Difference Test of Calcium between Neuron Control group and Infected Neurons Using t2 Free Sample Test

| Groups                        | Mean ± Standard Deviation | Significant value (p) | Description                  |
|-------------------------------|---------------------------|-----------------------|------------------------------|
| Control 2 hour (Ca Neuron)    | 1254.206 ±177.629         | 0.038                 |                              |
| Infected Neuron 2 hour (Ca Neuron) | 922.678 ±177.173         |                       |                              |
| Control 24 hour (Ca Neuron)   | 1388.460 ± 110.720        | < 0.0001              | significant difference (p < α) |
| Infected Neuron 24 hour (Ca Neuron) | 566.320 ± 94.669         |                       |                              |
| Control 48 hour (Ca Neuron)   | 1231.460 ± 196.058        | 0.002                 |                              |
| Infected Neuron 48 hour (Ca Neuron) | 405.523 ± 51.144         |                       |                              |

Table 2. shows free sample t test on both control and infected group (2-hour, 24-hour, and 48-hour infection). The result showed a significant difference between control group and infected group.

Table 3. Results of the Difference Test between Calcium Neurons and Calcium Toxoplasma gondii using t2 Free Sample Test

| Groups                        | Mean ± Standard Deviation | P         | Description                  |
|-------------------------------|---------------------------|-----------|------------------------------|
| Infected Neuron 2 hour (Ca Neuron) | 922.678 ±177.173         | 0.934     | Was not significantly different (p > α) |
| Infected Neuron 2 hour (Ca Toxo) | 912.550 ± 152.719        |           |                              |
| Infected Neuron 24 hour (Ca Neuron) | 566.320 ± 94.669         | 0.026     |                              |
| Infected Neuron 24 hour (Ca Toxo) | 743.130 ± 74.386         |           | significant difference (p < α) |
| Infected Neuron 48 hour (Ca Neuron) | 405.523 ± 51.144         | 0.004     |                              |
| Infected Neuron 48 hour (Ca Toxo) | 717.635 ± 130.578        |           |                              |

Based on the result presented in Table 3 with 2 free sample t test, there was no significant difference between control group and after 2 hours infection, yet the significant difference presented after 24 and 48 hours infection.
4. Discussion

4.1. Confocal laser microscopy, a fluorescent method to study intracellular calcium

A laser-scanning microscope converges the laser beam into a small spot using an objective and scans the specimen in the X-Y direction using the laser beam. The microscope then captures fluorescent light and reflected light from the specimen using light detectors, and outputs the specimen image on an image monitor. The confocal optics incorporate a confocal aperture on the optically conjugate position (confocal plane) with the focus position to eliminate light from other parts than the focus position. This causes the extraneous light to be viewed as darkness in the observation image. It is possible to slice optically a specimen tissue that has thickness. On the other hand, an ordinary optical microscope, the light from other part than the focus position is overlapped with the imaging light of the focus position so the image is blurred in overall. Laser beam that has been transmitted through the specimen is detected by the transmitted light detector and provides the transmitted image, which is not a confocal image.

In regards of its importance, numerous methods for analysing the mechanism of cellular and/or subcellular calcium activity have been established. Fluorescence dyes methods have been widely used in Toxoplasma gondii since it had high sensitivity (Gryniewicz et al., 1985; Tsien, 1989)[5]. Fura-2 loaded neuron was used in this study, for fura-2 measurement, excitation set at 340 and 360 nm and emission at 510 nm.

To study the calcium dynamic during Toxoplasma gondii infection on neuron, neural stem cell cultured from baby’s preputium was used to grow adult neuron. Figure 1 to figure 4 presents the step of neural cell development.

Confocal microscopy detected intracellular calcium on control group as well as infected group, as shown on figure 5 to figure 11. To separate calcium content inside the neuron and inside Toxoplasma gondii, antibody for Toxoplasma gondii was used. The intensity of both cells can be measured.

4.2. The influence of toxoplasma gondii infection to host cytosolic calcium

Calcium plays important roles during attachment of tachyzoite to the host-cell surface and invasion (Viera and Moreno, 2000; Bouchot, et al., 1999; Mondragon and Frixione, 1996). The sequential secretion of parasite organelles marks the tachyzoite invasion process. Micronemes, rhoptries, and dense-granule contents are released by invading parasites, and participate in attachment, vacuole formation and intracellular survival (Carruthers and Sibbley, 1997)[6]. In invasive phase, tachyzoites-neuron attachment begins with protein secretion that functions as enzyme from secretory organelles microneme, rhoptry, and dense granules from unique microtubules cytoskeleton called conoid. They are active PLA, rhoptry proteins (ROPs), and microneme protein (MICs). These enzymes influence host’s plasma membrane phospholipids to produce that cause local lysis, influence membrane fluidity or egress intracellular calcium store (Bonhomme, 1999; Lin, 1993)[7]. Arachydonic acid influenced calcium entry to host cell. MICs and ROPs also secreted from apical Toxoplasma gondii. After exocytosis MIC3, an epidermal growth factor (EGF-) like signal may activate inositol phosphate signal (IP), causing calcium to be released from inner pool, and increase calcium concentration. Figure 12 shows cytosolic calcium on infected neuron was lower than from uninfected group, but on tachyzoite was higher than from control and neural cytosol. It shows that increment of neural cytosolic calcium immediately present after invasion in 20 seconds (Morisaki et al., 1995) cannot be evaluated in this study because the observation was done in 2 hours. Higher calcium intensity on Toxoplasma gondii was due to calcium transfer from neural cytosol to Toxoplasma gondii. Calcium intensity was observed from 2 hours to 24 hours and 48 hours as shown on Figure 8, 9, 10 and may prove that cytosolic calcium in neuron decreased. The increment of calcium inside the tachyzoite and calcium transfer proceeded until 48 hours. T2 test resulted a significant difference between neural cytosolic calcium in control group than experimental group, proofing that calcium signalling worked on plasma membrane and extracellular influx to neuron.
4.3. *Toxoplasma gondii*’s calcium intensity transformation during infection

*Toxoplasma gondii* needs calcium during motility, conoid extrusion, microneme secretion, attachment, and invasion to the host cell (Debrowski, et al., 1997). Gliding motility depends on actomyosine motor and important for active invasion host cell (Debrowski and Sibley, 1996), involving *Toxoplasma gondii*’s intracellular calcium. Conoid extrusion exists during invasion and egress from host cell, and it is stimulated by material that can enhance calcium (Mondragon and Frixione, 1996; Monteiro et al., 2001) [8]. Microneme is the first secretory organelle that produced from conoid. This microneme secretion needs intracellular calcium from *Toxoplasma gondii*, and host attachment triggers microneme release. (Brecht et al., 2001).

Free calcium intracellular concentration in the infected host cell is variable (Bouchot A., et al., 1999). When *Toxoplasma gondii* enters the host cell, calcium pump is activated to their environment. Calcium may freely diffuse across membrane because of the porus of Parasitoporus vacuole membrane (PMV), implicating that product secreted to the vacuole can bind the calcium. The candidate for this activity is dense granule protein GRA 1, which is highly related to tubovesicular network from parasitoporus vacuole (PV) and high affinity for calcium. This study showed that calcium decreased after 2 hours infection (Figure 15) and even more decreased after 24 hours infection (Figure 16). The consequence, 48 hours after infection, when the parasites have more replicated and built rosette, host cytosolic calcium decreased significantly (Figure 17). Host intracellular calcium increment and egress of parasite from host cell are seemed to be correlated. Calcium increment on PV was a signal that was triggered the *Toxoplasma gondii* egress and parasites push the host cell membrane during this moment. Rupture of plasma membrane happened, parasites came out from the cell, and continued by host cell collapse. Thus, regulation of host intracellular calcium and parasitic vacuole compartment has a close relationship with the growth and egress of parasites from host cell.

4.4. Calcium signalling during neural infection by *Toxoplasma gondii*

According to Krebs and Michalak (2007), eukaryotic cell is surrounded by free calcium media more than 1mM. Yet, it regulates cytoplasmic intracellular calcium concentration maintained four times lower. This low concentration conditions calcium precipitation prevention, and phosphate calcium sodium low solubility. This condition also prevents the energy loss that is needed for targeted calcium concentration transformation. Total intracellular calcium is higher than mM, but it is reduced until ionic sub-μM. First bounding with phospholipids membrane (acidic) to lower the metabolite molecular weight, to be inorganic ion such as phosphate, and then built complex with certain protein. This protein is divided into some classes: one consists membrane intrinsic protein that operates as calcium messenger on plasma membrane and on organelle membrane. This protein plays an important role on cellular calcium homeostatic, to relocate calcium into and so on between cytosol, where the highest targeted calcium was located, extracellular space or organelle luminal space.

Calcium signalling coordinates the motility, cell invasion, and egress from host cell of this apicomplexan parasite that until recently the mediators was not known. Lourido et al., (2012) states that *Toxoplasma gondii* calcium-dependent kinase 1 (TgCDPK 1) is needed during invasion, TgCDPK1 and TgCDPK3 during egress from host cell. During host cell invasion, *Toxoplasma gondii* secret PLA2 with microneme3, activates phosphate inositol signalling and continues with calcium release from internal pool. From statistical test, this study has proven significant difference of calcium measurement between control and infected group, and the calcium intensity transformation during 2 hours, 24 hours and 48 hours of infection. This result of this research supported transformation of neural intracellular calcium and during *Toxoplasma gondii* infection, and showed that calcium signalling as a first messenger and second messenger works in this process.

5. Conclusion

The confocal laser microscopy is able to detect intracellular calcium on control group and infected group. It also detects intracellular calcium by capturing fluorescent light and reflecting light from the specimen using light detectors. The outputs of the specimen image then presented on image monitor.
Intracellular neuron calcium intensity was significantly different between the control and *Toxoplasma gondii* infected neuron groups. It was because *Toxoplasma gondii* may alter neural calcium concentration. Among the 3 groups of infected neuron (2 hours infection, 24 hours infection and 48 hours infection), there was significant difference of calcium intensity inside the neuron and parasite cells. The calcium intensity in *Toxoplasma gondii* was gradually increasing while the intensity of calcium in neurons was gradually declining. This condition caused significant difference between control and *Toxoplasma gondii* infected groups on neuron intracellular neuron calcium intensity.

**Acknowledgment**

This study was supported by Medical Faculty of Universitas Airlangga.

**References**

[1] Nagamune K et al 2008 Calcium Regulation and Signaling in Apicomplexan Parasites Molecular mechanism of Parasite Invasion pp 70-81.
[2] Dobrowolski, JM, Carruthers, VB and Sibley LD 1997 Participation of myosin in gliding motility and host cell invasion by *Toxoplasma gondii*. Mol. Microbiol. 26 pp 163-173.
[3] Lovett, JL, and Sibley L.D., 2003. Intracellular calcium stores in *Toxoplasma gondii* govern invasion of host cells. J. Cell Sci. 116 pp 3009-3016.
[4] Rozenfeld C 2005 *Toxoplasma gondii* Prevents Neuron Degeneration by Interferon-γ-Activated Microglia in a Mechanism Involving Inhibition of Inducible Nitric Oxide Synthase and Transforming Growth Factor-β1 Production by Infected Microglia *The Americal Journal of Pathology*, vol. 167 Issue 4. October 2005 1021-1031.
[5] Tsien, RY 1989 Fluorescent indicators of ion concentrations Methods Cell Biol 30 pp 127-156.
[6] Carruthers V B and Sibley L D 1997 Sequential protein secretion from three distinct organelles of *Toxoplasma gondii* accompanies invasion of human fibroblast Eur. J. Cell Biol. 73 pp 114-123.
[7] Bonhomme A 1999 Signaling during the invasion of host cells by *Toxoplasma gondii* FEMS Microbiol Rev 23 pp 551-561.
[8] Mondragon R and Frixione E 1996 Ca2+-dependence of conoid extrusion in *Toxoplasma gondii* tachyzoites. J. Eukaryot. Microbiol. 43 pp 120-127.
[9] Bouchot A, Zierold K, Bonhomme A, Killian L, Belloni A, Balossier G, Pinon J and Bonhomme P 1999 *Tachyzoite* calcium changes during cell invasion by *Toxoplasma gondii* Parasitol Res 85 pp 809-818.
[10] Brecht S, Carruthers V B and Ferguson D J 2001 The toxoplasma micronemal protein MIC4 is an adhesin composed of six conserved apple domains J. Biol Chem 276 pp 4119-27.
[11] Couvreur J, Desmonts G, 1962 Congenital and Maternal toxoplasmosis A review of 300 congenital cases Dev. Med. Child Neurol. 4 pp 519-530.
[12] Dobrowolski J M and Sibley L D 1996 *Toxoplasma* invasion of mammalian cells is powered by the actin cytoskeleton of the parasites Cell 84 pp 933-939.
[13] Dunn RB 1999 *Mother-to-child* transmission of toxoplasmosis: risk estimates for clinical counseling. Lancet. 353 pp 1829-1833.
[14] Grynkiewicz, G, Poeni M and Tsien RY 1985 A new generation of Ca2+ indicators with greatly improved fluorescent properties. J. Biol. Chem. 260 pp 3440-3450.
[15] Krebs J and Michalak M 2007 *Calcium : A Matter of Life or Death* first edition (Amsterdam:Elsevier) pp 5-12.
[16] Lin L L 1993 cPLA2 is phosphorylated and activated by MAP kinase Cell 72 pp 269-278.
[17] Lourido S, Tang K, and Sibley L D 2012 Distinct signalling pathways control *Toxoplasma* egress and host-cell invasion *The EMBO Journal* 31 pp 4524-34.
[18] Monteiro VG 2001 Morphological changes during conoid extrusion in *Toxoplasma gondii* tachyzoites treated with calcium ionophore J Strukt Biol 136 pp 181-189.
[19] Morisaki J H, Heuser J E and Sibley L D 1995 Invasion of *Toxoplasma gondii* occurs by active...
penetration of the host cell J Cell Sci 108 pp 2457–64.

[20] Viera M C, and Moreno S N 2000 Mobilization of intracellular calcium upon attachment of Toxoplasma gondii tachyzoites to human fibroblasts is required for invasion Mol Biochem. Parasitol 106 pp 157-162.