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Imaging marine virus *CroV* and its host *Cafeteria roenbergensis* with two-photon microscopy

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ABSTRACT

We use two-photon microscopy to monitor the infection process of marine zooplankton, *Cafeteria roenbergensis* (*C.roenbergensis*), by *Cafeteria roenbergensis* virus (*CroV*), a giant DNA virus named after its host. Here, we image *C.roenbergensis* in culture by two-photon excited NADH autofluorescence at video-rate (30 frame/s), and the movement of *C.roenbergensis* is recorded in live videos. Moreover, *CroV* is stained with DNA dye SYBR gold and recorded simultaneously with this two-photon microscope. We observed the initial infection moment with this method. The result demonstrates the potential use of two-photon microscopy to investigate the fast dynamic interaction between *C.roenbergensis* with virus *CroV*. After catching this initial moment, we will freeze the sample in liquid nitrogen for cryo-electron microscopy (EM) study to resolve the virus-host interaction at molecular level. The long-term goal is to study similar fast moving pathogen-host interaction process which could lead to important medical applications.

Keywords: Two-photon fluorescence microscopy, Cafeteria roenbergensis, Cafeteria roenbergensis virus

1. INTRODUCTION

In ocean, viral abundance ranges from approximate 10^7 to $10^{10}L^{-1}$, and 10^7 to 10^{10} g^{-1} of dry weight in marine sediments[1-2]. Marine viruses have been shown directly and indirectly to play critical roles in natural microbial communities in the oceans [2-5].Viruses are major causes of mortality of marine microorganisms. Therefore, marine viruses influence nutrient recycles, impact genetic diversity in oceans [2-6] and might also affect the climate changes [1].

Viral infections are extremely abundant in oceans, estimated to be 10^{23} per second [5]. Viral infection in marine are largely unknown, and very little research has been done on viewing the infection process between marine viruses and their hosts. It is still a mystery for the first step of the infection about how the viruses get into the host, whether it is going through the phagocytosis or penetrating the host cell membrane directly. *C.roenbergensis* is a eukaryotic organism which has been found in all ocean water, especially in coastal water. *CroV*, a giant virus named after its host, has significant influence on the mortality of *C.roenbergensis* population, *CroV* attacks *C.roenbergensis* only, and leave many other similar organisms unaffected [7], this specific infection between *C.roenbergensis* and *CroV* would be a perfect host-virus interaction system to explore viral infection. However, *C.roenbergensis* swims fast, in addition, the infection speed between *C.roenbergensis* and *CroV* are also very fast and complicated, therefore, there are many difficulties and challenges for traditional imaging techniques to view viral-host interaction.

Optical multiphoton microscopy (MPM) is opening new windows for biomedical research[8]. Two-photon fluorescence microscopy (TPFM) is one example of such novel optical imaging technology. TPFM is a very attractive tool for scientists to explore dynamic processes *in vivo* at a cellular level[9-11]. The benefits by using TPFM are from the ability of optical sectioning, deeper tissue penetration and less photobleaching and photodamage[12].The lateral resolution of TPFM could be as high as several hundred nanometers[13]; by using fast scanning approaches, the imaging speed of the TFPM could reach to real-time video rate[14]. Therefore, it is a suitable optical live imaging modality to monitor the interaction between fast moving viruses and hosts. To observe the interaction between *C.roenbergensis* and *CroV*, a two-photon laser scanning

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fluorescence microscopy was used to explore the viral infection process. We image *C.roenbergensis*in culture by two-photon excited NADH autofluorescence, and we stain *CroV* with SYBR gold dye, and image it simultaneously with two-photon by excited SYBR gold fluorescence. Both NADH and SYBR gold can be excited by using the same laser wavelength at 710nm. Combing a polygonal mirror with a galvanometer, the imaging speed of our TPFM system can be higher than video rate (120frame/s).

2. MATIERAL AND METHOD

2.1 *Sample preparation*

C.roenbergensis and *CroV* samples were prepared and cultured at Department of Biomolecular Mechanisms, Max Planck Institute for Medical Research (Heidelberg, Germany) by using published protocol [15]. 1mL *CroV* sample (1x10^11 particle per mL) was further concentrated by centrifugation at 5,000xg for 30 mins to remove majority of the bacteria. The supernatant was then centrifuged at 18,000xg for 2 hours and virus pellet was re-suspended in 20uL. SYBR Gold stain is superior sensitive for staining DNA and RNA due to the high fluorescence quantum yield of the dye-nucleic acid complexes (approximately 0.7) [16], Fig.1 shows the excitation and emission spectra of SYBR Gold nucleic acid gel stain bound to double-stranded DNA, the excitation peak wavelength of SYBR Gold is around 495nm for single-photon excitation, the emission peak wavelength is round 537nm. SYBR Gold stain is expensive and sensitive to photobleaching and freeze-thaw cycle [17], we used two steps to dilute the manufacturer's stock to avoid this problems. First, we diluted manufacturer's stock of SYBR Gold Nucleic Acid Gel Stain (life technologies, catalog number S-11494) concentration 1:40 by pipetting out 1ul from 40ul of 0.02μm filter-autoclaved MilliQ H_2O, and substitute in 1ul SYBR Gold Nucleic Acid Gel Stain manufacturer's stock, this stock can be stored at -20°C for up to 1 week; We further diluted 1:5 to make a 1:200 dilution of the manufacturer's stock SYBR Gold stain reagent. We stained *CroV* sample by using the same volume of 1:200 dilution of SYBR Gold stain in a dark room, and wait for 15mins until *CroV* were stained completely. In order to reduce SYBR gold dye's impact on *C.roenbergensis*, a dye remove procedure is used to remove SYBR gold dye in *CroV* culture before we mix *C.roenbergensis* and *CroV*. The dye in the *CroV* sample solution was removed by centrifugation at 18,000xg and pelleting as described above twice.

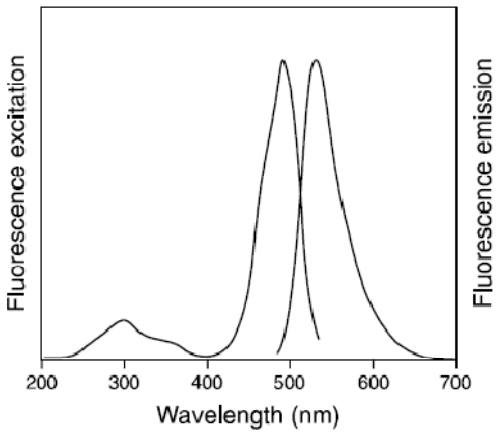


Figure 1. Spectra of SYBR Gold nucleic Acid Gel Stain bounding with double-stranded DNA.

2.2 *Two-photon fluorescence microscopy*

Fig. 2 shows the schematic drawing of our TPFM setup. A mode-locked Ti/sapphire laser(Newport, Spectra-Physics Maitai HP, Wavelength 700-1020nm, 100fs, 80MHz) is employed as the laser source; the laser beam is reflected to a fast spinning polygonal mirror (480 revolutions/s) and a fast swing galvanometer-mounted mirror (30Hz) to obtain the raster scan on sample. A diachronic beam splitter (Semrock, FF660-FDi01) is used to separate the excitation and emission beam; An Olympus water immersion microscope objective lens with a 60x, N.A.1.2 is used to focus the laser beam on the sample. The excitation wavelength can be tuned by the Maitai HP control programmer on computer, and the excitation power on the sample can be adjusted by combining a quarter wave plate and a linear polarizer as a laser attenuator. A blue detection channel and a green detection channel are built in order for detecting the fluorescence signals from *C.roenbergensis* and *Cro.V* separately, and the signals are separated by another diachronic beam splitter (Semrock, FF495-FDi01). Blue range

filters (2 Edmund 447/60nm, 1 Chroma 445/50nm) in blue detection channel and green range filters (2 Chroma HQ522/70nm, 1 Semrock FF01-650sp-25) in green detection channel are used respectively to filter out the residual pump. The green fluorescence signal is detected by a photo multiplier tube PMT A (Hamamatsu R3896), and the blue fluorescence signal is detected by another multiplier tube PMT B (Hamamatsu R10699). The voltage of these two PMTs are controlled by two customized power suppliers, correspondingly. The *C.roenbergensis* and *CroV* fluorescence signals are acquired simultaneously by a frame grabber (MatroxSolioseA/XA) and recorded on personal computer at 30 frames/s. A reflection channel is built by placing a beam splitter before the polygonal mirror, an avalanche photo-diode (HAMAMATSU, part No. S2381) is used to collect the reflection signal from the sample, and the reflection signal is acquired by the frame grabber and recorded on computer. A light source from a 660nm-680nm laser diode hits on the polygonal mirror and is detected by a bi-cell to synchronize the polygonal mirror and galvanometer.

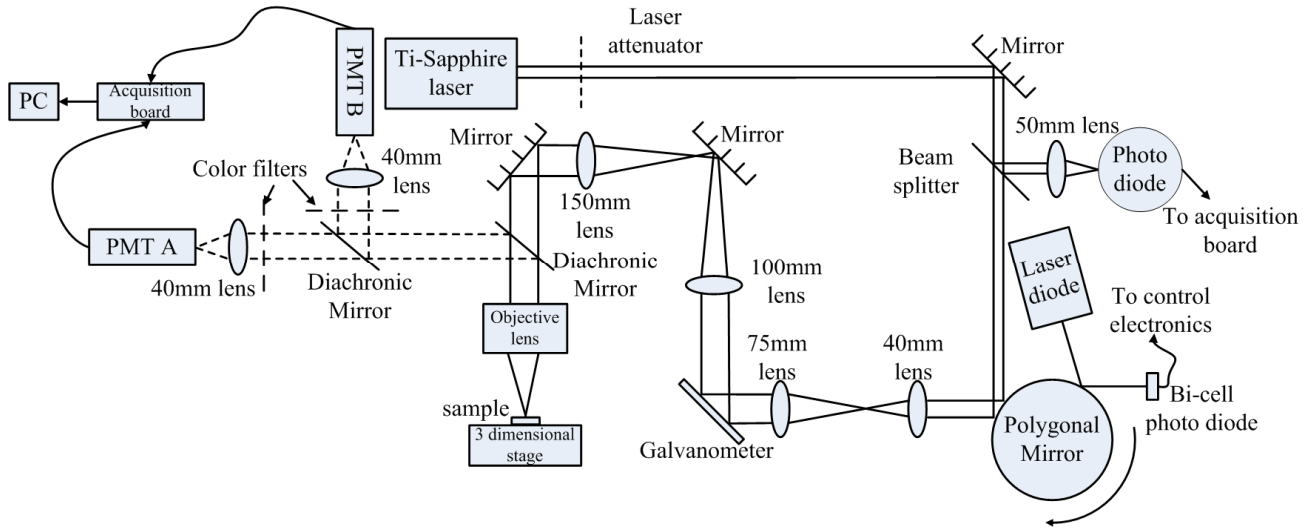


Figure2. Schematic drawing of two-photon laser scanning fluorescence microscopy. PMT: photo multiplier tube. PC: personal computer.

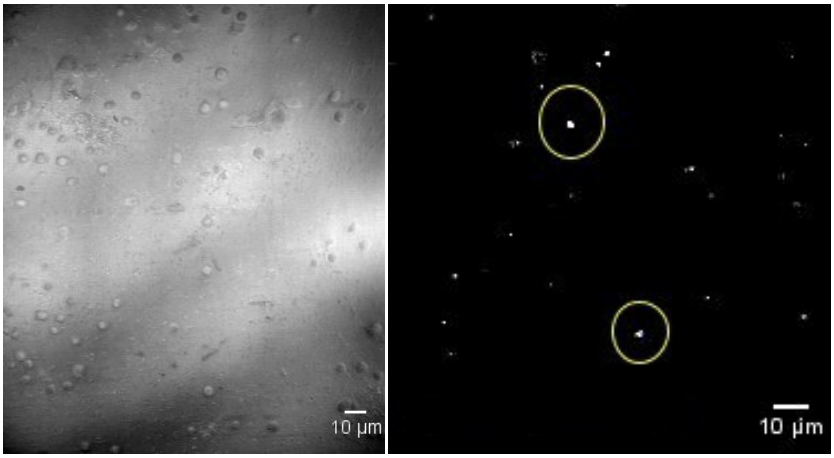
3. RESULTS

3.1 *C.roenbergensis*imaging

We dripped 4uL *C.roenbergensis* sample on slide glass and covered it with cover glass. The images of *C.roenbergensis* are shown in Fig.3 (a) and Fig.3 (b). Fig.3 (a) is the image obtained in reflection channel, the laser wavelength is 710nm, the laser power is about 20mW on the sample, and the voltage of the photodiode is about 142V. Fig.3 (b) shows the NADH autofluorescence signal from *C.roenbergensis* in the blue detection channel, the excitation wavelength is 710nm, the laser power is about 20mW on the sample and the voltage of the PMT A is 3.3V. From Fig.3 (b), it can be seen clearly that the NADH signal from *C.roenbergensis* can be obtained. The focal plane of the fluorescence channel in axial direction is about 1um, which is much thinner than that of the reflection channel, hence, although the sample contains many *C.roenbergensis* which are shown in Fig.3(a), only a few of them can be viewed in blue detection channel in Fig.3(b).

3.2 *CroV* imaging

In *CroV* imaging, the slide glass and cover glass were cleaned by 90% Acetone to reduce potential environmental contamination. Fig. 4 shows the SYBR gold stained *CroV* image obtained from the green detection channel. In this image, the excitation laser wavelength is 710nm, the power on the sample is about 20mW, and the PMT A is about 1.84V. The size of *CroV* is about 280nm [7]. The circle indicates *CroV* and the rectangle indicates bacteria in the sample.



(a) (b)

Figure 3. *C.roenbergensis* imaging. (a) Image of *C.roenbergensis*in reflection channel, and (b) NADH autofluorescence image of *C.roenbergensis*. Circle indicates *C.roenbergensis*.

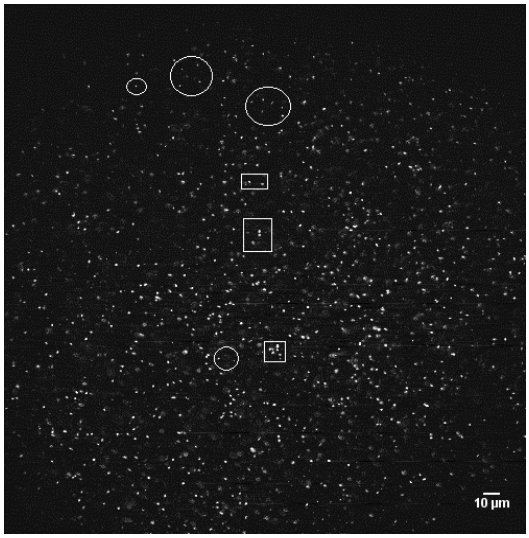


Figure 4. SYBR gold stains *CroV* imaging.Circle indicates *CroV* and square indicates bacteria.

3.3 *Interaction between C.roenbergensis and CroV*

We cleaned the slide glass and cover glass by using 90% Acetone to reduce potential environmental contamination, and dripped 4uL *C.roenbergensis* and 2uL *CroV* on the slide glass and covered it with cover glass. We put and recorded the sample under our two-photon fluorescence microscopy immediately after the sample is ready. The interaction process between *C.roenbergensis* and *CroV* are shown in Fig.5(a) to (d) and media 1. In Fig. 5(a) to (d), the excitation laser wavelength is 710nm, the excitation power on the sample is about 20mW, the voltage of PMT A and PMT B is 1.75V and 3.3V respectively. Video 1 is movie recorded at video-rate (30 frames/second). Fig.4(a) is one frame captured at time 0.57s. In Fig.4(a), a *C.roenbergensis* which is shown in the circle, is approaching to a *CroV*, which is shown in the ellipse; Fig. 4(b), which was captured at time 0.60s, shows the *C.roenbergensis* is contacting with the *CroV*; Fig 4(c) and (d), which were captured at time 1.17s and 1.43s respectively, show the *CroV* is traveling with *C.roenbergensis*, which means the *C.roenbergensis* has been infected by the *CroV*. Video 1 shows the complete infection procedure between *C.roenbergensis* and *CroV*.

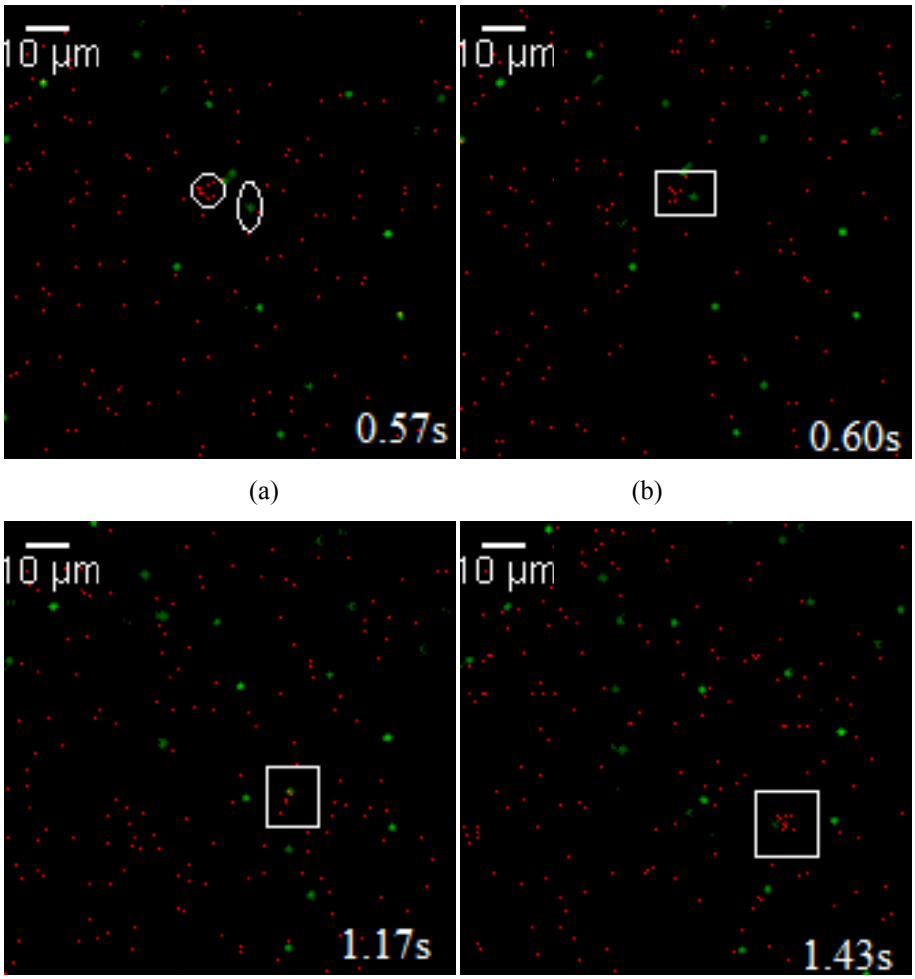


Figure 5. Interaction between *C.roenbergensis and Cro.V.* Image (a)-(d) are single frame captured at time 0.57s, 0.6s, 1.17s and 1.43s, respectively; (a) Image of a *C.roenbergensis* is moving to a *CroV;* Circle indicates the *C.roenbergensis*, ellipse indicates the *CroV*; (b) Image of interaction moment between the *C.roenbergensis and* the *CroV*; Rectangular indicates both the *C.roenbergensis* and *CroV*; (c) and (d) Images of the *C.roenbergensis and* the *CroV* are travelling together, which indicate that the *C.roenbergensis* has been attached by the *CroV*. Rectangular indicates both the *C.roenbergensis* and *CroV*.

Video 1. A Movie, which recorded at 30 frames per second, shows the complete interaction between *C. roenbergensis* and *CroV*. http://dx.doi.org/10.1117/12.2041121.1

4. DISCUSSION AND CONLUSION

In this study, *cafeteria roenbergensis* and *cafeteria roenbergensis virus* are visualized by using two-photon fluorescence microscopy, the *C.roenbergensis* was imaged by exciting and detecting the NADH autofluorescence signal; and the *CroV* was viewed by staining with SYBR gold nucleic acid gel stain. Both *C.roenbergensis* and *CroV* can be imaged by using 710nm excitation wavelength. Moreover, we have demonstrated the initial interaction between *C.roenbergensis* and *CroV* by using two-photon fluorescence microscopy, making this a potentially useful technique to track the viral replica process longitudinally in the living host and even in humans. The initial interaction time between *C.roenbergensis* and *CroV* is so short (<0.57s), which means it is highly possible that the *C.roenbergensis* was infected by *CroV* through the phagocytosis, but further studies are required to confirm this infection processing. Furthermore, by combining TPFM with transmission electron microscopy, which could resolve the virus-host interaction at molecular level, the complete process of host-viral interaction system can be demonstrated, which will significantly expand the applications of two-photon fluorescence in immunology research and lead to important medical applications.

5. REFERENCES

[1] Danovaro, R., Corinaldesi, C., Dell'Anno, A., Fuhrman, J. A., Middelburg, J. J., Noble, R. T. and Suttle, C. A.,"Marine viruses and global climate change," FEMS microbiology reviews, 35(6), 993-1034(2011).

[2] Wilhelm, S. W. and Suttle, C. A., "Viruses and nutrient cycles in the sea." Bioscience, 49(10), 781-788(1999).

[3] Fuhrman, J. A., "Marine viruses and their biogeochemical and ecological effects." Nature,399(6736), 541-548(1999).

[4]Wommack, K. E. and Colwell, R. R., "Virioplankton: viruses in aquatic ecosystems." Microbiology and Molecular Biology Reviews, 64(1), 69-114(2000).

[5] Suttle, C. A., "Viruses in the sea." Nature, 437(7057), 356-361(2005).

[6] Wilhelm, S. W., Weinbauer, M. G., Suttle, C. A., Pledger, R. J. and Mitchell, D. L., "Measurements of DNA damage and photoreactivation imply that most viruses in marine surface waters are infective." Aquatic microbial ecology, 14(3), 215-222(1998).

[7] Massana, R., Javier D. C., Christian D. and Ruben S., "Crash of a population of the marine heterotrophic flagellate Cafeteria roenbergensis by viral infection." Environmental microbiology, 9(11), 2660-2669(2007).

[8] Hoover, E. E. and Jeff A. S., "Advances in multiphoton microscopy technology." Nature Photonics, 7(2): 93-101(2013).

[9] Li, C., Costas P., Judith M. R., Daniel C. and Charles P. L., "Multiphoton microscopy of live tissues with ultraviolet autofluorescence." IEEE Journal of Selected Topics in Quantum Electronics, 16(3): 516-523(2010).

[10] Li, C., Pastila, R. K., Pitsillides, C., Runnels, J. M., Puoris'haag, M., Côté, D. and Lin, C. P., "Imaging leukocyte trafficking in vivo with two-photon-excited endogenous tryptophan fluorescence." Optics express, 18(2), 988-999(2010).

[11] Li, C., Pastila, R. K. and Lin, C. P., "Imaging immune response of skin mast cells in vivo with two-photon microscopy." Proc. SPIE 8207, 82070F-82070F(2012).

[12] Zipfel, W. R., Williams, R. M. and Webb, W. W., "Nonlinear magic: multiphoton microscopy in the biosciences." Nature biotechnology, 21(11), 1369-1377(2003).

[13]James E.N. J., Jim S., Holger K., Alexander R. and Ernst H.K. S., "Resolution in Optical Microscopy." Methods in Enzymology, 360(Biophotonics Part A), 416-446(2003).

[14]Veilleux, I., Spencer, J. A., Biss, D. P., Cote, D. and Lin, C. P., "In vivo cell tracking with video rate multimodality laser scanning microscopy." IEEE Journal of selected topics in quantum electronics, 14(1), 10-18(2008).

[15] Noble, Rachel T. and Jed A. Fuhrman., "Use of SYBR Green I for rapid epifluorescence counts of marine viruses and bacteria." Aquatic Microbial Ecology, 14(2): 113-118(1998).

[16]Tuma, R. S., Beaudet, M. P., Jin, X., Jones, L. J., Cheung, C. Y., Yue, S. and Singer, V. L., "Characterization of SYBR Gold nucleic acid gel stain: a dye optimized for use with 300-nm ultraviolet transilluminators." Analytical biochemistry, 268(2), 278-288(1999).

[17] Patel, Anand, Rachel T. Noble, Joshua A. Steele, Michael S. Schwalbach, Ian Hewson and Jed A. Fuhrman. "Virus and prokaryote enumeration from planktonic aquatic environments by epifluorescence microscopy with SYBR Green I." Nature protocols, 2(2): 269-276(2007).