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Dual-color fluorescence imaging in a nude mouse orthotopic glioma model

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Abstract

We sought to establish a new orthotopic glioma model of nude mice by transfer of DsRed2, a red fluorescent protein gene, to malignant glioma cells and to perfuse the tissue with fluorescein isothiocyanate (FITC) dextran in vivo, which would permit the concurrent detection of brain tumor invasion and angiogenesis in vivo by fluorescence microscopy. 9L or U87 malignant glioma cells with DsRed2 expression were intracerebrally injected into the nude mice. FITC dextran was administered intravenously to the mice bearing DsRed2-9L or DsRed2-U87 cells immediately before they were sacrificed at 10 days or 15 days after the implantation, respectively. Coronal vibratome sections were examined using 2D and 3D fluorescence microscopy and the results were compared with those examined by routine hematoxylin and eosin (H & E) staining. Angiogenesis induced by glioma was confirmed by two-dimensional and three-dimensional imaging analysis. DsRed2 fluorescence clearly demarcated the primary tumor margins and readily allowed for the visualization of local invasion at the single-cell level in the brain adjacent to tumor. We found that a few tumor cells migrated from the tumor mass along the aberrant microvasculature, but did not extend out of the angiogenic areas. However, locally invasive foci were very difficult to detect by H & E staining. We demonstrated, for the first time, that abnormal vascular structure and glioma cells can be visualized concurrently by fluorescence microscopy. This method is superior to H & E staining for the detection and study of physiologically relevant patterns of brain tumor invasion and angiogenesis in vivo.

Keywords

Glioma; tumor model; angiogenesis; invasion; fluorescence

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Introduction

Despite decades of advancement in neurosurgery and neuro-oncology, malignant brain tumors still have a high recurrence rate and confer a dismal clinical prognosis. Malignant gliomas, the most aggressively invasive of adult brain tumors, have extremely high levels of neovascularization and rank among the most angiogenic of all human tumors (Bogler and Mikkelsen, 2003; Lopes, 2003). Angiogenesis has become one of the most intense areas of cancer research since studies pioneered by Judah Folkman showed that the development of tumor-induced vasculature was essential for tumor growth beyond an initial small size (Folkman, 2003). This remarkable finding afforded important new insights into the mechanisms regulating tumor growth and, perhaps most importantly, indicated that newly induced blood vessels offered promising new therapeutic targets (Yang et al., 2003).

Diffuse infiltration of tumor cells into normal brain is another major problem in the treatment of gliomas (Holtkamp et al., 2005). These invasive tumor cells are clonogenic and can be isolated and grown in tissue culture (Silbergeld and Chicoine, 1997). This implies that after treatment of a glioma, a single invasive cell can give rise to a recurrent tumor (Giese and Westphal, 2001). Identification of individual or even small numbers of invading tumor cells in the brain adjacent to tumor (BAT), however, may be impossible by standard immunohistochemistry or routine hematoxylin and eosin (H & E) techniques (MacDonald et al., 1998). A major problem encountered in studies of brain tumor invasion has been the inability to establish an experimental in vivo model that could clearly facilitate the detection and subsequent analysis of brain tumor micrometastasis and physiologically relevant stages of local spread and neovascularization.

The green fluorescent protein (GFP) or red fluorescent protein (RFP) gene can be stably transduced into cancer cell lines, which subsequently express GFP or RFP at high levels in vitro and in vivo, including primary and metastatic tumor deposits. This technology has been applied in fluorescent orthotopic models of pancreatic cancer (Bouvet et al., 2002; Bouvet et al., 2000; Sun et al., 2003), lung cancer (Rashidi et al., 2000), prostate cancer (Yang et al., 1999), breast cancer (Yang et al., 2001), and cervical cancer (Cairns and Hill, 2004) (Lu et al., 2003). These GFP and RFP models have enabled the analysis of the dynamics of a wide variety of tumor behaviors, such as growth, invasion, and metastasis, which could not otherwise be easily detected and monitored in vivo. However, there are very few orthotopic tumor models that can precisely visualize the interaction between tumor cells and the hyperproliferative tumor vasculature amid the surrounding tissue.

In this study, we established a new orthotopic malignant glioma model of nude mice by transfer of the RFP gene to 9L or U87 malignant glioma cells and intravenously administered fluorescein isothiocyanate (FITC) dextran immediately before they were killed, which enables visualization of angiogenesis and tumor through dual-color fluorescence imaging. The stable high-level DsRed2 expression by the brain tumor cells allows for the easy visualization of single-cell invasion in the BAT under fluorescence microscopy, without the addition of further staining or histological preparation. Two-dimensional (2D) and three-dimensional (3D) analytical software provide novel images of clear demarcation of primary and satellite tumor tissue in conjunction with precise quantification of cerebral microvasculature, including tumor-induced microvasculature in tumor-bearing brain.

Materials and Methods

All experimental procedures have been approved by the Institutional Animal Care and Use Committee of Henry Ford Hospital.

DsRed2-9L and DsRed2-U87 malignant glioma cell culture

New stable U87 and 9L cell lines (ATCC, Manassas, VA) expressing a red fluorescent protein, modified from *Dicosoma* sp, under the control of the CMV promoter were obtained by transfection with pDsRed2-N1 vector (Clontech, Mountain View, CA), complexed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Clones were selected for stable plasmid DNA integration in G418 for two (9L) or three weeks (U87). Several clones were amplified and screened for high DsRed expression and normal proliferation rate.

In vitro invasion assay

Matrigel invasion assays were used to assess the ability of the DsRed2-9L and DsRed2-U87 cells to penetrate the ECM compare to their parent cells. Invasion of cells through Matrigel was determined using 24-well BD invasion chambers (8.0µm pore size with polycarbonate membrane; BD Biosciences, Cowley, UK) in accordance with the manufacturer's instructions with the following modifications (Zheng et al., 2007; Zheng et al., 2009). BD invasion chambers were pre-hydrated with serum-free DMEM (500µl/well) for 2h of incubation at 37°C in 5% CO₂. After trypsinization, 9L, DsRed2-9L, U87, and DsRed2-U87 were suspended in medium without serum (500µl) in a concentration of 1×10^5 cells/well and immediately placed onto the upper compartment of the plates. Subsequently, the lower compartment was filled with complete medium (750µl). The cells were incubated for 24 h. Following incubation, the non-invading cells were removed from the upper surface of the membrane by wiping with cotton-tipped swabs. 9L and U87 cells on the lower surface of the membrane were stained with CellTracker Green (Molecular Probes, Eugene, OR) for 45 minutes and fixed in 4% paraformaldehyde. DsRed2-9L and DsRed2-U87 cells on the lower surface of the membrane will be taken pictures without staining under red channel. Five fields of adherent cells were randomly counted in each well under a fluorescent microscope at 4X magnification, and the results were counted and averaged.

DsRed2-9L or DsRed2-U87 malignant glioma cell implantation in nude mice

Athymic mice (n=10; 20–22g) employed in this study were obtained from the National Cancer Institute (Frederick, MD). Nude mice were anesthetized with ketamine (80mg/kg) and xylazine (13mg/kg) administered intramuscularly (i.m.). Atropine (0.02ml) was injected (i.m.) at the time of anesthesia induction. After fixing the mouse's head in the stereotaxic device, a 5–6 mm incision was made directly down the midline, and the scalp was retracted and the cranium was exposed. Using a drill, a 3-mm craniectomy was made over the right hemisphere anterior to the coronal suture. A 10µL Hamilton syringe was introduced to a depth of 2.5 mm beneath the dura and 5×10^4 DsRed2-9L cells or 5×10^5 DsRed2-U87 cells in a 5µL volume were implanted through the burr hole during a 5-minute period (n=5 per group). The craniectomy was covered with a piece of polyvinyl chloride film glued to the surrounding intact bone and the incision was then closed with 4-0 silk sutures (Ethicon, Somerville, NJ).

Tissue preparation

FITC dextran (2×10^3 kDa molecular weight, Sigma, St. Louis, MO; 0.1 ml of 50 mg/ml) was administered intravenously to the mouse at 10 days and 15 days after the implantation of DsRed2-9L and DsRed2-U87, respectively. FITC-dextran remains dissolved and free in plasma. Two minutes after the injection of FITC-dextran, animals were sacrificed. The brains were rapidly removed and placed in 4% of paraformaldehyde at 4°C for 48 hours. Coronal sections (100 µm thick) were cut on a vibratome. Every other section was stained with H & E for light microscopic examination and the other vibratome sections were prepared for fluorescent microscopic examination.

Tumor volume examined by H & E staining

The tumor volume was measured by a blinded experimenter to the treatment groups. Each H & E section was evaluated under a 4× objective (BX40; Olympus Optical Co. Ltd., Nagano, Japan) using a 3-CCD color video camera (DXC-970MD; Sony Corp., Tokyo, Japan) interfaced with the microcomputer imaging device (MCID) image analysis system (Imaging Research; St. Catharines, Canada). On each coronal section, the area of the tumor was measured by tracing the demarcation of the tumor on the computer screen, and the tumor volume (mm^3) was determined by the sum of the values produced by multiplying the appropriate tumor area by the section interval thickness.

Fluorescence of vascular structure and tumor in cerebral tissue

Using 2D imaging of vibratome sections, vascular structure and tumor in the mouse brain were studied. FITC and DsRed2 fluorochromes on the sections were excited by a laser beam at 488 nm and 568 nm, respectively, and emissions were simultaneously acquired with two separate photomultiplier tubes through 522 nm and 585 nm emission filters, respectively. Coronal sections at 100 μm interval were digitized under a 4× objective (Olympus BX40) using a 3-CCD color video camera (Sony DXC-970MD) interfaced with MCID image analysis system. In each coronal section, the areas of the entire tumor and aberrant microvasculature were measured by tracing the respective tissue boundaries on the computer screen. The aberrant microvasculature was identified by enlarged vascular diameters and decreased vessel lengths compared to adjacent normal microvasculature. The tumor and aberrant microvasculature volumes (mm^3) were calculated by the sum of multiplying the respective areas by the section interval thickness.

Three dimensional image acquisition and analysis

To examine dynamic changes in cerebral blood vessels in the tumor area, we performed morphologic analysis of vessels in the area of tumor as well as in the contralateral hemisphere of the mouse brain in three dimensions. Our 3D quantitative analysis program has features to measure number of vessels, number of branch points, segment lengths, and vessel diameters (del Zoppo, 1994; Jiang et al., 2008; Zhang et al., 2002). The vibratome sections were analyzed with a Bio-Rad MRC 1024 (argon and krypton) laser-scanning confocal microscopy (LSCM) imaging system mounted onto a Zeiss microscope (Bio-Rad; Cambridge, MA), as previously described (Zhang et al., 1999). Briefly, a series of 100- μm -thick coronal sections were selected from the beginning to the end of tumor microvessels. Every 5th coronal section per mouse was selected for 3D image analyses. Three fields in the tumor area (Figure 1a, b, and c) and another three in the homologous area of the contralateral hemisphere (Figure 1a', b' and c') were scanned in 512 × 512 pixel (279 × 279 m^2) format in the x-y direction using a 4× frame-scan average and twenty-five optical sections along the z-axis with a 1 μm step-size were acquired under a 40× objective. The number of vascular branch points, segment lengths and vessel diameters were measured. Abnormal vasculature was identified in 3D analysis by increases in mean vessel diameter and number of vessel branch points, coincident with a decrease in mean vessel length.

Results

Results obtained using the 24h in vitro invasion assay revealed that the invasiveness of DsRed2-9L was not significantly different compare to 9L cells. Also, DsRed2-U87 did not show a significant difference in the vitro invasion assay compared to U87 cells (Figure 2). This result indicates that DsRed2 transfection does not change the invasiveness of 9L or U87 glioma cells.

Red color tumor cells (Figure 3a and b) and prominent green abnormal vascular structure (Figure 3c and d) were demonstrated by the 2D imaging analysis method in the tumor-containing vibratome sections from either the DsRed2-9L bearing (Figure 3a, c, and e) or the DsRed2-U87 bearing mice (Figure 3b, d and f). Substantial angiogenesis occurred in the tumor area. To confirm our observation from the 2D imaging analysis, we measured the reconstructed 3D cerebral vessels in both the tumor area and the homologous contralateral area derived from the original images obtained from LSCM imaging system. Red, green and blue colors in Figure 4 code for blood vessels with diameters smaller than 7.5 μ m, between 7.5 μ m and 30 μ m, and between 30 μ m and 50 μ m, respectively. Quantification of the 3D fluorescent imaging revealed a significant increase in number of vessel branch points and vessel diameters, and a significant decrease in segment length of the cerebral vessels (Table 1, 2) in the tumor regions (Figure 4a) compared to the vessels in the homologous regions of the contralateral hemisphere (Figure 4b).

There was no significant difference in the tumor volume measured on the H & E sections and on the fluorescent vibratome sections (data not shown). The red tumor mass was embedded within the green excessively proliferative blood vessels in the fluorescent images from all of the 10 mice (Figure 5a). The volume of the aberrant microvasculature was larger than that of the tumor in both the DsRed2-9L bearing mice and the DsRed2-U87 bearing mice (Figure 5a & Figure 6). From the merged fluorescent images (Figure 7c and d), we found that a few tumor cells migrated from the tumor mass along the aberrant microvasculature, but did not extend out of the angiogenic areas. Therefore, we propose that angiogenesis is a prerequisite for the invasion of DsRed2-9L and DsRed2-U87 gliosarcoma cells.

By using dual-color fluorescence imaging, we were able to clearly demarcate the BAT (Figure 5a and Figure 7), which is the tumor-induced angiogenic area (outlined by the purple line in Figure 5a and Figure 7b and c) outside the tumor mass (outlined by the blue line in Figure 5a and Figure 7c). Cerebral vessels in this area exhibited significantly increased vessel diameters. Three-dimensional images obtained from laser scanning confocal microscopy showed sprouting and splitting vessels. Numbers of vessels, numbers of branch points were significantly increased in this area compared to contralateral tissue. However, with the routine H & E staining, we were unable to observe the single invading tumor cells surrounding the tumor mass in the BAT, which showed same brain structures in morphology compared to contralateral brain tissue and no tumor foci present (Figure 5b). Therefore, compared to routine H & E histopathology, which failed to recognize most of the small local foci of early invasion in the BAT, using DsRed2 to label tumor cells followed by vessel perfusion with a green fluorescent dye increased the sensitivity of detecting single invading cells, and their relationship to brain microvasculature.

Discussion

Green fluorescent protein (GFP), produced from the jellyfish *Aequorea Victoria*, has been used for repetitive, non-invasive reporter-gene imaging in living cultured cells, single cell organisms and multi-cellular organisms transparent to light (Chalfie, 1995; Chalfie et al., 1994), allowing direct visualization of proteins without the need for harsh fixation methods, which can be plagued by artifacts. A red fluorescent protein gene with homology to GFP (drFP583) was afterwards cloned from *Discosoma coral* (Matz et al., 1999), and a humanized version of drFP583, called DsRed, was then made commercially available. Even though DsRed shares only 30% amino-acid similarity with GFP, the two crucial residues (tyrosine-66 and glycine-67) that contribute to the chromophore of GFP are conserved. DsRed protein as a fluorescent marker has distinct advantages over GFP due to the wavelength of its emission spectrum (575–625 nm vs 500–550 nm). Tissue penetration of light at this wavelength is greater, and the auto-fluorescence of mouse tissues is less intense, resulting in an enhanced signal to noise ratio compared to results obtained with GFP (Cairns and Hill, 2004). DsRed1,

a humanized version of drFP583, has been the subject of extensive research to determine its biochemical and photophysical properties. DsRed2 used in the present study is a new variant of DsRed1. DsRed2 contains six amino acid substitutions: A105V, I116T, S197A, R2A, K5E, and K9T. These mutations improve the solubility of DsRed2 by reducing its tendency to form aggregates and also reduce the time from transfection to detection to only 24 h (Lu et al., 2003). DsRed2 retains the benefits of a red fluorescent protein, such as a high signal-to-noise ratio and distinct spectral properties for use in multicolor labeling experiments. Furthermore, the DsRed2 signal was also shown to be brighter than GFP to permit detection from stably introduced transgenes in viable mammalian cells by a fluorescent microscopic methodology (Katz et al., 2003). Therefore, we selected DsRed2 gene to transfect 9L and U87 cell lines.

Angiogenesis is an essential prerequisite for tumor growth and development (Folkman, 1995). Angiogenesis is quantitatively most prominent in glioblastoma compared to malignancies elsewhere in the body (Brem et al., 1972), and the patterns of growth of invading malignant glioma cells and angiogenesis in this study suggest that these processes are fundamentally related. Our dual-color, tumor-vascular interaction, malignant glioma animal model offers the opportunity to concurrently visualize the red fluorescent tumor cells and the tangled and interdigitating network of green fluorescent tumor vessels amid the surrounding brain parenchyma. The application of our 3D microvasculature analysis program in conjunction with this model allows us to precisely measure the vascular changes, and thus provides additional insight into the angiogenic process and its interaction with tumor invasion. Our observations confirm the critical role of tumor-induced angiogenesis in the further invasion of tumor cells into the peritumoral area (Vajkoczy et al., 2000). Furthermore, we demonstrated that angiogenesis—the major hallmark of malignant glioma—seems to be permissive for invasion, and our finding is consistent with previous studies that the acquisition of the angiogenic phenotype occurs at the same time the cells migrate away from the main tumor mass (Friedlander et al., 1995).

In tumor-bearing brain, dark purple tumor masses are clearly identified on paraffin sections stained with H & E. We considered that BAT was an area around the main tumor mass of approximately 0.5 to 1 mm in depth, that consists of mixed small multi-focal tumors and non-tumored brain tissue which is slightly darker than the surrounding normal tissue (Jiang et al., 1997). However, it is very difficult to demarcate the BAT area because BAT looks just like normal brain tissue surrounding the tumor mass in H & E slides. Immunohistochemistry may also fail to produce positive staining of tumor cells if there is insufficient intact antigen present or if the antigen is heterogeneously expressed (MacDonald et al., 1998). By stereotactically implanting these DsRed2-expressing cells intracerebrally into nude mice, we are now able to clearly visualize local invasion down to the single-cell level by fluorescence microscopy, which otherwise is difficult or impossible to be seen using either routine H & E staining or standard immunohistochemistry. Our study demonstrates that DsRed2 gene transduction of tumor cells can be a simple but powerful tool for the detection and subsequent analysis of experimental brain tumor invasion and metastasis in vivo.

Some tumor treatments such as surgical resection and clinically relevant doses of photodynamic therapy (PDT) were shown to significantly increase the expression of vascular endothelial growth factor (VEGF) in the BAT (Zhang et al., 2006; Zhang Xuepeng) and thus may induce angiogenesis in the BAT which subsequently contributes to tumor recurrence, since VEGF is an essential angiogenic factor orchestrating glioblastoma angiogenesis (Plate et al., 1994) and neovascularization has been demonstrated to support tumor survival, growth, and likely invasiveness (Jiang et al., 2004). However, with previous methods, the area of BAT could not be clearly demarcated. By using this dual-color fluorescent imaging in the orthotopic malignant glioma model, BAT can be demarcated as the area of aberrant vasculature outside the tumor boundary. Cerebral vessels in this area exhibited significantly increased vessel

diameters. Three-dimensional images obtained from laser scanning confocal microscopy showed sprouting and splitting vessels. Numbers of vessels, numbers of branch points were significantly increased in this area compared to contralateral tissue. The area within the boundary of the tumor mass appears on H&E slides like normal brain tissue, however the area contains angiogenic vessels and invading tumor cells. Therapeutic options targeting tumor blood flow and angiogenesis-related tumor invasiveness in the peripheral tumor regions may offer new hope for glioma management. By measuring the angiogenesis in the tumor-bearing brain, we can establish correlation between tumor growth and the extent of neovascularization and angiogenic activity present at the time of sacrifice.

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Figure 1. Schematic diagram of a representative coronal section from a tumor-bearing mouse. a, b, and c are three fields selected from the tumor area, while a', b' and c' are three fields selected from the homologous area of the contralateral hemisphere for 3-dimensional analysis of tumor-induced angiogenesis.

Figure 2.

Results obtained using the 24h in vitro invasion assay revealed that the invasiveness of DsRed2-9L was not significantly different from 9L cells; DsRed2-U87 did not show significant difference in the vitro invasion assay compare to U87 cells.

Figure 3.

Representative fluorescent images under 40 times magnification collected from the sections with the maximum tumor area of the brain. Panels a and b are the fluorescent images of DsRed2-9L (a) and DsRed2-U87 (b) tumor cells (red), collected from mice sacrificed at 10 days and 15 days after tumor implantation, respectively. Panels c and d show the cerebral vessels (green) in the DsRed2-9L (c) and DsRed2-U87 (d) tumor regions, collected from the mice perfused with FITC-dextran immediately before they were sacrificed. Panel e is merged from a and c. Panel f is merged from b and d. Panel g and h show the cerebral vessels (green) in the contralateral regions, Bar = 400 μ m.

Figure 4.

Representative LSCM images of the microvasculature of nude mouse brain bearing DsRed2-U87 tumor cells. Mice were sacrificed 15 days after DsRed2-U87 cell implantation. Panel a was obtained from the tumor area. Panel b was obtained from the contralateral homologous tissue. Color-coding highlights different vessel diameters.

Figure 5.

Panel a is a representative fluorescent image under 40 times magnification collected from a DsRed2-9L tumor-containing section. Nude mice implanted with DsRed2-9L cells (red) were sacrificed 10 days after tumor cell implantation. Cerebral vessels were perfused with FITC-dextran (green) before animals were sacrificed. Panel b is a representative image of H & E staining under 40 times magnification collected from the contiguous section of panel a. In panel b, the boundary of the tumor mass is highlighted by the white line. The area of the tumor mass in panel a is approximately equal to that in panel b. The BAT can be defined as the area demarcated between the boundary of the tumor mass (outlined by the blue line in panel a) and the boundary of angiogenesis (outlined by the purple line in panel a), however this area appears like normal brain tissue in the H & E-stained sections. Bar=1mm.

Figure 6.

The volume of the tumor mass was significantly smaller than the volume of the tumor-associated vasculatures in the nude mice bearing either 10 days of DsRed2-9L tumors or 15 days of DsRed2-U87 tumors. * $p < 0.05$; ** $p < 0.05$.

Figure 7.

Representative fluorescent images collected from the sections with the maximum tumor area of the brain. Panel a (40 times magnification) shows a few red DsRed2-9L cells (arrows) migrating from the tumor mass (red). Panel b (40 times magnification) shows cerebral vessels (green) after perfusion with FITC-dextran in the same region with panel a. The left lower area in panel b is a tumor-induced angiogenic region (outlined by the purple line). Panel c is merged from panel a and b and demonstrates that migrating cells spread from the tumor mass (outlined by the blue line) along the angiogenic vessels (outlined by the purple line) in the BAT. Panel d shows migrating red tumor cells (arrows) and green angiogenic vessels at higher magnification

Table 1
Indices of the vessels in both the tumor area and the contralateral homologous tissue in LSCM images. Cerebral vessels in the tumor area exhibited significantly increased numbers of branch points, significantly increased vessel diameters, and significantly decreased segment lengths, compared to those in the homologous regions of the contralateral hemisphere.

	Contralateral	9L, 10 days	p	Contralateral	U87, 15 days	p
Segment length (μm)	35.44 ± 3.16	28.37 ± 2.55	<0.05	36.28 ± 2.1	27.89 ± 3.12	<0.05
No. of branch points	24.93 ± 2.85	31.47 ± 4.26	<0.05	23.27 ± 3.57	33.14 ± 5.07	<0.05
Branch diameter (μm)	4.53 ± 1.07	6.82 ± 1.16	<0.05	4.38 ± 1.43	7.93 ± 2.02	<0.05

Table 2

Percentages of the vessels in different diameter ranges in LSCM images. The tumor area exhibited significantly increased in percentages of cerebral vessels with 7.5 μ M above in diameter.

Diameter (percentage %)	Contralateral	9L, 10 days	Contralateral	U87, 15 days
<7.5 μ m	89.5 $\pm$ 10.4	55.7 $\pm$ 9.3	87.4 $\pm$ 11.9	60.4 $\pm$ 10.0
7.6–30 μ m	10.5 $\pm$ 3.2	36.1 $\pm$ 5.9	12.6 $\pm$ 2.8	32.4 $\pm$ 3.7
30–50 μ m	0	5.8 $\pm$ 1.0	0	5.6 $\pm$ 1.2
>50 μ m	0	2.4 $\pm$ 0.4	0	1.6 $\pm$ 0.3