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Quantification of cell infection caused by *Listeria monocytogenes* invasion

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ABSTRACT

Listeria monocytogenes causes a life-threatening food-borne disease known as Listeriosis. Elderly, immunocompromised, and pregnant women are primarily the victims of this facultative intracellular Gram-positive pathogen. Since the bacteria survive intracellularly within the human host cells they are protected against the immune system and poorly accessed by many antibiotics. In order to screen pharmaceutical substances for their ability to interfere with the infection, persistence and release of *L. monocytogenes* a high content assay is required. We established a high content screen (HCS) using the RAW 264.7 mouse macrophage cell line seeded into 96-well glass bottom microplates. Cells were infected with GFP-expressing *L. monocytogenes* and stained thereafter with Hoechst 33342. Automated image acquisition was carried out by the Scan^R screening station. We have developed an algorithm that automatically grades cells in microscopy images of fluorescent-tagged Listeria for the severity of infection. The grading accuracy of this newly developed algorithm is 97.1% as compared to a 74.3% grading accuracy we obtained using the commercial Olympus Scan^R software.

1. Introduction

Listeria monocytogenes is a Gram-positive pathogenic bacterium that causes a food-borne disease called Listeriosis in both humans and animals. Listeriosis is a rare but serious disease with a high overall mortality rate of 30%, most common in pregnant women or immunocompromised individuals (Ramaswamy et al., 2007). The bacteria cause severe gastroenteritis and central nervous system infections and are able to cross the intestinal, materno-fetal and blood–brain barrier (Barbuddhe and Chakraborty, 2009; Drevets and Bronze, 2008).

L. monocytogenes is a facultatively intracellular pathogen, which has evolved several mechanisms for exploiting the hosts' cellular machinery for infection and proliferation (Portnoy et al., 2002). Because of its ability to transit from extracellular environments to the intracellular milieu of infected eukaryotic cells, it is an important model organism for infection, intracellular proliferation and host–pathogen interactions and presently represents one of the most well studied pathogens (Cossart, 2007). Several Listeria strains with varying virulence and pathogenicity are known (Liu et al., 2003; Roche et al., 2003).

The pathogen can provoke its internalisation in normally non-phagocytic cells such as fibroblasts and can survive the engulfment by macrophages (Birmingham et al., 2008). The cellular invasion and proliferation of cells occurs in consequential stages (Portnoy et al., 2002). First the pathogen is internalised or provokes its internalisation in host cells with virulence factors called internalins (Seveau et al., 2007). Inside the cell, the bacterium lyses the vacuole and escapes to the cytoplasm, where it proliferates. By recruitment of the hosts' actin cytoskeleton, the bacteria move in the cytoplasm and spread from one cell to another (Vázquez-Boland et al., 2001). Those intracellular bacteria are sheltered from the host immune system and are poorly accessible for treatment with antibiotics. Therefore, the invasion of the host cells is an important and crucial step in Listeria pathogenesis and virulence (Ireton, 2007).

This pathogenesis and infection can be elucidated using in vivo or in vitro cell culture infection assays (Liu et al., 2007). In conventional in vitro cell assays, a defined number of Listeria is incubated with a confluent mammalian cell layer (Roche et al., 2001). After a defined incubation time extracellular bacteria are washed away or killed by gentamycine treatment. The remaining intracellular bacteria are enumerated on solid agar medium after lysis of the

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host cell. An average number of infected bacteria per cell can be calculated by this procedure.

However, not all macrophage cells are infected by bacteria equally, and the asymmetry and heterogeneity of cellular cultures and bacteria contribute to this variability. Furthermore, the infection and intracellular replication is a dynamic process with different infection time points for each cell. For this reason we have set up a high content screen using automated microscopy and *L. monocytogenes* expressing the green fluorescent protein (GFP). The interesting feature of high content imaging is more than one grey value is associated to a pixel, so the GFP signal can be linked to individual cells or cell compartments, depending on the interpretation of the other grey values. However, this increase in information comes with a necessity to apply computational tools to analyze the data, since the human observer is not able to visually integrate the information from several grey value images on his/her own.

In this paper, we show how image processing can be applied to fully automatically analyze high content screen images including a full segmentation of the image into individual cells and classification of each cell regarding its degree of *L. monocytogenes* infection. To the best of our knowledge, no work has been done so far to quantify the severity of cell invasion by *L. monocytogenes* from microscopic images. The automation of image segmentation and classification in microscopy has been considered before within the computer science/bioinformatics community and just recently, the term bioimage informatics has been introduced. Although automated cell segmentation still remains to be a difficult task and only a small number of approaches have been proposed that try to make segmentation obsolete in classification contexts (such as Lessmann et al., 2007), a variety of well-performing algorithms have been proposed for the problem of automated cell segmentation in microscopy images (Bamford and Lovell, 1998; Chen et al., 2006; Coelho et al., 2009; Garrido and Perez de la Blanca, 2000; Herold et al., 2010b; Malpica et al., 1997; Nattkemper et al., 2001; Nattkemper, 2004a; Yang et al., 2006). Since new imaging platforms and sophisticated labelling techniques allow high content imaging, new algorithmic approaches are needed that combine segmentation with classification based on the interpretation of more than one channel (Herold et al., 2010a; Nattkemper, 2004b). We describe two algorithms for automatic image analysis and compare their performance to a gold standard derived by an expert (N. Jensen, one of the authors). The first method is based on the commercially available Olympus Scan^R analysis software and reaches a grading accuracy of 74.3%. The LiMISGA (*L. monocytogenes* Infection Screening and Grading Algorithm) method proposed in this article achieves a grading accuracy of 97.1%.

2. Materials and methods

2.1. Sample preparation

Mouse macrophages RAW 264.7 were seeded at a density of 20,000 cells per well in a 96 well glass bottom microplate. *L. monocytogenes* EGDe Serotype 1/2a (Leimeister-Wächter and Chakraborty, 1989) expressing the green fluorescent protein (GFP) (Zelmer et al., 2005) was used. After 24 h, around 10^6 GFP expressing *L. monocytogenes* were introduced in each well for 60 min. Following the processes of washing, fixation and permeabilisation, nuclei and cytoplasm are stained using 10 μM Hoechst 33342 (Sigma) and a 1:500 dilution of Whole Cell Stain Red (Thermo Scientific) in PBS for 30 min. After further washing steps the cells were used for microscopy (see Fig. 1 for a system overview).

2.2. Image acquisition

The image acquisition was done with the Scan^R screening station (Olympus). Images were taken with a 40× objective (N.A. 0.9) and standard filter sets for bisbenzimide Hoechst 33342 (ex. 360–370 nm, em. 420–460 nm), GFP (ex. 451–490 nm, em. 500–530 nm) and WCS Red (ex. 590–650 nm, em. >650 nm). The software autofocus was used in the bisbenzimide channel. Throughout this manuscript, we will use the term nuclei channel to refer to the bisbenzimide channel image showing the nuclei. The term cell channel is used to refer to the cytoplasm channel image labelled with WCS red and the term Listeria channel refers to the GFP signal (see Fig. 2).

2.3. Preprocessing and thresholding using Scan^R

In Scan^R analysis, an automated background correction was applied for every channel. Objects (cells) and the sub-objects (nuclei and Listeria) were identified with an intensity threshold based algorithm. The Scan^R approach allows two options, one of which is an automatic determination of the threshold which is based on evaluation of the grey value histogram. Since these results were not convincing, we applied another approach, which was simply based on tuning threshold parameter on one example image and keeping this constant for the entire set of images. Please note that we do not propose to do that in practice and use this approach just as baseline for evaluating our image processing tool LiMISGA. The threshold for recognition of the cells was manually tuned to 480 in the WCS channel, objects between 1000 and 100,000 pixel size were detected. Objects on the border were excluded from analysis. An area of 30 pixels around the cell borders was used to define the extracellular area for every cell. Nuclei were recognized with a threshold of 740 in the nuclei channel. Objects between 300 and 50,000 pixel size were classified as an object.

3. Results and discussion

3.1. Functional staining and automated microscopy of the infection process

If four images are taken per well, the screening of an entire 96-well microplate took about 40 min. In vivo imaging was possible over several hours using only the bisbenzimide and GFP channels (data not shown). To obtain the best image quality, cells were fixed and permeabilised. All images used for the quantification of the infection process were obtained ex vivo. The acquired images were then used for object and sub-object recognition by the Olympus Scan^R software (see Sections 2.3 and 3.2 for details) or the algorithm proposed in this paper (see Section 3.3 for details). The recognized objects are counted and classified by their area (Fig. 1). Using a 40× objective (N.A. 0.9) turned out to be the best compromise between field of view and resolution of details needed to monitor the infection process.

3.2. Grading infection states in the *L. monocytogenes*–macrophage interaction manually and by the Scan^R software

Automated microscopy resulted in well resolved micrographs consisting of three channels (Fig. 2a–d). Intracellular enumeration of Listeria and their penetration depth determines the aggressiveness and the degree of infection. Therefore, the ultimate goal would be an exact counting of all *L. monocytogenes* within one host cell. Unfortunately, counting of all bacteria would require 3D-imaging that slows down the throughput and results in huge amounts of data. For this reason, the exact number of *L. monocytogenes* cells