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Hyperspectral microscope imaging methods for multiplex detection of *Campylobacter*

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Campylobacter **is an emerging zoonotic bacterial threat in the poultry industry. The current methods for the isolation and detection of *Campylobacter* are culture-based techniques with several selective agars designed to isolate *Campylobacter* colonies, which is time-consuming, labour intensive and has low sensitivity. Several immunological and molecular techniques such as enzyme-linked immunosorbent assay (ELISA) and Latex agglutination are commercially available for the detection and identification of *Campylobacter*. However, these methods demand more advanced instruments as well as specially trained experts. A hyperspectral microscope imaging (HMI) technique with the fluorescence *in situ* hybridisation (FISH) technique has the potential for multiplex foodborne pathogen detection. Using Alexa488 and Cy3 fluorophores, the HMI (450–800 nm) technique was able to identify *Campylobacter jejuni* stains with high sensitivity and specificity. In addition, HMI was able to classify six bacteria using scattering intensity from their spectra without a FISH fluorophore. Overall classification accuracy of quadratic discriminant analysis (QDA) method for six bacteria including *Bifidobacter longum*, *Campylobacter jejuni*, *Clostridium perfringens*, *Enterobacter cloacae*, *Lactobacillus salivarius* and *Shigella flexneri* using the HMI technique without fluorescent markers was approximately 88.6 % with pixel-wise classification.**

Keywords: acousto-optic tuneable filters, *Campylobacter*, detection, poultry, food safety, hyperspectral microscope imaging

Introduction

Foodborne illnesses are a burden on public health and contribute significantly to the cost of health care. Each year foodborne illnesses sicken 48 million Americans (approximately 17 % of people in the United States) and lead to 128,000 hospitalisations and 3000 deaths,[1] so reducing foodborne illness by improving food safety practices is a high priority of the US Department of Agriculture (USDA). Among many foodborne pathogens, the highest incidence of infections include *Campylobacter*, *Cryptosporidium*, *Salmonella*, *Shiga* toxin-producing *Escherichia coli* O157 (STEC), *Shigella* and *Yersinia*.[2] *Campylobacter* are members of the family campylobacteraceae. These bacteria are

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Gram-negative and can be spiral-shaped, S-shaped or curved, which exhibit characteristics of corkscrew motility mediated by polar flagella.[3] A cell may possess either a single polar flagellum or two at both ends of the cell.[4] *Campylobacter* is a bacterium that can cause an illness called campylobacteriosis in humans. Also, *Campylobacter* is the major group of bacteria responsible for foodborne gastroenteritis in humans worldwide.[5] In the United States, there are about 845,000 cases per year.[6] Although these illnesses are rarely lethal, fatalities can occur when immunocompromised individuals are infected. In terms of *Campylobacter*, 209 foodborne outbreaks were reported, resulting in 2234 illnesses from 2010 to 2015 in the United States.[6] In the European Union (EU), *Campylobacter* is the most frequently reported foodborne illness with over 190,000 human cases annually. However, the actual number of cases is around nine million each year, which resulted in an economic burden to the EU to be estimated around EUR 2.4 billion per year due to campylobacterosis.[7] In particular, poultry, raw milk and untreated water have been the most commonly identified sources of *Campylobacter* outbreaks. The UK Food Standards Agency[8] (FSA) has launched a programme together with the Department for Environment, Food & Rural Affairs, the UK poultry industry and major retailers to reduce *Campylobacter* levels in chickens.[8] Their target is to reduce the numbers of the highest contaminated birds in UK poultry houses from 27 % to 10 % by 2015.[7]

Culture-based methods

Culture-based techniques for isolation and detection of *Campylobacter* from foods are described in detail.[9] In brief, the pathogen is isolated by culturing on selective media[10] followed by incubation at 41.5 °C for 44 hours under microaerobic conditions. Food and environmental samples need an additional pre-enrichment step designed to facilitate the recovery of damaged cells. Enrichment can be done using a selective enrichment broth medium, which is then incubated at 37 °C for five hours. Clinical samples can be cultured directly onto selective agar media. Several selective agars designed to isolate *Campylobacter* colonies are commercially available. These media contain various selective agents and most of them are antibiotics that suppress the growth of other enteric bacteria. Pre-enrichment media contain ingredients that protect the cells from the damaging effects of toxic oxygen derivatives.[11] These include lysed or defibrinated blood, charcoal, a combination of ferrous sulphate, sodium metabisulphite and sodium pyruvate (FBP).

After isolation, similar to other pathogens, *Campylobacter* identification is carried out based on their morphological, biochemical and growth characteristics. Most identification methods include Gram staining and biochemical tests such as catalase, oxidase, hippurate hydrolysis and nitrate/nitrite reduction. Although these culture-based methods are relatively cost effective and require no sophisticated equipment, they have several limitations. Most significant drawbacks include the time required to obtain the final results and the limited response of *Campylobacter* to biochemical tests. Moreover, these techniques are labour intensive and have lower sensitivity compared to serological and molecular methods. There is also the possibility of *Campylobacter* cells entering the viable but not culturable (VBNC) state under unfavourable conditions, resulting in false negative results.

Various methods of culture preparation including cell separation and concentration by filtration or centrifugation can be done to accelerate the enrichment process.[12]

Rapid detection methods

Several immunological and molecular techniques are commercially available for the detection and identification of *Campylobacter*.[13] These techniques offer rapid, accurate and more sensitive results compared to the traditional methods. Another advantage of these methods is that these techniques can detect *Campylobacter* cells in the VBNC state. However, some of these methods demand more advanced instruments as well as specially trained individuals. They also fail to distinguish between dead and live cells.[14] Various immunoassay systems based on antibody/antigen interactions such as enzyme-linked immunosorbent assay (ELISA)[15] and Latex agglutination[16] are also commercially available. Nucleic acid-based methods such as polymerase chain reaction (PCR) and real-time PCR are commonly available as commercial kits.[17] Other molecular techniques including pulsed field gel electrophoresis (PFGE)[18] and random amplified polymorphic DNA (RAPD)[19] can also be applied for detection and identification of *Campylobacter spp*. Furthermore, a combination of traditional and modern techniques can be used to further enhance the reliability and speed of the result.

Multiplex fluorescence *in situ* hybridisation (FISH) methods

Fluorescence *in situ* hybridisation (FISH) is a molecular cytogenetic technique that uses fluorescent markers, which bind to only those parts of the chromosome with a high degree of sequence complementarity. FISH has been used to detect and localise the presence or absence of specific DNA sequences on chromosomes and specific RNA targets in bacterial cells.[20] Fluorescence microscopy can be used to find out where the fluorescent probe is bound to the chromosomes. With their binding ability to specific targets, multiplex FISH enables us to assay multiple targets and visualise co-localised signals in a single specimen. Using spectrally distinct fluorophore labels for each hybridisation probe, this approach gives us the power to resolve several genetic elements or multiple gene expression patterns through multicolour visual display.[21]

Optical detection without FISH probe

Optical detection has the potential to non-destructively assess food products for the presence of microbial life.[22–24] This technique identifies bacteria based on a spectral profile that is inherently unique to the bacteria. A spectral signature from hyperspectral imaging is composed of two-dimensional spatial and one-dimensional spectral data, resulting in three-dimensional data, the so-called hypercube or datacube. Each pixel within the hypercube generates a spectral pattern from the light scattering intensity values collected at each wavelength. Similarly, hyperspectral microscope imaging (HMI) can acquire hypercubes from live microorganisms mounted on a glass microscope slide. Recently, HMI has been used in bacterial detection for differentiating species[25–27] and serogroups as well as serotypes.[28,29] Using the scattering intensity from microorganisms at the cellular level, HMI with optimal lighting sources (metal halide or tungsten halogen) could differentiate and classify foodborne pathogens without FISH probes. The objectives of this research are 1) to evaluate HMI methods for detection of *Campylobacter jejuni* stains in conjunction with FISH probes and 2) to classify foodborne bacteria using their spectral signatures without FISH probes. Specifically, determining if HMI (450–800 nm) can differentiate between six bacterial species, including *Campylobacter jejuni* at the cellular level.

Materials and methods

Bacterial sample preparation

All cultures were obtained from ATCC (American Type Culture Collection), except *Lactobacillus salivarius* that was from NRRL (Northern Regional Research Laboratory) and *Campylobacter jejuni* 11168 from NCTC (National Collection of Type Cultures), for the experiments. Six bacterial strains, *Lactobacillus salivarius* PVD-32, *Shigella flexneri* A12022, *Campylobacter jejuni* (11168 and 81-176), *Clostridium perfringens* A13124, *Bifidobacterium longum* A15708 and *Enterobacter cloacae* A13047, were streaked on blood agar plates and incubated at 37 °C. Following incubation, microscope sample slides were prepared, similar to the protocol described in Reference 26. In brief, 3–4 colonies from each plate were placed in 100 µL of sterile water, followed by vortexing. 3 µL of the bacterial suspension was spread onto the centre of a glass slide, and allowed to air dry in a biosafety cabinet (Baker, BSC, Sanford, ME, USA) for 15 min. After drying, 0.8 µL of sterile water was added on top of the dried suspension and the glass cover slip was applied by firmly pressing the cover slip to the slide. A drop of immersion oil was added to the top of the cover slip. The total time for slide preparation and image collection was approximately 20 min. In order to collect quality images, all slides were scanned on two different gains of 1.6 % and 3.5 % with the same exposure time of 250 ms.

Campylobacter jejuni with FISH probe

Figure 1 shows a *Campylobacter jejuni* cell image collected by HMI with 250 ms exposure and 3.5 % gain. For multiplex detection of *Campylobacter jejuni*, two strains (11168 and 81-176) were used with two FISH probes including *Eubacteria* attached Alexa488 fluorophore (Eub-Alexa488) and *Campylobacter* attached Cy3 fluorophore (Campy-Cy3).

Table 1 shows two *Campylobacter jejuni* strains with FISH probes including Alexa488 and Cy3. An Alexa488 FISH probe was attached to Eubacteria and a specific Cy3 probe was attached to *Campylobacter jejuni* 11168 strain for both FISH epifluorescence microscopy as well as hyperspectral microscopy to compare their performance.

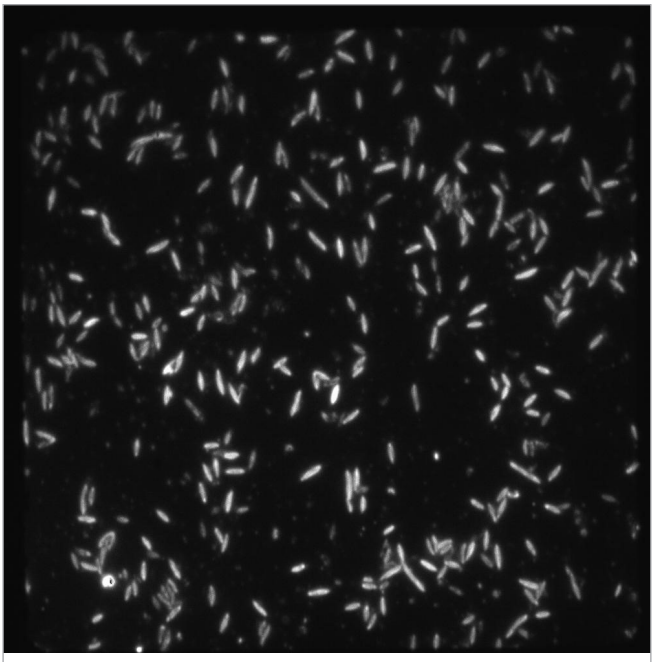


Figure 1. Hyperspectral image from *Campylobacter jejuni* 33560 with 250 ms exposure and 3.5 % gain.

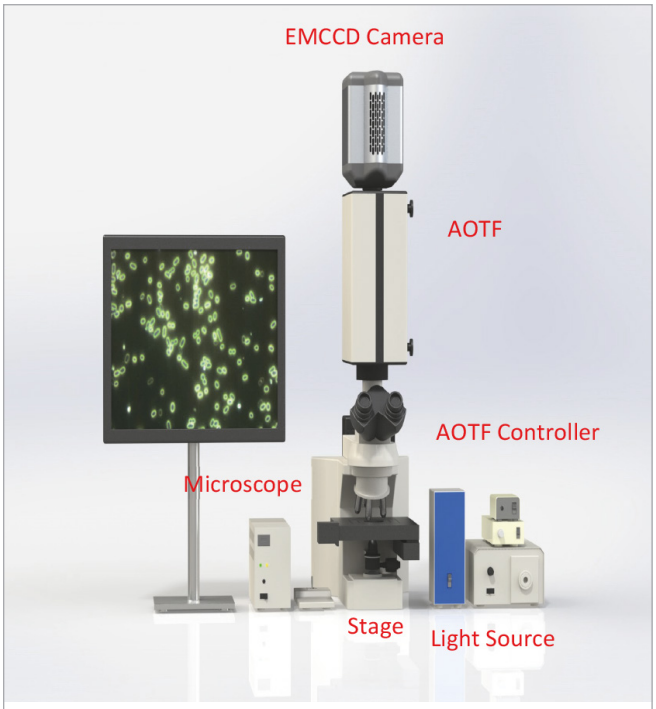


Figure 2. The hyperspectral microscope imaging system. Note: EMCCD = electron multiplying charge coupled device, AOTF = acousto-optic tuneable filter.

Alexa fluor488 and Cy3 have absorbance at 494 nm and 550 nm wavelengths and emission at 517 nm and 570 nm, respectively (Table 2).

HMI system and lighting sources

Figure 2 shows the HMI system. The HMI system consists of an upright microscope (Eclipse e80i, Nikon, Lewisville, TX, USA), mounted with an acousto-optic tuneable filter (AOTF) (HSI-400, Goouch & Housego, Ilminster, UK), a high-performance cooled electron multiplying charge coupled device (EMCCD) 16-bit camera (iXon, Andor Technology, Belfast, UK) mounted on top of the AOTF,

Table 1. *Campylobacter jejuni* with FISH probes.

Well no.	Sample ID	Tag	Number of sample pixels
W9	*C. jejuni* 11168 and *C. jejuni* 81-176	*Eubacteria* probe Alexa488	80,994
W10	*C. jejuni* 11168 and *C. jejuni* 81-176	*Campylobacter* Cy3	161,822
W11	*C. jejuni* 11168 and *C. jejuni* 81-176	*Eubacteria* and *Campylobacter* and competitor	212,091

Table 2. Fluorophore for multiplexing.

Dye	Absorbance wavelength (nm)	Emission wavelength (nm)	Visible colour
Alexa fluor488	494	517	Green (light)
Cy3	550	570	Yellow

and dark-field illuminating light sources: metal halide (MH) or tungsten halogen lamps (TH). The AOTF filter has the capability for a high-speed, high-throughput, random-access solid-state optical filter with an adjustable optical pass-band and exceptionally high rejected light levels.[26] The AOTF delivers diffraction-limited image quality with variable bandwidth resolution within 2 nm. In comparison with other hyperspectral imaging platforms, the system has advantages of wavelength and bandwidth that are changed less than 100 µm with bandwidth 1.5 nm at 450 nm and 3 nm at 800 nm producing linear polarised outputs.

A 24 W MH lighting source with MR-11 reflector (Ushio America, Cypress, CA, USA) was used for the illuminating source. The lighting source has a colour temperature of 5600 K, and operates between 0.3 amps and 0.4 amps. The lighting source was housed in enclosed boxes and kept next to the microscope setup, instead of underneath the slide stage. A fibre optic cable ran from the lighting houses to the base of the microscope, emitting light upward through the sample, and into the AOTF. This was done to avoid bacterial cell injury or death as a result of the heat generated from the lamps with dark-field illumination.[26]

Image and data processing

Hypercubes were collected from the HMI system with dimensions of 1002 (x axis) × 1002 (y axis) × 89 (wavelengths), resulting in over 89 million data points per sample. The x- and y-dimensions represent spatial coordinates of the cells, while the z-dimension represents the light scattering intensity from the cells at each observed wavelength in the spectrum. The high signal-to-noise ratio allows for information from the cells to be extracted from the image using the environment for visualising images (ENVI) software (Harris Geospatial, Boulder, CO, USA) with a global threshold method. Two hyperspectral microscope images were collected from each of the six bacteria. In order to extract useful spectral information of regions of interests (ROI) from bacterial cells, we specified the minimum and maximum intensity threshold values and extracted only useful pixels, discarding the spectra from background or non-cellular objects, as well as oversaturated pixels. Extracted pixels were then randomised. Calibration and validation sets of 5000 pixels each were extracted from the first hyperspectral microscope images, while the test sets of 5000 pixels per image were extracted from the second hyperspectral microscope images. Since images with MH light have a strong excitation peak at 546 nm, all images were max-peak normalised to 546 nm. A mean-centred and cross-validated principal component analysis (PCA) was performed on each of the calibration, validation and test sets using JMP Pro (v.14.0, Cary, NC, USA). Hotellings T^2 values representing each pixel's distance to the model's centroid were used as an outlier detection method, calculated from principal components (PCs) 1–20. Outliers were identified above the test's critical limits and found to be less than 1% for each data set, calibration = 52 (0.17 %), validation = 299 (0.99 %) and test = 120 (0.40 %). PCs 1–20 were extracted for further analysis, and explained 97.5, 97.6 and 95.7% of the PCA's variance for the calibration, validation and test sets, respectively.

Quadratic discriminant analysis

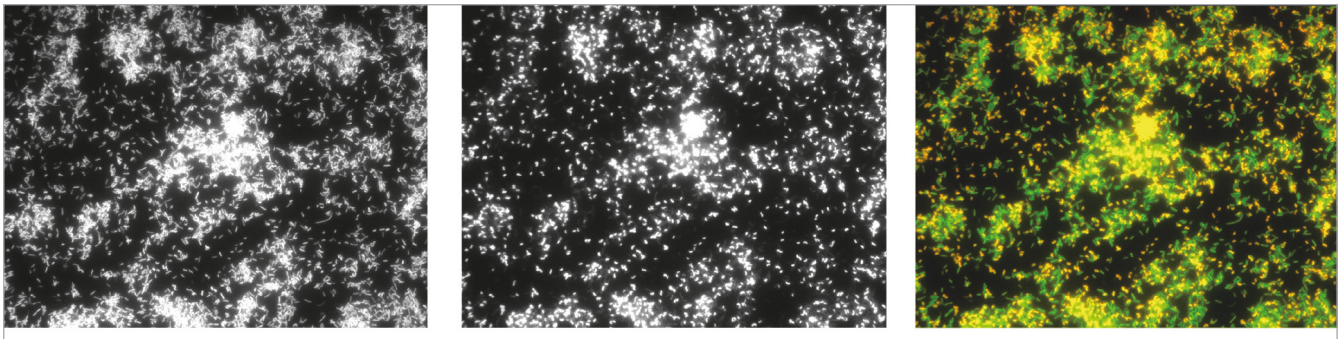
For the classification of six bacterial species from HMI data, quadratic discriminant analysis (QDA) was employed, because QDA is a general version of the linear classifier. Usually QDA is used in statistical classification to separate two or more classes of objects by a quadric surface. Similar to linear discriminant analysis (LDA), QDA assumes that the measurements from each class are normally distributed. However, unlike LDA, there is no assumption in QDA that the covariance of each class is identical.[26] Similar to LDA, QDA uses the Mahalanobis distance to measure the discriminating information between classes.[30] In contrast, even if the distributions are significantly non-Gaussian in the hyperbolic space, the QDA projections may preserve the complex structures in the data needed for classification. Here, the QDA was applied to each of the calibration, validation and test datasets, with leave-one-out cross-validation. PCs 1–20 were used as the input variables, while the bacterial species were used as the prediction variable.

Results and discussion

Fluorophore for multiplexing

Figure 3 shows FISH images from mixture of *Eubacteria* with Alexa488 fluorophore and *Campylobacter* with Cy3 fluorophore using an epifluorescence microscope.

Since the *Eubacteria*-Alexa488 fluorophore was attached to both *Campylobacter jejuni* 11168 and 81-176 strains, more fluorescent cells were observed (Figure 3a) than fluorescence from *Campylobacter jejuni* 11167 strain



(a) (b) (c)

Figure 3. FISH with epifluorescence microscope: (a) W9 *Eubacteria* Alexa 488, (b) W10 *Campylobacter* Cy3, (c) W11 *Eubacteria* and *Campylobacter* competitive (green represents *Campylobacter jejuni* 11168).

only attached to a *Campylobacter*-Cy3 fluorophore (Figure 3b). The colour composite to demonstrate competitive *Campylobacter jejuni* 11167 (green) with *Campylobacter jejuni* 81-176 (yellow) strain is displayed in Figure 3c.

Hyperspectral microscope images from FISH fluorescence

Figure 4 shows fluorescence images with two fluorophores of Alexa488 (Figure 4a) and Cy3 (Figure 4b). The samples (W11) contained mixture of both *Campylobacter jejuni* strain 11168 and strain 81-176. The sample was stained with both *Eubacteria* with Alexa488 and *Campylobacter* with Cy3, and an unlabelled competitor probe, which will block one *Campylobacter* sequence, was added. Images were captured at the same field-of-view with 520 nm for *Eubacteria* (Figure 3a) and 590 nm for *Campylobacter* (Figure 3b). As seen in Figure 3a, more bacteria with the *Eubacteria* probe were observed, because *Eubacteria* with Alexa488 bound to both 11168 and 81-176 strains and bacteria from both *Campylobacter* strains were included. In contrast, *Campylobacter* with Cy3 bound to the 11168 strain only, because the competitor probe blocked another *Campylobacter* strain, resulted in staining only one *Campylobacter*. When these two images were superimposed, two strains of 11168 and 81-176 were observed.

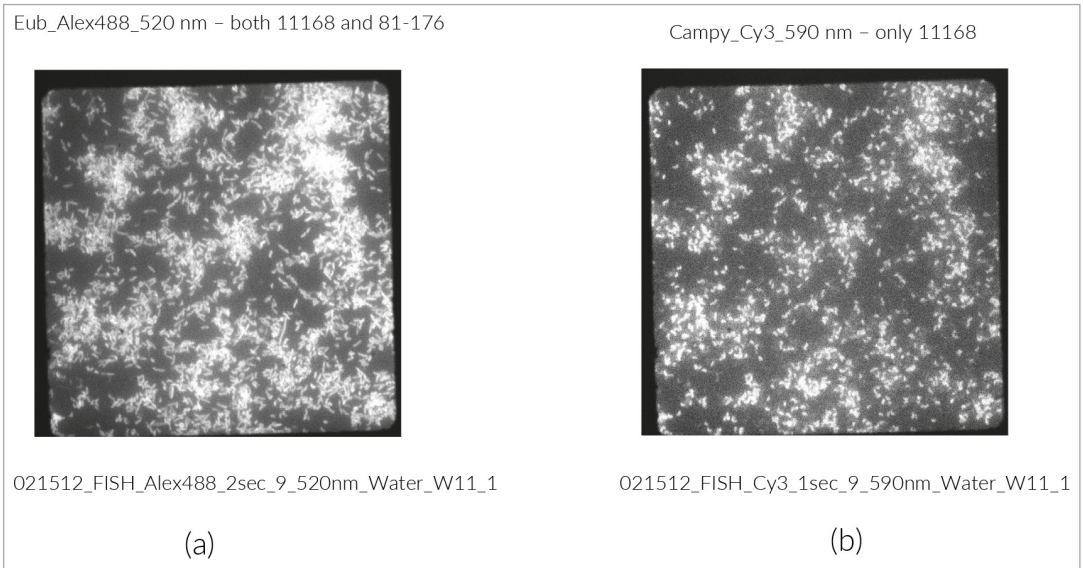
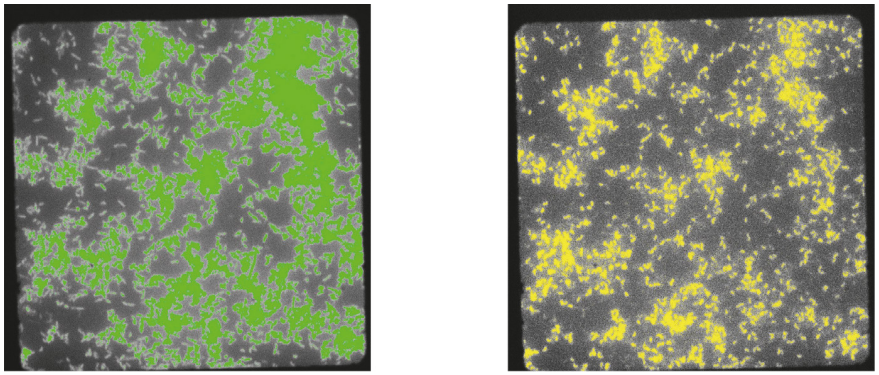


Figure 4. Fluorescence images with two fluorophores for multiplexing *Campylobacter jejuni* detection.



(a) (b)

Figure 5. Mixture of *Campylobacter jejuni* two strains (11168 and 81-176) with (a) specific *Campylobacter*_Cy3 and (b) *Eubacteria*_Alexa488 probe. Note: HMI from *Campylobacter jejuni* (left) and corresponding ROIs to isolate cell only (right) of each sample.

Figure 5 shows mixture of *Campylobacter jejuni* two strains (11168 and 81-176) with specific *Campylobacter*_Cy3 (Figure 5a) and *Eubacteria*_Alexa488 probe (Figure 5b). In both images, HMI images from *Campylobacter jejuni* are on the left and corresponding ROIs to isolate the cell only are on the right of each sample. The number of pixels from Alexa488 and Cy3 fluorophores were detected as 210,701 and 129,400 pixels, respectively. Assuming each cell contains approximated 500 pixels,[29] about 421 *Campylobacter jejuni* cells were observed. Among them, 259 cells were identified as 11168 strain and 162 cells were identified as 81-176 strain. Thus, HMI was able to approximately quantify bacterial cells in a sample.

Samples tested with HMI without FISH

Although FISH has been used as a promising tool for multiplex bacterial detection,[31,32] the more detecting targets, the more difficulties to identify correctly. Also, the performance of the fluorophore could be limited if too many fluorophores are used simultaneously in addition to the question of their cost effectiveness and the extra time required. In contrast, HMI could be a tool for detecting multiple bacteria at the cellular level without a FISH fluorophore. Table 3 shows bacterial samples tested with HMI. All isolates were collected from a blood agar plate. All isolates were grown for 24 h except *Campylobacter*, which were grown for 48 h. Comparing with other bacteria, *Lactobacillus salivarius*_PVD-32 plate colonies were stuck to the agar which was unable to be

Table 3. Samples tested using HMI without FISH.

Isolates	Threshold (min)	Threshold (max)	Number of pixels	Number of cells[a]
Bifidobacter longum A15708	14,000	16,000	149,302	298
Campylobacter jejuni 11168	10,000	16,000	78,553	157
Clostridium perfringens A1312	11,000	16,000	90,590	181
Enterobacter cloacae A13047	9500	16,000	89,594	179
Lactobacillus salivarius PVD-32	12,000	16,000	50,008	100
Shigella flexneri A12022	10,000	16,000	69,713	139

[a]The number of cells was calculated based on one cell containing approximately 500 pixels.

scraped from the colonies (Figure 6f). All colonies were resuspended in deionised water with 3 µL placed on a glass slide for the HMI scan with 250 ms exposure and gains of 3.5 %.

Images and corresponding spectral with MH lighting source

For the purpose of identifying bacteria at the cellular level, dark-field microscopy offers a high contrast method with a large signal-to-noise ratio, by illuminating the unstained live cells onto a dark background from every azimuth, with only light scattered from the living cells collected by the microscope objective.[33] The average cell was found to have approximately 500 pixels, when reconstructing images.

Figure 6 demonstrates the procedure of classification model development using hyperspectral microscope images collected from six pathogenic bacteria including *Bifidobacter longum*, *Clostridium perfringens*, *Enterobacter cloacae*, *Lactobacillus salivarius*, *Shigella flexneri* and *Campylobacter jejuni*. Hyperspectral microscope images were collected from each species (Figure 6a) followed by pixel extraction from cells (Figure 6b) for further processing with randomised pixel data for calibration, validation and test model development using spectral characteristics from six species including *Campylobacter* (Figure 6c). The spectral data were normalised to the max peak at 546 nm with a MH lighting source.

Figure 7 shows six pathogen samples with the *Campylobacter jejuni* strain and their corresponding ROIs for analysis. According to scattering intensity distribution of each species acquired by HMI at same condition, minimum values of thresholding target cells varied between 4500 for the *Campylobacter jejuni* 81-176 strain and 14,000 for the *Bifidobacter longum* A15708 strain. However, maximum thresholding value was 16,000 for all species. More detail minimum thresholding values for selecting ROIs of each species were summarised in Table 3.

After thresholding values were applied to hyperspectral microscope images, the ROIs from each bacterial sample produced the following numbers of pixel data: 149,302 pixels from *Bifidobacter longum* A15708; 78,553 pixels from *Campylobacter jejuni* 11168; 90,590 pixels from *Clostridium perfringens* A1312; 89,594 pixels from *Enterobacter cloacae* A13047; 50,008 pixels from *Lactobacillus salivarius* PVD-32; and 69,713 pixels from *Shigella flexneri* A12022. For HMI data collection, *Lactobacillus* was noted as containing extracellular debris.

A benefit of HMI is the sensitivity of detection, potentially classifying individual bacterial cells. Given that the hyperspectral microscope images collected in this experiment were generated from pure lab-grown cultures of the six bacteria, we can be reasonably confident that all

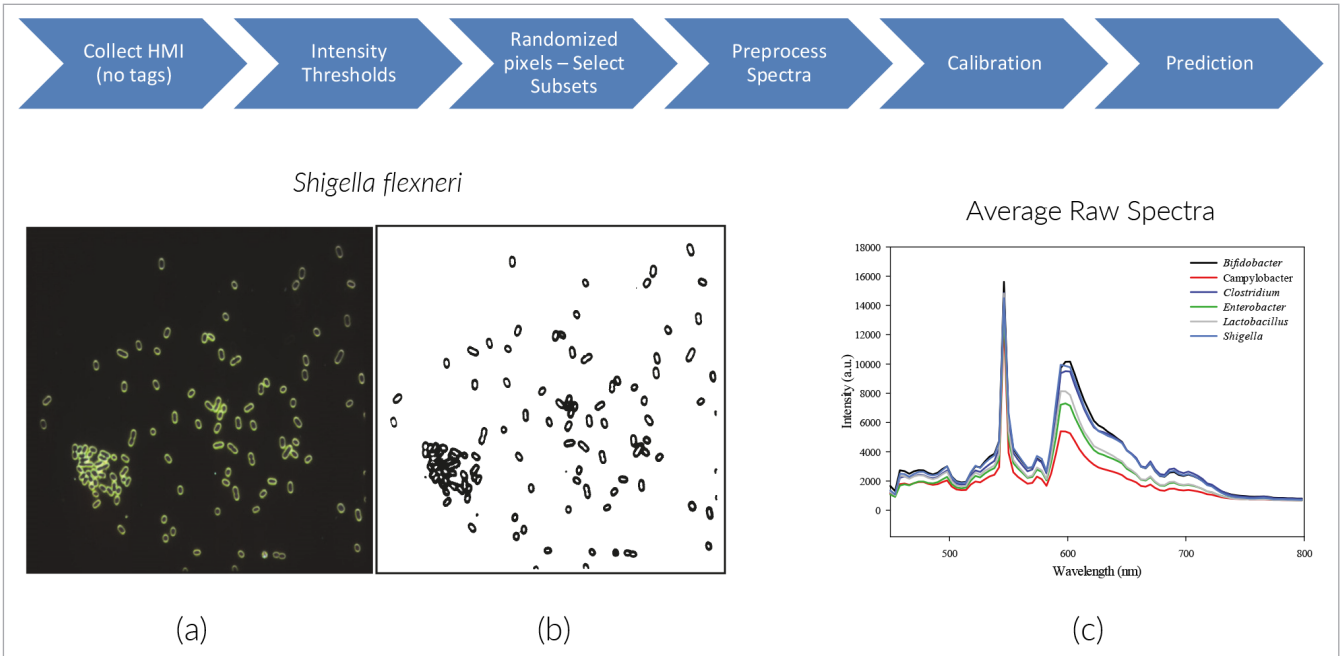


Figure 6. Procedure of hyperspectral microscope image analysis for model development. (a) Hypercube slice at 590 nm, (b) 590 nm with ROI selected and (c) mean raw spectra.

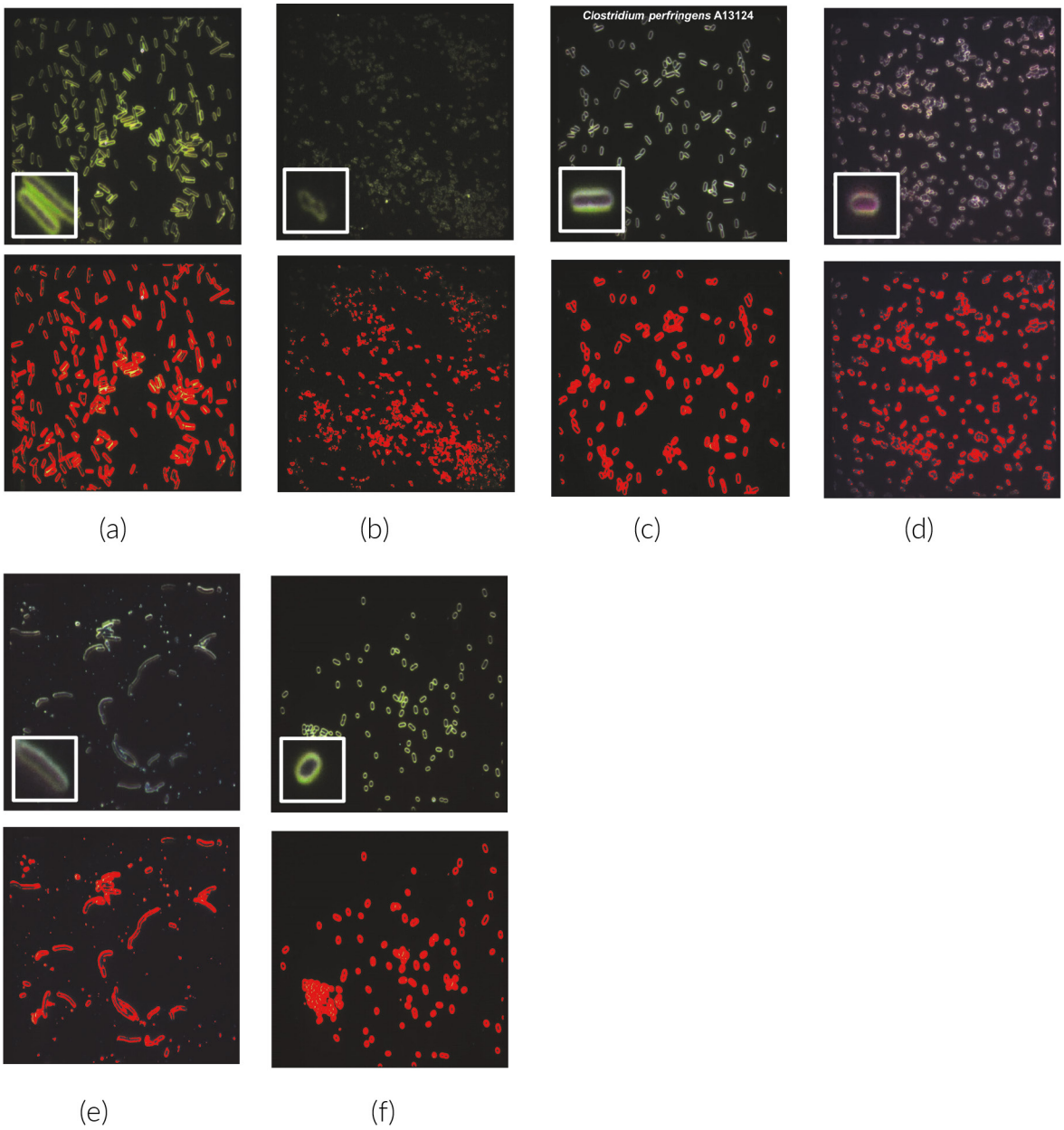


Figure 7. Pathogen samples with ROI from a hypercube at 590 nm for analysis. (a) *Bifidobacter longum* A15708, (b) *Campylobacter jejuni* 11168, (c) *Clostridium perfringens* A1312, (d) *Enterobacter cloacae* A13047, (e) *Lactobacillus salivarius* PVD-32 and (f) *Shigella flexneri* A12022.

the cells in the image are of the same species. Because each cell contains hundreds of pixels, randomisation of the pixels in the hyperspectral microscope images was necessary to avoid subsets representing only a few cells.

Figure 8 shows spectral patterns of six bacterial species including *Bifidobacter*, *Campylobacter*, *Clostridium*, *Enterobacter*, *Lactobacillus* and *Shigella*. In general, peak intensity was observed at 546 nm and 590 nm (Figure 8a) and the variability of scattering intensity was highest between 590 nm and 700 nm. *Shigella* resulted in the highest pixel-wise based variance in spectra. Individual bacterial cells can have similar physiological traits between species. With one cell containing hundreds of pixels, it is possible that the MH backscatter is interacting with similar traits such as pili, membrane pores or flagelli between bacteria. Generating a single cell mean spectrum can remove variance and yield a broader overall representation of the cell. More research in terms of

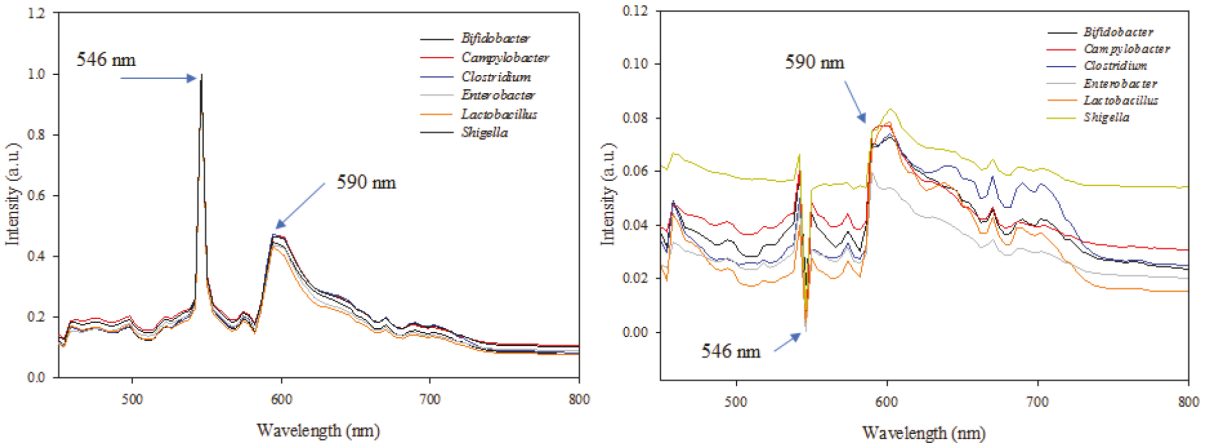


Figure 8. Spectral pattern of species from single-cell ROIs. (a) Normalised mean spectra, (b) spectral standard deviation.

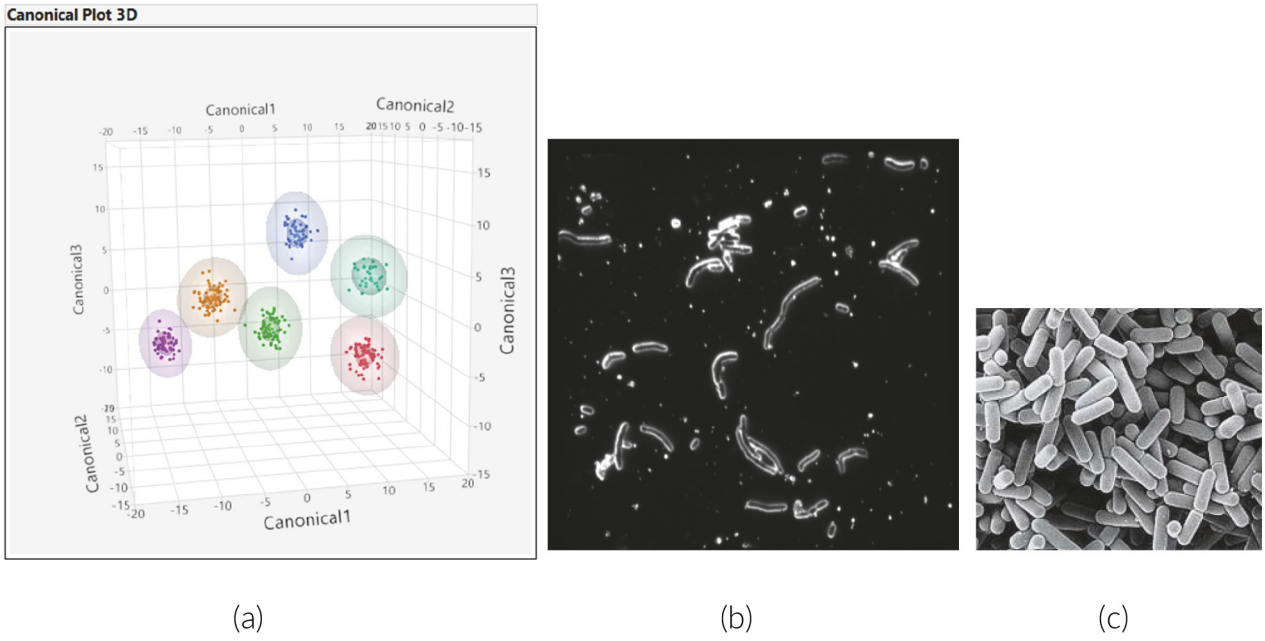


Figure 9. (a) Three-dimensional canonical plot to classify six bacterial species, (b) *Lactobacillus* cells at 590 nm spectral images collected by HMI and (c) typical *Lactobacillus* bacteria.

understanding the relationship between lighting and scattering characteristics considering sizes and shapes of bacteria needs to be investigated.

Figure 9 is a three-dimensional canonical plot to classify six bacterial species (Figure 9a) and *Lactobacillus* cells at 590 nm spectral images collected by HMI (Figure 9b). Using QDA with a total of 89,539 pixels from six different species, as mentioned in the sample preparation section, an overall accuracy of 88.6 % was achieved (Table 4). *Clostridium perfringens* had the lowest classification accuracy at 80 %, while *Campylobacter jejuni* had the highest accuracy at 97.2 %.

Conclusion

A hyperspectral microscope imaging system with the FISH technique has the potential for multiplex foodborne pathogen detection. Using Alexa488 and Cy3 fluorophores, HMI was able to identify two different *Campylobacter jejuni* strains with corresponding emission

Table 4. Principal component-quadratic discriminant analysis (PC-QDA) pixel-wise classification results of six bacteria.

Bacteria	Calibration	Validation	Test	Total
Bifidobacter	4977 (82.3)	4907 (82.1)	4942 (87.3)	14,826 (94.0)
Campylobacter	5000 (97.0)	4994 (97.2)	5000 (97.4)	14,994 (97.2)
Clostridium	4998 (80.7)	4980 (80.4)	4979 (78.9)	14,957 (80.0)
Enterobacter	5000 (93.2)	4997 (93.6)	5000 (92.3)	14,997 (93.0)
Lactobacillus	4999 (87.3)	4986 (88.4)	4994 (80.2)	14,979 (85.3)
Shigella	4974 (85.4)	4837 (86.3)	4975 (74.7)	14,786 (82.1)
Total	29,948 (87.8)	29,701 (88.0)	29,890 (85.1)	89,539 (88.6)

wavelengths of 520 nm and 590 nm with high sensitivity and specificity. In addition, HMI was able to classify six bacteria with scattering intensity from their spectra without a FISH fluorophore. The classification accuracy of six bacteria along with *Campylobacter jejuni* was approximately 97 %. In particular, the classification accuracy of *Lactobacillus salivarius* was low (85 %) due to an unexpected growth condition with agar media, which needs to be understood for future experiments. Overall classification accuracy of the QDA method for six different bacteria using a HMI technique without fluorescence markers was 89 % at the pixel level. Further study to identify the maximum number of bacteria using HMI with a FISH fluorophore and to classify mixture samples from a food matrix needs to be done. Moreover, additional analysis of HMI data from bacteria can be carried out for the development of cell-based classification models instead of pixel-based models for real applications.

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