Dual-type hyperspectral microscopic imaging for the identification and analysis of intestinal fungi

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Abstract: A method based on a dual-type (transmission and fluorescence) hyperspectral microscopic image system was developed to identify species of intestinal fungi. Living fungi are difficult to identify via transmission spectra or fluorescence spectra alone. We propose an identification method based on both fluorescence and transmission spectra that employs a series of image processing methods. Three species of intestinal fungi were used to evaluate the method. The results demonstrate that the specificity of the model trained with dual-type spectra was 98.36%, whereas the specificities achieved by training with fluorescence spectra and transmission spectra alone were 94.04% and 92.88%, respectively.

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References and links

1. E. Angelakis, M. Million, M. Henry, and D. Raoult, “Rapid and accurate bacterial identification in probiotics and yogurts by MALDI-TOF mass spectrometry,” J. Food Sci. 76(8), M568–M572 (2011).
2. K. J. Welham, M. A. Domin, K. Johnson, L. Jones, and D. S. Ashton, “Characterization of fungal spores by laser desorption/ionization time-of-flight mass spectrometry,” Rapid Commun. Mass Spectrom. 14(5), 307–310 (2000).
3. F. Cappa and P. S. Cocconcelli, “Identification of fungi from dairy products by means of 18S rRNA analysis,” Int. J. Food Microbiol. 69(1-2), 157–160 (2001).
4. M. Perea Vélez, K. Hermans, T. L. A. Verhoeven, S. E. Lebeer, J. Vanderleyden, and S. C. J. De Keersmaecker, “Identification and characterization of starter lactococcal bacteria and probiotics from Columbian dairy products,” J. Appl. Microbiol. 103(3), 666–674 (2007).
5. M. Candela, F. Perina, P. Carnevali, B. Vitali, R. Ciati, P. Gionchetti, F. Rizzello, M. Campieri, and P. Brigidi, “Interaction of probiotic Lactobacillus and Bifidobacterium strains with human intestinal epithelial cells: adhesion properties, competition against enteropathogens and modulation of IL-8 production,” Int. J. Food Microbiol. 125(3), 286–292 (2008).
6. A. Oust, T. Morett, C. Kirschner, J. A. Narvhus, and A. Kohler, “FT-IR spectroscopy for identification of closely related lactococci,” J. Microbiol. Methods 59(2), 149–162 (2004).
7. M. C. Curk, F. Peledan, and J. C. Hubert, “Fourier transform infrared (FTIR) spectroscopy for identifying Lactobacillus species,” FEMS Microbiol. Lett. 123(3), 241–248 (1994).
8. H. Liang, “Advances in multispectral and hyperspectral imaging for archaeology and art conservation,” Appl. Phys., A Mater. Sci. Process. 106(2), 309–323 (2012).
9. A. H. Sivertsen, T. K. V. Kimiya, and K. Heia, “Automatic freshness assessment of cod (Gadus morhua) fillets by Vis/Nir spectroscopy,” J. Food Eng. 103(3), 317–323 (2011).
10. D. Lau, C. Villis, S. Furman, and M. Livett, “Multispectral and hyperspectral image analysis of elemental and micro-Raman maps of cross-sections from a 16th century painting,” Anal. Chim. Acta 610(1), 15–24 (2008).
11. A. Robles-Kelly and C. P. Jaynh, Imaging Spectroscopy for Scene Analysis (Springer, 2013).
12. R. A. Schultz, T. Nielsen, J. R. Zavaleta, R. Ruch, R. Wyatt, and H. R. Garner, “Hyperspectral imaging: a novel approach for microscopic analysis,” Cytometry 43(4), 239–247 (2001).

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1. Introduction

Intestinal fungi are important to human health, and disorders of intestinal fungi can cause many diseases. Accurate species identification is important in the study of intestinal fungi. Several methods of identifying species of intestinal fungi have been developed. The traditional methods are based on mass spectrometry (MS) technology. For example, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) has been used for the ionization of the components of spores to identify prohibits [1] and several strains of Aspergillus species [2]. With the development of gene technology, several different polymerase chain reaction (PCR) amplifications of identifying fungi have been put forward. PCR amplification and sequencing of 18S rDNA have been used to identify yeasts and molds [3], and this combined with SYBER Green I fluorophore [5] made the species of fungi easier to identify. Although the application of these methods makes identification with high accuracy possible, all of them require destruction of the samples, which makes them unsuitable for continuous observation of samples. Fourier transform infrared spectroscopy is a nondestructive method used in fungi research, such as the analysis of four closely related species of Lactobacillus [6] and the discrimination of Lactobacillus species in breweries [7]. However, this method cannot provide morphology information about fungi and cannot identify a specific species of fungi in mixed conditions.
Hyperspectral imaging technology, which can provide both sample morphology information and spectral information, has been widely used in various fields for analysis and identification, such as archaeological and art conservation [8,9], food freshness assessment [10], and plant disease detection [11]. The combination of spectroscopy and microscopy shows great potential in biomedical research. In 2001, a hyperspectral microscopic imaging system was presented that permits the acquisition and identification of different spectral signatures [12]. Various hyperspectral microscopic imaging systems have since been reported [13–16]. These systems have been used in cancerous tissue detection [17–20], classification of bee pollen grains [21], and identification of microalgae [22]. There have been some applications of hyperspectral imaging technology in fungi research, such as the detection of toxigenic fungi on maize. Assays of fungi have revealed significant differences at the 95% confidence level according to Fisher's least significant difference test [23]. However, this research only classified fungi into large categories according to their transmission spectra; it did not identify specific species of fungi.

Because living intestinal fungi entwine around each other in the culture medium and because their metabolic material contains abundant fluorescent materials that disturb the fluorescent signal from the fungi, they are difficult to identify via conventional single-type hyperspectral imaging technology. In a previous study, we applied a method based on two types of hyperspectral images (fluorescence and transmission spectra) to pollen identification [24], and the results showed that this dual-type hyperspectral imaging technique can achieve higher specificity. In the present study, this dual-type hyperspectral imaging technique was applied to the identification of intestinal fungi for the first time, to the best of our knowledge. The specificity of identification achieved was to 98.03%. Furthermore, to take full advantage of the imaging technique, the species distinction and distribution of three species of fungi in a mixed sample were observed intuitively after a series of imaging processing steps.

2. Dual-type hyperspectral microscopy imaging system

The hyperspectral microscopy imaging system (HMIS) is shown in Fig. 1. The main imaging system consists of an infinity-corrected 5X microscope objective, a tube lens, and a 16-bit grayscale Complementary Metal Oxide Semiconductor (CMOS, HAMAMATSU Inc., ORCA-Flash4.0 LT C11440-42U). Used in combination with a liquid-crystal tunable filter (LCTF, CRI Inc., VariSpec VIS), it can acquire a series of two-dimensional (2-D) images of the sample at each wavelength and form a spectral data cube containing both morphological and spectral information. To perform the dual-type hyperspectral imaging, we placed two light sources in the system: a halogen lamp for transmission spectra acquisition and a xenon lamp with a narrow band-pass filter centered at 361 nm for fluorescence excitation. A dichroic mirror (Thorlabs Inc., DMLP425R) with a cutoff wavelength of 425 nm was employed to separate the excitation light and signal light.

![Fig. 1. Experimental setup of self-built hyperspectral microscopic imaging system.](image-url)
The software system consists of a control program and a data processing program. The control program is used to coordinate the CMOS with the LCTF, enabling wavelength scanning. The data processing program is used for spectral feature extraction, model training, and sample classification, as shown in Fig. 2. The small data cubes shown inside the training/test arm represent different sets of hyperspectral images. Each set of hyperspectral images was obtained from one sample, which constituted data cubes of training data and testing data. In our experiment, we built three classified models, one of these was trained using only the fluorescence spectral data, one was trained using only the transmission spectral data, and one was trained using both fluorescence and transmission spectral data.

![Hyperspectral data processing procedure](image)

**Fig. 2.** Hyperspectral data processing procedure. I and I represent spatial dimensions. $\lambda$ represents the spectral dimension. K, K, K, and K represent the numbers of the data cubes of each group. X represents the collection of the hyperspectral data. $X^{(1)}$, $X^{(2)}$, $X^{(3)}$, $X^{(4)}$, and $X^{(5)}$ represent different sets of hyperspectral data cube.

### 3. Samples

Three different species of fungi were used in our experiment: Aspergillus Flavus Link BNCC 336156 (Aspergillus Flavus), Aspergillus Fumigatus BNCC340016 (Aspergillus Fumigatus), and Candida Utilis Henneneberg Lodder et Kreger-van Rij BNCC 336517 (Candida Utilis). Candida Utilis is a probiotic, whereas Aspergillus Flavus and Aspergillus Fumigatus are harmful fungi. These three species of fungi are usually found in the human intestinal tract. Because these three species of fungi are relatively large in size and contain abundant pigment and fluorescent substances, they are easy to detect and analyze using our system. All original samples were provided by Beijing Beina Science & Technology Co., Ltd. We performed the fungi culture in our laboratory at a temperature of 28°C.

A classical Enterobacteria Enrichment (EE) Broth Moss obtained from the Kunming Huangbao Business Trading Co., Ltd. was used as the culture medium in our experiment. The enrichment broth powder was mixed with distilled water at a ratio of 1:3 to achieve a pH of 7.2 for the broth. The broth was then heated in a water bath at 100°C for 30 min. When it had cooled down, we poured the broth into test tubes and inoculated the broth with fungi. Because all of the samples were aerobic fungi and required an environment rich in oxygen, the test
tubes were wobbled continuously by an oscillator to dissolve more oxygen in the broth. It also enabled uniform distribution of fungi, and thus the samples that were extracted from test tubes had the same density as that in the broth.

Each of the three species of fungi was used to inoculate the broth in six different test tubes, resulting in a total of 18 test tubes. We also mixed the three species of fungi together at a ratio of 1:1:1 in one test tube. The experiment was conducted over a period of 48 h, which we considered to begin (0 h) after a 12-h period of cultivation. We extracted a sample from each of the 18 single-species test tubes at the beginning of the experiment, and we extracted a sample from the test tube of mixed fungi at the beginning of the experiment and after 24 and 48 h.

4. Transmission spectrum and fluorescence spectrum

Obtaining transmission spectral images only required turning on the halogen lamp. When the broadband-emitted light passed through the sample, a portion of it was absorbed by the sample and the rest carried the transmission information of the sample and was received by the CMOS.

Based on the spectral images we obtained, the transmittance of a certain pixel at a certain wavelength was defined as follows:

$$T(\lambda) = \frac{I(\lambda)_{\text{sample}}}{I(\lambda)_{\text{background}}}.$$  

(1)

Here, $T(\lambda)$ represents the transmittance of the pixel at a wavelength of $\lambda$. Because the grayscale of the pixel is proportional to the light intensity, $I(\lambda)_{\text{sample}}$ and $I(\lambda)_{\text{background}}$ represent the intensity of the pixel with and without the sample, respectively.

To obtain the fluorescence spectra, it was necessary to turn on the xenon lamp with a narrow band-pass filter at 361 nm and switch off the halogen lamp. This short-wavelength radiation was easily able to excite the fluorescence of the fungus. The fluorescence passed through the dichroic mirror and was detected by CMOS, yielding the fluorescence spectra of the fungus. The fluorescence intensity was normalized as follows before the analysis:

$$I_n(\lambda) = \frac{I(\lambda)}{I_{\lambda_{\text{max}}}}.$$  

(2)

Here, $I_n(\lambda)$ represents the normalized intensity of the pixel at the emission wavelength of $\lambda$, $I(\lambda)$ is the intensity of the pixel at the emission wavelength of $\lambda$, and $I_{\lambda_{\text{max}}}$ is the highest intensity of the pixel over the whole wavelength range.

The transmission and fluorescence spectral images of the three species of fungi were obtained in the spectral range from 460 nm to 660 nm at increments of 2 nm. The exposure time of each transmission and fluorescence spectral image was 0.03 seconds and 10 seconds respectively. Examples of different single-wavelength images are shown in Fig. 3. We randomly selected 100 pixels from each of the standard samples (three single species of fungi). Their average transmittance curves and normalized fluorescence intensity curves are shown in Figs. 3(a) and (b), respectively. Candida utilis can easily be distinguished from the other two species of fungi by the fluorescence characteristic peaks, whereas the fluorescence spectra of the Aspergillus flavus and Aspergillus fumigatus are too similar to be distinguished from each other. However, their transmission spectra curves are clearly different. Thus, the dual-type spectra were used for identification.

Because the signal-to-noise ratios of the transmission spectral images ranging from 460 nm to 558 nm were too low, only the transmission images ranging from 560 nm to 660 nm were used in the classification. Hence, the transmission spectra ranging from 560 nm to 660 nm (a total of 51 bands), together with the fluorescence spectra from 460 nm to 660 nm (a total of 101 bands), constituted a dual-type spectral data set for use in identification in our study.
5. Results and analysis

We obtained six sets of images from the six test tubes for each species of fungi. Each set of images contained 51 single-band transmission images and 101 single-band fluorescence images. For each set of images, the spectral information of 100 randomly selected pixels was obtained. This constituted a spectral data set of 600 pixels of each species of fungi, as shown in Table 1. For each species of fungi, the spectral data set of 300 pixels was used for standard samples, and the spectral data set of the other 300 pixels was used for testing samples. The spectral data set of 900 selected pixels of the standard samples (300 pixels from each single species) was used to train the Fisher linear classifier [26, 27], and the spectral data set of the other 900 pixels selected from the testing samples (300 pixels from each single species of fungi) was used to evaluate the effect of the classification. Three Fisher classifiers were built up for the purpose of comparative analysis. One of these was trained using only the fluorescence spectral data, one was trained using only the transmission spectral data, and one was trained using both fluorescence and transmission spectral data. Both the standard samples and the testing samples were classified using these three models. The results of the classifiers trained using the pixels of the standard samples are shown in Tables 2, 3, and 4. The results of the classifiers trained using the pixels of the test samples are shown in Tables 5, 6, and 7.
Table 1. Experimental data for single-species fungi

| Species of fungi | Number of test tubes | Number of training samples | Number of testing samples | Number of images per sampling | Total number of images per single-species fungi | Number of selected pixels per image | Total number of selected pixels per single-species fungi |
|------------------|----------------------|----------------------------|---------------------------|-------------------------------|-----------------------------------------------|-----------------------------------|-------------------------------------------------------------|
| Candida utilis   | 6                    | 3                          | 3                         | 152                           | 912 (6×152)                                  | 100                               | 600                                                         |
| Aspergillus flavus | 6                    | 3                          | 3                         | 152                           | 912 (6×152)                                  | 100                               | 600                                                         |
| Aspergillus fumigatus | 6                  | 3                          | 3                         | 152                           | 912 (6×152)                                  | 100                               | 600                                                         |

Table 2. Results of cross-validation of the standard samples based on the fluorescence spectra information.

| Prediction of Fisher classifier | Actual  | Candida utilis | Aspergillus flavus | Aspergillus fumigatus | SEN (%) |
|---------------------------------|---------|----------------|-------------------|-----------------------|---------|
| Candida utilis                  | 300     | 0              | 0                 | 100%                  |         |
| Aspergillus flavus              | 0       | 293            | 7                 | 97.67%                |         |
| Aspergillus fumigatus           | 0       | 0              | 300               | 100%                  |         |
| SPEC (%)                        | 100%    | 100%           | 97.71%            |                       |         |

^SEN: sensitivity; SPEC: specificity.

Table 3. Results of cross-validation of the standard samples based on the transmission spectra information.

| Prediction of Fisher classifier | Actual  | Candida utilis | Aspergillus flavus | Aspergillus fumigatus | SEN (%) |
|---------------------------------|---------|----------------|-------------------|-----------------------|---------|
| Candida utilis                  | 300     | 0              | 0                 | 100%                  |         |
| Aspergillus flavus              | 0       | 286            | 14                | 95.33%                |         |
| Aspergillus fumigatus           | 0       | 0              | 300               | 100%                  |         |
| SPEC (%)                        | 100%    | 100%           | 95.54%            |                       |         |

^SEN: sensitivity; SPEC: specificity.
Table 4. Results of cross-validation of the standard samples based on the fluorescence spectra information and the transmission spectra information.

| Actual        | Candida utilis | Aspergillus flavus | Aspergillus fumigatus | SEN (%) |
|---------------|----------------|--------------------|-----------------------|---------|
| Candida utilis| 300            | 0                  | 0                     | 100%    |
| Aspergillus flavus | 0     | 298                | 2                     | 99.33%  |
| Aspergillus fumigatus | 0     | 0                  | 300                   | 100%    |
| SPEC (%)      | 100%          | 100%               | 99.33%                |         |

cSEN: sensitivity; SPEC: specificity.

Table 5. Results of the Fisher identification of the test samples based on the fluorescence spectra information.

| Actual        | Candida utilis | Aspergillus flavus | Aspergillus fumigatus | SEN (%) |
|---------------|----------------|--------------------|-----------------------|---------|
| Candida utilis| 300            | 0                  | 0                     | 100%    |
| Aspergillus flavus | 0     | 281                | 19                    | 93.67%  |
| Aspergillus fumigatus | 0     | 0                  | 300                   | 100%    |
| SPEC (%)      | 100%          | 100%               | 94.04%                |         |

dSEN: sensitivity; SPEC: specificity.

Table 6. Results of the Fisher identification of the test samples based on the transmission spectra information.

| Actual        | Candida utilis | Aspergillus flavus | Aspergillus fumigatus | SEN (%) |
|---------------|----------------|--------------------|-----------------------|---------|
| Candida utilis| 300            | 0                  | 0                     | 100%    |
| Aspergillus flavus | 0     | 277                | 23                    | 92.33%  |
| Aspergillus fumigatus | 0     | 0                  | 300                   | 100%    |
| SPEC (%)      | 100%          | 100%               | 92.88%                |         |

eSEN: sensitivity; SPEC: specificity.
Table 7. Results of the fisher identification of the test samples based on the fluorescence spectra information and the transmission spectra information.

| Actual          | Candida utilis | Aspergillus flavus | Aspergillus fumigatus | SEN (%) |
|-----------------|----------------|--------------------|-----------------------|---------|
| Candida utilis  | 300            | 0                  | 0                     | 100%    |
| Aspergillus flavus | 0            | 295                | 5                     | 98.33%  |
| Aspergillus fumigatus | 0           | 0                  | 300                   | 100%    |
| SPEC (%)        | 100%           | 100%               | 98.36%                |

SEN: sensitivity; SPEC: specificity.

The sensitivity (SEN) is a measure of the proportion of positives correctly identified, and the specificity (SPEC) is a measure of the proportion of negatives correctly identified, as defined by the formulae below [21]:

\[
SEN = \frac{TP}{TP + FN} \tag{3}
\]

\[
SPEC = \frac{TN}{TN + FP} \tag{4}
\]

The number of true positives (TP) is the number of times the classifier correctly labeled a positive identification as positive, the number of true negatives (TN) is the number of times the classifier labeled a negative identification as negative, the number of false negatives (FN) is the number of times the classifier erroneously labeled a positive identification as negative, and the number of false positives (FP) is the number of times the classifier erroneously labeled a negative identification as positive.

The results show that the SPEC of Candida utilis and Aspergillus flavus according to the three models were all 100%. However, the SPEC of Aspergillus fumigatus according to the first model was 94.04%, that according to the second model was 92.88%, and that according to the last model was up to 98.36%. The SEN of Candida utilis and Aspergillus flavus according to the three models were also 100%, but the SEN of Aspergillus fumigatus according to the first model was 93.67%, that according to the second model was 92.33%, and that according to the last model was 98.33%. This shows that the model trained using both fluorescence and transmission spectral data had a higher sensitivity and specificity than the model trained using only fluorescence spectral data or transmission spectral data. Therefore, we chose the dual-type spectral data to classify the fungi in the mixed sample.

Compared with the Fourier transform infrared spectroscopy method for fungi identification, whose average specificity was 91.5% [25], our method had a higher specificity of up to 98%. To take full advantage of the hyperspectral imaging technology and enhance the visualization quality, a series of image processing methods was designed and added to the identification process.

The identification process for the mixed sample was as follows. The first step was to obtain the spatial information of the fungi pixels so as to release the calculation burden. If the spectra data of all the pixels in the image was used for identification, the calculation would be very large which contains more than 39 million of data. Therefore, to obtain the spatial information of the fungi pixels, the grayscale image of the mixed sample was converted to a binary image using a certain threshold. Pixels with intensities beyond the threshold were set to 65535; otherwise they were set to 0. As Fig. 4 shows, both the fluorescence image at 546
nm (Fig. 4(a)) and the transmission image at 660 nm (Fig. 4(c)) of the mixed sample were converted to binary images (Figs. 4(b) and (d)) using thresholds of 1930 and 30040, respectively. One can see that the binary image based on the transmission image was not satisfactory; the fluorescence image was more suitable for binary image conversion. Unlike the fluorescence image, there was considerable noise in the binary image based on the transmission image, because of the uneven illumination. Furthermore, some fluorescence of metabolic material and nutrients was relatively strong, resulting in a high grayscale threshold. If only one threshold (Fig. 5(b)) were set for binary image conversion, the threshold would have been too high and resulted in the loss of some fungi pixels. Thus, to improve the signal-to-noise ratio of the binary image based on the fluorescence image, the image segmentation method was used to find suitable thresholds. The size of the images obtained by the CMOS was 512 × 512 pixels, which we divided into 4096 equal regions 64 pixels (8 × 8) in size.

As Fig. 5(a) shows, for each equal region (squares labeled in different grayscale in Fig. 5(a)), the largest between-class variance method was used to find the grayscale threshold. For the part without noise, the grayscale threshold was relatively low, ensuring that the pixels of the fungi could be preserved. The improved binary image is shown in Fig. 6(b). Compared with the single-threshold binary image (Fig. 6(a)), although the improvement was accompanied by an increase in noise, more complete spatial information ensured more rigorous results. To eliminate the noise caused by the fluorescent metabolites of the fungi and fluorescent nutrients of the broth, a Gaussian low-pass filter was applied. As Fig. 6(c) shows, the noise in the binary image was greatly reduced. This method of

![Fig. 4. Procedure for obtaining binary images. (a) is the fluorescence image of the mixed sample at a wavelength of 546 nm and (b) is the binary image based on the fluorescence image. (c) is the transmission image of the mixed sample at a wavelength of 660 nm and (d) is the binary image based on the transmission image. The scale bar is 500 μm.](image)

![Fig. 5. Schematic illustration of the image segmentation method. (a) illustrates the principle of the image segmentation method for obtaining binary images. (b) illustrates the principle of the single threshold method for obtaining binary images.](image)
When the object area was separated from the background (Figs. 7(a) to (c)), we removed the circular parts at the upper right and bottom right of the image, which represented the agar block in the broth, whose spectral curve was quite different from those of the three species of fungi (Fig. 7(b)). All of the pixels in the object area were the classified using the Fisher classifier, which was trained using the previously obtained dual-type spectral information. Spatial information for the fungi pixels was obtained from the binary image (Fig. 7(c)). With precise spatial information, all the fungi pixels could be precisely identified using the transmission information (Fig. 7(d)) and fluorescence information (Fig. 7(e)). The pixels were then labeled with different grayscale values that corresponded to the identification results (Fig. 7(f)). For the purpose of more intuitive observation, the pixels were filled with corresponding pseudo color using grayscale thresholds of 120 and 200, according to the grayscale histogram (Fig. 7(g)). Further, the connected-region labeling algorithm [28, 29] was used to remove the erroneously identified pixels at the edge of the sample. As shown in the region in blue ellipse of Figs. 7(h) and (i), the green pixels were erroneously identified pixels. We calculated the proportion of these erroneously identified pixels (labeled in green) in the whole region that should be labeled in yellow. The results showed the proportions with and without the application of connected-region labeling algorithm were 2.6% and 6.7%, respectively.

Fig. 6. Binary image processing procedure. (a) is a binary image that based only on a single threshold and (b) is a binary image based on different thresholds in different regions. (c) is a binary image obtained after application of the Gaussian low-pass filter. The regions in the red boxes are the cropped and magnified images of the regions to which the arrows point. The scale bar is 500 μm.

Fig. 7. Flow chart of image processing algorithm. (a) is the original fluorescence image of the mixed sample. (b) is the normalized intensity curves of the agar block and three species of fungi. (c) is the binary image, (d) and (e) are the transmission and fluorescence images, respectively. (f) is the classified grayscale image. (g) is the grayscale histogram of the classified grayscale image from which the background pixels were removed. (h) and (i) are the pseudo color classified images without and with correction using the connected-region labeling algorithm, respectively. Candida utilis, Aspergillus flavus, and Aspergillus fumigatus are indicated in yellow, green, and red, respectively. The regions in the yellow boxes are the cropped and magnified images of the regions in the dotted boxes. The scale bar is 500 μm.
Based on the method using dual-type spectral information (Fig. 8), the growth competition in the mixed sample could be observed. Because the method was nondestructive and fast (it took 17 min), we were able to reserve the fungi repeatedly or continuously. As Fig. 9 shows, the growth competition of the fungi at different times (0, 24, and 48 h), the distribution of the fungi, and the morphology information of the three species of fungi could be observed intuitively, which proved that our system makes continuous observation of mixed samples possible. According to the results, we could see that the growth trends of three species of fungi were different. The Candida Utilis and Aspergillus Flavus both soon disappeared since the growth of them was inhibited, while Aspergillus Fumigatus dominated in the mixed condition, which indicated that they do exist competition in the mixed samples.

![Fig. 8. Diagram of the whole processing algorithm](image)

![Fig. 9. Growth competition in the mixed sample at 0, 24, and 48 h. (a), (b), and (c) are the pseudo color classified images of the mixed sample at 0, 24, and 48 h, respectively. (d), (e), and (f) are the transmission images of the mixed sample at 0, 24, and 48 h, respectively. The scale bar is 500 μm.](image)
6. Conclusions

It has been verified in this study that the application of dual-type spectrum technology to the identification of fungi yields higher specificity (98.03%) than the single-type spectrum technology (94.04% for fluorescence spectra and 92.88% for transmission spectra). The image segmentation method for binary image acquisition ensured more rigorous results, and the connected-region labeling algorithm improved the specificity of identification. This method is very effective in the identification of fungi in mixed samples. We believe that this fast, nondestructive method has great potential for use in studying fungi and will contribute to further research and development in the identification of biological samples.

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Disclosures

The authors declare that there are no conflicts of interest related to this article.