Visualization and Quantification of Three-Dimensional Distribution of Yeast in Bread Dough

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A three-dimensional (3-D) bio-imaging technique was developed for visualizing and quantifying the 3-D distribution of yeast in frozen bread dough samples in accordance with the progress of the mixing process of the samples, applying cell-surface engineering to the surfaces of the yeast cells. The fluorescent yeast was recognized as bright spots at the wavelength of 520 nm. Frozen dough samples were sliced at intervals of 1 µm by an micro-slicer image processing system (MSIPS) equipped with a fluorescence microscope for acquiring cross-sectional images of the samples. A set of successive two-dimensional images was reconstructed to analyze the 3-D distribution of the yeast. The average shortest distance between centroids of enhanced green fluorescent protein (EGFP) yeasts was 10.7 µm at the pick-up stage, 9.7 µm at the clean-up stage, 9.0 µm at the final stage, and 10.2 µm at the over-mixing stage. The results indicated that the distribution of the yeast cells was the most uniform in the dough of white bread at the final stage, while the heterogeneous distribution at the over-mixing stage was possibly due to the destruction of the gluten network structure within the samples.

Key words: bread dough; distribution; enhanced green fluorescent protein (EGFP); fluorescent microscope; yeast

In recent years, 3-D images of biological materials have been frequently presented owing to rapid progress in image processing technologies.¹ Do et al.² reported that the micro-slicer image processing system (MSIPS) is useful for measuring the morphology of agricultural products having complex forms, and presented equations to determine both the surface area and the volume of samples of broccoli from their mass. The MSIPS has been also applied to observe the ice crystal structures formed in frozen beef³ and frozen dilute solutions,⁴ and to measure bubble size distributions in dough.⁵ On the other hand, Ueda et al. have proposed a cell-surface engineering concept.⁵,⁶ This technology utilizes information on protein molecules, the transport of which is localized to cell surfaces. This method features surface display of foreign protein molecules, and has also made it possible to grant new functions to conventional cells without disturbing their inner metabolic systems. Maeda has applied the cell-surface engineering to visualize enhanced green fluorescent protein (EGFP) yeasts in bread dough,⁷ and then Maeda et al. developed a novel technique for monitoring the change in the three-dimensional distribution of yeast in frozen bread dough in accordance with progress in the mixing process.⁷ However, the 3-D distribution of yeast in frozen bread dough has not been quantified yet, due to the difficulty in distinguishing EGFP yeasts from the auto-fluorescent background of bread dough.

The objective of this work is to develop a novel technique for visualizing and quantifying 3-D distribution of yeast in frozen bread dough samples, melding the cell-surface engineering technique with the MSIPS.

Materials and Methods

Micro slicer image processing system (MSIPS). Figure 1 shows a schematic diagram of the MSIPS, composed of a multi-slicing section for exposing sample cross-sections, a computer, and image processing software. The PC is used to control the timing of slicing a sample and recording image data. After the slicing operation, the 3-D sample image is reconstructed based on the image data for a frozen sample with a minimum thickness of 1 µm. The image processing software has functions displaying the internal structure and an arbitrary cross section of the sample by varying observation angles.

The sample, fixed in a sample holder, is pushed up with a linear actuator using a stepping motor and then sliced by a cutting blade that is installed on the rotating arm with an AC motor and timing belt. The maximum rotational speed of the cutting blade is 90 rpm. The slicing thickness can be adjusted mechanically, with a minimum thickness of 1 µm. The cross-sectional images of the exposed surfaces after slicing are captured directly with a CCD camera (Cool Snap, Nippon Roper, Tokyo 103-0026, Japan).

Abbreviations: EGFP, enhanced green fluorescent protein; MSIPS, micro-slicer image processing system

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KpnI fragment of pICAS1, containing the secretion signal sequence vector pCAS1 was constructed by introducing the 1.48 kbp Eco RI-
pMEG1 was constructed as follows: The multicopy-type cassette strain MT8-1 11) was used as the host strain in the yeast cell-surface Saccharomyces cerevisiae strain for recombinant DNA manipulation.

As shown in Table 1. All the samples were mixed in a SK mixer (DTM-)
(0.5% on a flour weight basis was also used.

14% moisture basis) and 13.6% moisture. EGFP-displayed yeast of
0.003% L-leucine, and 0.002% uracil). 12) 2-[4-(2-Hydroxyethyl)-1-
acids, 2% glucose, 0.002% adenine sulfate, 0.002%L-histidine-HCl,
11) The fragment of gene encoding EGFP was
section of pGA11. 13) The fragment of gene encoding EGFP was
[58x330]Saccharomyces cerevisiae was cultivated in Luria-Bertani (LB) medium (1% tryptone,
[58x340]E. coli display system to display enhanced green fluorescent protein (EGFP).

Results and Discussion

3-D visualization and quantification. Figure 2 shows a flow diagram of 3-D visualization and quantification of yeast in frozen bread dough. First, test samples collected at the various mixing stages were rapidly frozen in liquid nitrogen to form the samples into sample holders. Frozen samples (φ8 mm × 30 mm) were embedded and fixed with optimal cutting temperature (OCT) compound in heat shrinking tubes (φ8 mm × 30 mm). The samples with sample holders were frozen again in liquid nitrogen.

Frozen samples (n = 10 for each mixing stage) were sliced together with the holders at a rate of 60 rpm with a thickness of 1.0µm to capture cross-sectional images of the sliced samples with a CCD camera through a fluorescent microscope at the wavelength of 520 nm in a synchronized manner. Image binarization was performed for all the 2-D images to extract EGFP yeasts using general-purpose software. Then 3-D images were reconstructed by the volume rendering method using 3-D visualization software (AVS Express, Advanced Visual System, Waltham, MA). The yeasts in the reconstructed 3-D images were pigmented in different colors for visualization. The average shortest distances between the centroids of the EGFP yeasts were measured for 10 samples at each mixing stage.

Fig. 2. Flow Diagram of Experimental Procedure.

3-D visualization and quantification.

3-D Visualization of yeast in bread dough

Since the EGFP yeasts in the 2-D images were clearly distinguished from the auto-fluorescent green background of bread dough (Maeda et al.15), the EGFP yeasts in the reconstructed 3-D images were pigmented in different colors to identify different yeast cells. Figures 3–6 shows 3-D images of yeast distribution in bread dough at each stage of the mixing process: (a) pick-up stage, (b) clean-up stage, (c) final stage, and (d) over-mixing stage. The 3-D images were reconstructed with 300 2-D images at the center of the shaped samples. The scale of the images was 260 µm in length (X-axis), 206 µm in width (Y-axis), and 300 µm in height (Z-axis). The resolution of the 2-D images was 1,300 × 1,000 pixels, corresponding to a square 0.2 µm on a side for each pixel. For each mixing stage, the upper two images

3-D Quantification of yeast

Table 1. Ingredients of White Bread Samples

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Flour basis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wheat Flour (Cameria: Nisshin Flour Milling)</td>
<td>100.0</td>
</tr>
<tr>
<td>Yeast (EGFP)</td>
<td>0.5</td>
</tr>
<tr>
<td>Sugar</td>
<td>5.0</td>
</tr>
<tr>
<td>Salt</td>
<td>2.0</td>
</tr>
<tr>
<td>Defatted skim milk</td>
<td>2.0</td>
</tr>
<tr>
<td>Shortening</td>
<td>5.0</td>
</tr>
<tr>
<td>Water</td>
<td>68.0</td>
</tr>
</tbody>
</table>

Tokyo) through a fluorescent microscope (BX-FLA, Olympus, Tokyo), and recorded into a computer (Precision 360, Dell Japan, Tokyo) with image processing software (Image-Pro PLUS Ver 4.0, Media Cybernetics, Bethesda, MD). A 3-D image is generated from these 2-D images, and is reconstructed using volume analysis software (TRI 3D Volume, Ratoc System Engineering, Tokyo). To prevent the frozen sample from melting during slicing, the sample holder in the multi-slicing section is cooled to about −40°C.

Strains and media.9) Escherichia coli DH5α10) was used as host strain for recombinant DNA manipulation. Saccharomyces cerevisiae strain MT8-11) was used as the host strain in the yeast cell-surface display system to display enhanced green fluorescent protein (EGFP). E. coli was cultivated in Luria-Bertani (LB) medium (1% tryptone, 0.5% sodium chloride, and 0.1% glucose). The yeast S. cerevisiae was cultivated in YPD medium (1% yeast extract, 2% polypepton, and 2% glucose) or SD-W medium (0.67% yeast nitrogen base without amino acids, 2% glucose, 0.002% adenine sulfate, 0.002%L-histidine-HCl, 0.003% L-leucine, and 0.002% uracil).12) 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) was added to SD-W medium at a concentration of 50 mM to bufferize the medium during cultivation.

Construction of plasmids and transformation of the yeast.9) Plasmid pMEG1 was constructed as follows: The multicopy-type cassette vector pCAS1 was constructed by introducing the 1.48 kbp Eco RI-KpnI fragment of pCAS1, containing the secretion signal sequence gene of the glycolalysyme gene, the multi-cloning site, the 3′-half of the coding region encoding 320 amino acids of α-agglutinin, and 446 bp of the 3′-flanking region of the α-agglutinin gene, into the Eco RI-KpnI section of pGA11.13) The fragment of gene encoding EGFP was inserted into the SacII site of pCAS1 to construct pMEG1. Plasmid pMEG1 was transformed to S. cerevisiae by the lithium acetate method.14)

Flour and yeast. The wheat flour used in this study was a commercial strong breadmaking flour (Cameria, Nisshin Flour Milling, Tokyo), containing 12.8% protein (N × 5.7) and 0.33% ash (both on a 14% moisture basis) and 13.6% moisture. EGFP-displayed yeast of 0.5% on a flour weight basis was also used.

Dough formulation and mixing stages. Bread dough samples (n = 40) were prepared using the no-punch straight-dough method, as shown in Table 1. All the samples were mixed in a SK mixer (DTM-
were still pictures obtained by X-axis rotation, and the lower two were obtained by Y-axis rotation.

At the pick-up stage, the yeast cells were heterogeneously distributed and some yeast cells were localized. During the transition of mixing from the clean-up stage to the final stage, the distribution of the yeast cells gradually became homogeneous throughout the dough samples. At the over-mixing stage some yeast cells were localized, probably due to destruction of the gluten network structure within the samples. In other words, the relevance of the distribution inhomogeneity of the yeast cells to the destruction of the gluten network structure induced by over-mixing was suggested by the mechanism whereby some proteins on the surface of the yeast

Fig. 3. Reconstructed 3-D Images of EGFP Displayed on Yeast Cell Surfaces (Pick-Up Stage).

Fig. 4. Reconstructed 3-D Images of EGFP Displayed on Yeast Cell Surfaces (Clean-Up Stage).
cells were taken up in the network structure by the interactions of intermolecular force among the hydrophobic molecules.

3-D quantification of average shortest distances between the centroids of the yeasts

Prior to 3-D quantification, all the yeast cells observed in the 3-D images for each mixing stage were replaced by particles to calculate the average shortest distances between the centroids of the particles. The shortest distance between A and B was selected after measuring the distances between the centroids of all the yeast cells surrounding yeast cell B.

Table 2 shows the average shortest distances between the centroids of the EGFP yeasts for 10 reconstructed 3-D images (L: 260 μm × W: 206 μm × H: 300 μm) at

Fig. 5. Reconstructed 3-D Images of EGFP Displayed on Yeast Cell Surfaces (Final Stage).

Fig. 6. Reconstructed 3-D Images of EGFP Displayed on Yeast Cell Surfaces (Over-Mixing Stage).
each mixing stage. The distances were 10.7 μm at the pick-up stage, 9.7 μm at the clean-up stage, 9.0 μm at the final stage, and 10.2 μm at the over-mixing stage. The results revealed that the average shortest distance was shortest at the final stage among the four mixing stages, and its variation was smallest.

Figure 7 shows the frequency distribution of the shortest distances between the centroids of the EGFP yeasts for 10 reconstructed 3-D images (L: 260 μm × W: 206 μm × H: 300 μm) at each mixing stage. As the stages of mixing progressed, the frequency in a range of 5–25 μm decreased, and then the frequency at around 5 μm, clearly increased at the final stage. A length of 5 μm in shortest distance between the centroids of the particles means an acute line in the frequency distribution. Consequently, the distribution of the yeast cells was found to be most uniform in the dough of white bread at the final stage.

The proposed methodology should be useful to improve the bread-making process, which has been dependent on experience and intuition had only by well-trained bread-makers, providing quantitative indicators that can be reflected in further improvement of the quality of white bread on the manufacturing floor. The methodology as an analytical tool is also expected to develop food histology further to visualize internal 3-D structures as well as the existence of specified substances.

### Table 2. Average Shortest Distances between Centroids of the EGFP Yeasts

<table>
<thead>
<tr>
<th></th>
<th>Pick-up</th>
<th>Clean-up</th>
<th>Final</th>
<th>Over-mixing</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average shortest distance (μm)</td>
<td>10.7</td>
<td>9.7</td>
<td>9.0</td>
<td>10.2</td>
</tr>
<tr>
<td>Standard deviation (μm)</td>
<td>7.6</td>
<td>6.8</td>
<td>5.0</td>
<td>6.2</td>
</tr>
<tr>
<td>Number (n)</td>
<td>15,940</td>
<td>16,282</td>
<td>16,157</td>
<td>15,986</td>
</tr>
</tbody>
</table>

**Fig. 7.** Frequency Distribution of Shortest Distances between Centroids of EGFP Yeasts.

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### References