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Isolation of Cells with Morphological and Spatial Information from Oral Submucous Fibrosis Samples by Laser Capture Microdissection

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Abstract

Oral submucous fibrosis (OSF) is a common type of potentially malignant disorder in the oral cavity. The atrophy of epithelium and fibrosis of the lamina propria and the submucosa are often found on histopathological slides. Epithelial dysplasia, epithelial atrophy, and senescent fibroblasts have been proposed to be associated with the malignant transformation of OSF. However, because of the heterogeneity of potentially malignant oral disorders and oral squamous cell carcinoma, it is difficult to identify the specific molecular mechanisms of malignant transformation in OSF. Here, we present a method to obtain a small number of epithelial or mesenchymal cells carrying morphological data and spatial information by laser capture microdissection on formalin-fixed paraffin-embedded tissue slides. Using a microscope, we can precisely capture microscale (~500 cells) dysplastic or atrophic epithelial tissue and fibrotic subepithelial tissue. The extracted cells can be evaluated by genome or transcriptome sequencing to acquire genomic and transcriptomic data with morphological and spatial information. This approach removes the heterogeneity of bulk OSF tissue sequencing and the interference caused by cells in non-lesioned areas, allowing for precise spatial-omics analysis of OSF tissue.

Introduction

Oral submucous fibrosis (OSF) is a chronic, insidious disease that develops mainly in the buccal mucosa and results in restricted mouth opening[1]. While OSF is a multifactorial disease, areca nut or betel nut chewing is the main cause of OSF[2,3]. Because of this geographically specific habit, OSF is predominantly concentrated in populations in Southeast and South Asia[3]. The common histological features of OSF include abnormal collagen deposition in the connective tissue

beneath the oral mucosal epithelium, vascular stenosis, and occlusion[1]. OSF epithelial tissue can present with manifestations of atrophy or hyperplasia and even dysplasia when concomitant with oral leukoplakia[4,5].

OSF is defined by the World Health Organization as a common oral potentially malignant disorder (OPMD) that exhibits the potential to progress to oral squamous cell carcinoma with a malignant transformation rate of 4%-6%[6,7,8,9]. The mechanism underlying the malignant transformation of OSF is complex[10]. Abnormal growth of the epithelium, including both dysplasia and atrophy, increases the potential for carcinogenesis, and senescent fibroblasts in the stroma may be involved in the malignant progression of OSF by inducing epithelial-mesenchymal transition (EMT) through reactive oxygen species (ROS) and other molecules[10].

Technologies for spatial-omic analyses generated multi-omic data with morphological and spatial information that have provided insights into cancer mechanisms[11,12,13]. Here, we present a protocol to capture morphology-related cell populations from formalin-fixed paraffin-embedded OSF tissue by laser microdissection. Multi-omic analyses of these samples can overcome challenges with intratissue heterogeneity and increase understanding of the molecular pathology and mechanisms of malignant transformation in OSF[14].

Protocol

This study was approved by the institutional review board of Peking University School and Hospital. Informed consent was obtained from the patients. The tissue samples used in this study were deidentified. The study scheme is shown in **Figure 1**.

1. Sample preparation

1. Cut formalin-fixed paraffin-embedded oral submucous fibrosis tissues into continuous sections of 3 μm and 10 μm thickness on a microtome.
2. Unfold the sections in water and then fish out onto the slides. Fix the 3 μM and 10 μm sections on adhesion microscope slides (slide A) and PEN-membrane slides (slide B), respectively.
3. Place the slides on a hot plate at 60 °C for 2 h.

2. Hematoxylin-eosin staining

1. Place slides A and B in xylene (CAUTION) for 10 min and repeat this step thrice.
2. Hydrate slides A and B in graded ethanol solutions in the order of 100%, 100%, 90%, 80%, and 70% for 1 min in each concentration.
3. Place slides A and B in ultrapure distilled water for 30 s
4. Place slides A and B in Harris hematoxylin dye solution for 90 s.
5. Place slides A and B in ultrapure distilled water for 30 s and repeat this step thrice.
6. Place slides A and B in div-hematoxylin solution (CAUTION) for 2 s.
7. Place slides A and B in ultrapure distilled water for 30 s and repeat this step thrice.
8. Place slides in A and B in 1% re-blue solution for 2 s.
9. Place slides A and B in ultrapure distilled water for 30 s and repeat this step thrice.
10. Place slides in eosin solution for 2 s.

11. Place slides A and B in ultrapure distilled water for 30 s and repeat this step thrice.
12. Dehydrate the slides in graded ethanol solutions in the order of 70%, 80%, 90%, 100%, and 100% for 2 s in each concentration.
13. Place slide A in xylene for 3 min and repeat this step twice.
14. Add a drop of mounting medium (CAUTION) to slide A and cover it with a cover glass.

3. Observation of histological morphology and laser capture microdissection

1. Initiate the laser microdissection system and software (**Figure 2**).
2. Place slide A on the slide stage and observe the histological morphology to identify the area of interest for laser microdissection.
3. Remove slide A and place slide B inversely on the slide stage.
4. Click the **Unload** button (**Figure 2**) to push out the collection device. Insert a polymerase chain reaction (PCR) tube into the collection device and ensure that the tube is held fixed. Click the **OK** button (**Figure 3**).
5. Select a cap by clicking the corresponding red circle marking the **Collector Device: Tube Caps**. The selected circle will turn green.
6. Click the **Draw** button and **Draw + Cut** button and use the mouse to sketch the area of interest.
7. Click the **Start Cut** button to capture the area of interest; the captured sample will be collected by the cap (**Figure 4**).
8. Click the **Lower** button to ensure that the captured sample is collected (**Figure 5**).
9. Click the **Specimen** button to capture the next sample.
10. Click the **Unload** button to unload the PCR tube with the captured sample.

Representative Results

By performing laser microdissection of OSF tissues, we captured samples of dysplastic epithelium, stroma beneath the dysplastic epithelium, atrophic epithelium, and stroma beneath atrophic epithelial tissue (**Figure 1**).Through extracting DNA and low-depth whole genome sequencing, we were able to analyze morphology-related copy number alterations (CNA)[15]. CNA is a common form of genomic instability associated with an increased risk of malignant transformation in OPMD[15,16]. We detected different CNA patterns among four kinds of samples. As shown in **Figure 6**, CNA was present in epithelial samples but not in stroma samples. Although the samples originated from the same patient, the CNA pattern in the dysplastic epithelium was not the same as that in the atrophic epithelium. CNA in chromosomes 3 and 8 were detected in dysplastic epithelium, while CNA was detected at a lower frequency in chromosome 8 in atrophic epithelium.

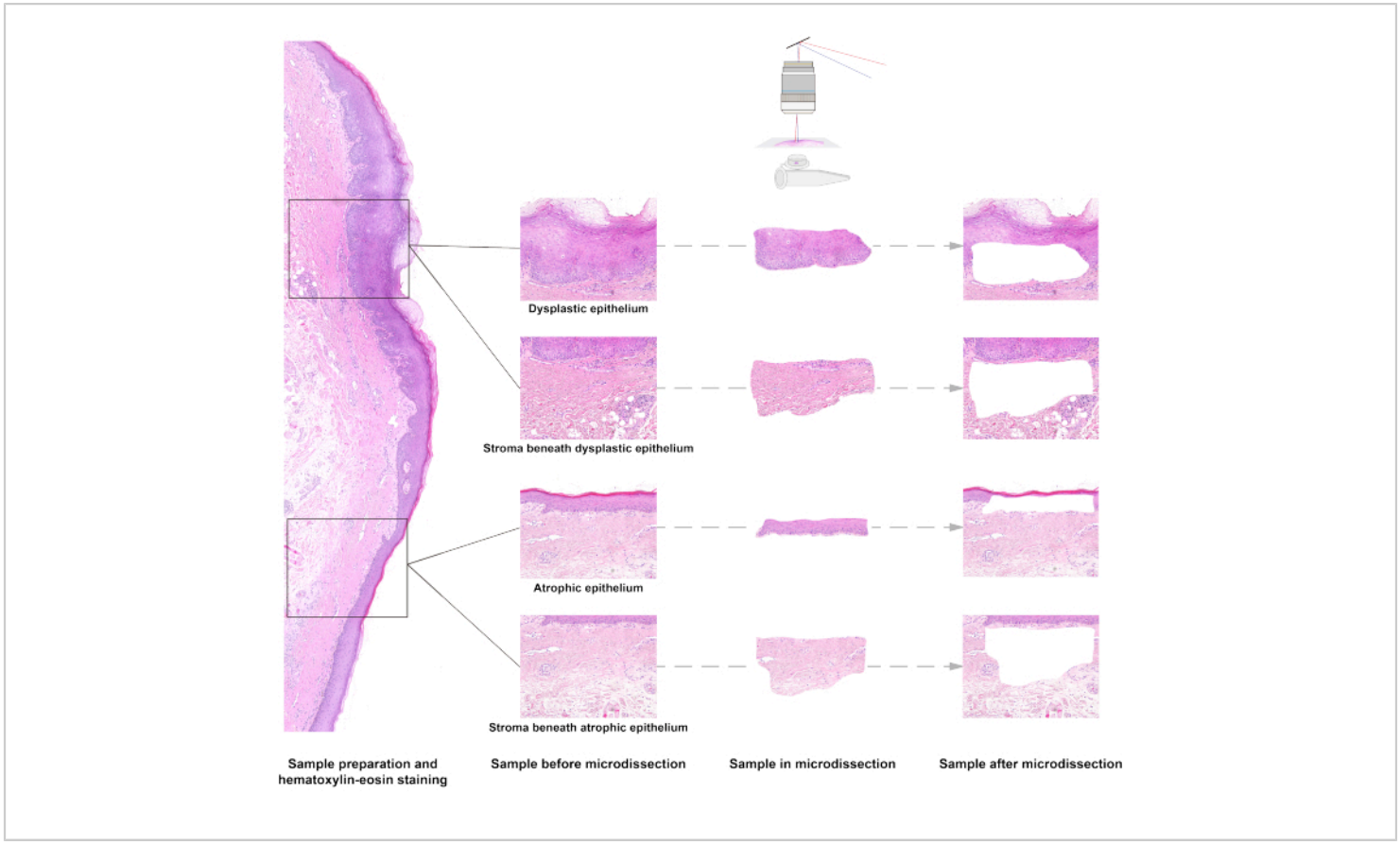

Figure 1: The scheme of laser capture microdissection of oral submucous fibrosis samples. The tissues of epithelium and stroma with different patterns are captured by laser under a microscope. Please click here to view a larger version of this figure.

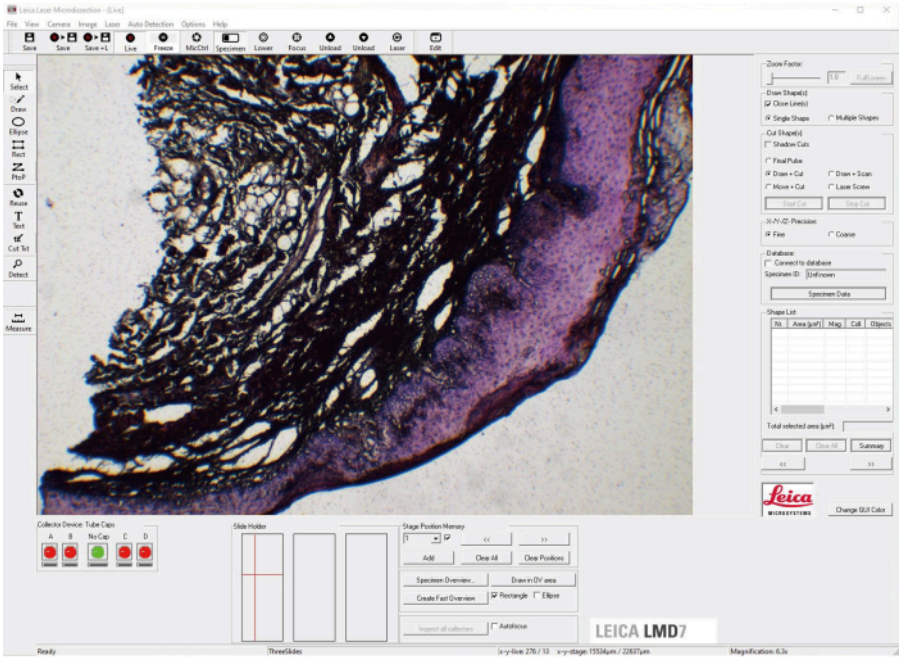

Figure 2: Laser microdissection software. A screenshot of the software showing the live panel. Please click here to view a larger version of this figure.

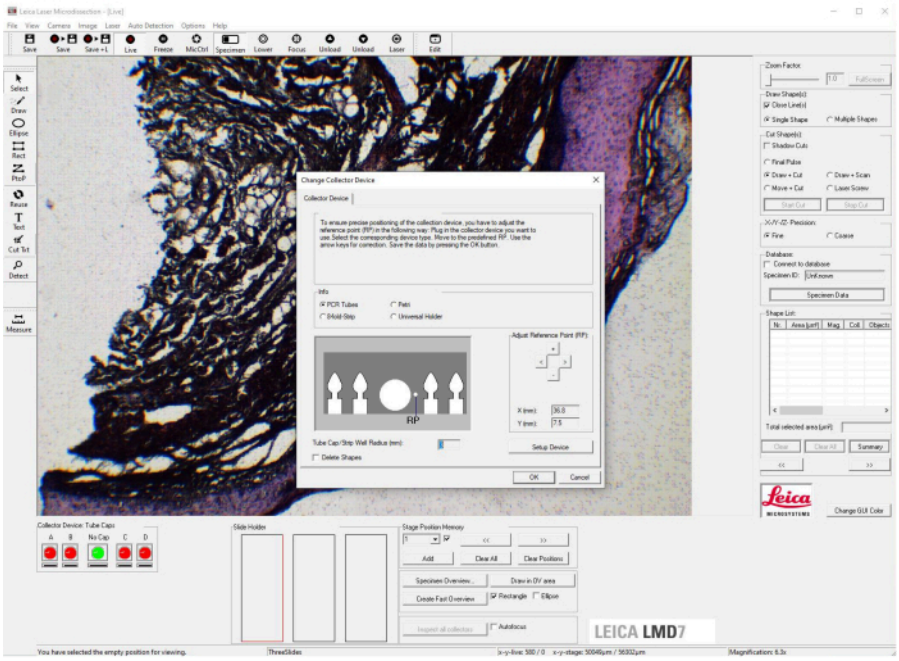

Figure 3: Collector device window. A screenshot of the software showing the collector device window for selecting the collection device. Please click here to view a larger version of this figure.

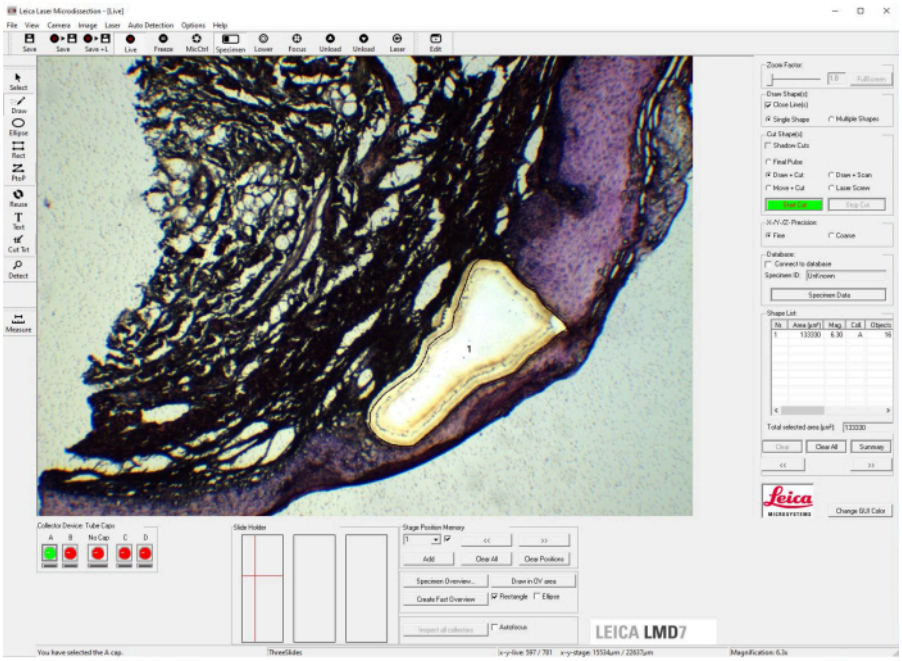

Figure 4: Capturing the sample in the area of interest. Clicking on the **Start Cut** button will allow capturing the area of interest. Please click here to view a larger version of this figure.

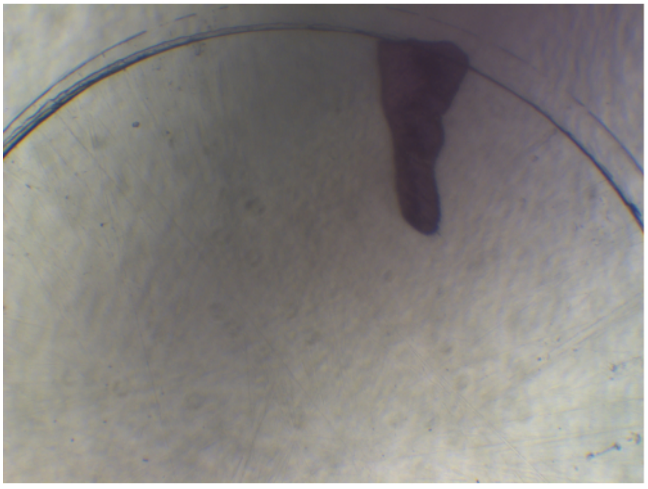

Figure 5: Captured sample. The captured sample can be seen in the cap of the PCR tube. Please click here to view a larger version of this figure.

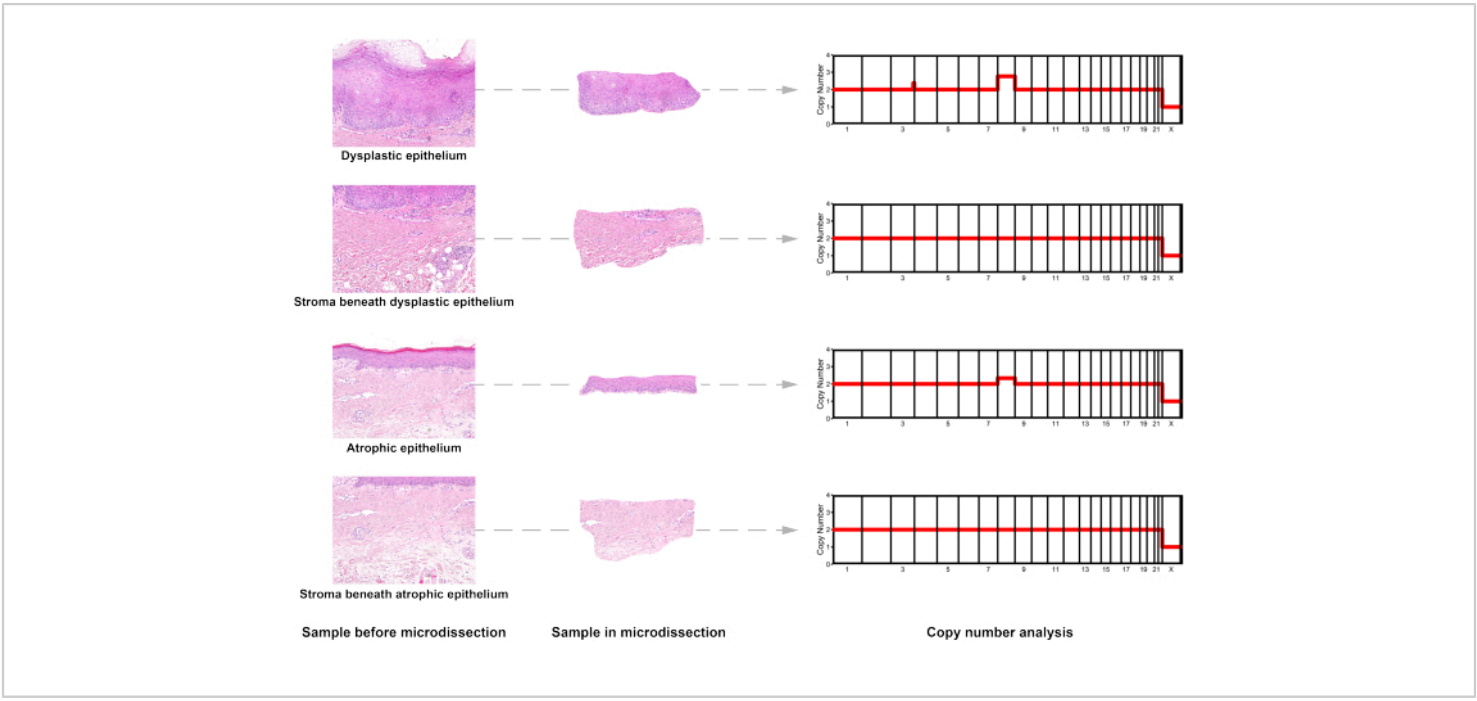

Figure 6: Copy number alterations. The representative results of different patterns of copy number alterations among various samples. There are different copy number alterations between dysplastic epithelium and atrophic epithelium. Please click here to view a larger version of this figure.

Discussion

This protocol reported a pipeline to capture OSF tissue samples with morphological and spatial information for further spatial-omic analyses through laser microdissection. From the representative results, we identified different CNA patterns among various morphology-related samples.

OSF, a type of OPMD, is a common precancerous condition of oral squamous cell carcinoma[6]. Genomic instability has been reported to be associated with the development and malignant transformation of OPMD[17,18]. Multiple studies have reported chromosome alterations, differential gene expression, and epigenetic variations associated with the progression and malignant transformation of OSF from genomics, transcriptomics, and proteomics data[19,20,21,22,23,24]. The carcinogenic components of areca nut, such as arecoline, not only led to the development of OSF, but the long-term stimulation also induced the senescence of fibroblasts and EMT and continuous production of reactive oxygen species (ROS), thus resulting in malignant transformation by regulating the immune microenvironment and signal pathways such as TGF-β and NF-κB signals[5,10,21]. However, both abnormal epithelial and stromal histological changes are present in OSF tissues, and which tissue or cellular alterations drive the malignant transformation in OSF remains unclear. Previous studies have mostly used bulk sequencing analyses, in which DNA or RNA was extracted from a block of tissue to further analyze the molecular expression and involved pathways. However, this analysis lacked a morphological correlation with histology and ignored the intratissue heterogeneity; furthermore, this approach cannot identify whether there

are differential molecular alterations in different histological areas.

Recent studies have reported spatial-omics analysis in many diseases[11,13,25]. Through precise spatial-omics analysis, increasing numbers of molecules and targets are being discovered and cellular interactions and clustering are being elucidated[11,12,13]. However, there is still a lack of spatially resolved omics analysis of OSF. OSF samples were usually taken from biopsies of oral mucosa and were prepared as small volumes of formalin-fixed, paraffin-embedded samples, making it difficult to perform single-cell spatial omics sequencing, which needs large samples. Therefore, the development of methods with spatial resolution using formalin-fixed paraffin-embedded samples might be a significant benefit. We expect the current protocol to benefit researchers working on OSF and other types of OPMD and contribute to understanding the mechanisms of the development and malignant transformation of OPMD.

This method has some limitations. First, the protocol was performed using formalin-fixed paraffin-embedded samples, making it difficult to extract RNA for sequencing analysis; however, morphology-related RNA sequencing analysis can be performed when using crystalline violet staining and maintaining aseptic practices[15,26]. The currently captured samples could not meet the level of single-cell resolution because when the number of cells is too small, the cells might be broken down by the laser beam, affecting DNA extraction.

Disclosures

The authors declare no conflict of interest.

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