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# Digitally guided microdissection aids somatic mutation detection in difficult to dissect tumors

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Molecular genetic testing on formalin fixed, paraffin embedded (FFPE) tumors frequently requires dissection of tumor from tissue sections mounted on glass slides. In a process referred to as "manual macrodissection," the pathologist reviews an H&E stained slide at the light microscope and marks areas for dissection, and then the laboratory performs manual dissection from adjacent sections without the aid of a microscope, using the marked reference H&E slide as a guide. Manual macrodissection may be inadequate for tissue sections with low tumor content. We compared manual macrodissection to a new method, digitally guided microdissection, on a series of 32 FFPE pancreatic cancer samples. *KRAS* hotspot mutation profiling was performed using the Sequenom MassARRAY system (Agena Bioscience). Digitally guided microdissection was performed on multiple smaller areas of high tumor content selected from within the larger areas marked for manual macrodissection. The *KRAS* mutant allele fraction and estimated neoplastic cellularity were significantly higher in samples obtained by digitally guided microdissection ($p < 0.01$), and 7 of the 32 samples (22%) showed a detectable mutation only with digitally guided microdissection. DNA quality and yield per cubic millimeter of dissected tissue were similar for both dissection methods. These results indicate a significant improvement in tumor content achievable with digitally guided microdissection.

**Keywords** Microdissection, macrodissection, digital pathology, formalin fixed paraffin embedded


## Introduction

Molecular genetic testing is increasingly performed on material extracted from formalin fixed, paraffin embedded (FFPE) tissue sections to aid histopathologic diagnosis, to define molecular subtypes for treatment planning, and to provide relevant prognostic information. Most current molecular assays on solid tumors are designed to tolerate a mixture of tumor and non-tumor cells, but higher neoplastic cellularity maximizes analytical sensitivity. Histologic examination of FFPE tissue sections is essential to confirm sufficient neoplastic cellularity prior to testing.

Different molecular testing approaches have different thresholds for mutation detection. Sanger sequencing is unreliable for cases with a neoplastic cellularity of 40% or lower, since somatic mutations are often heterozygous and the lower threshold for mutation detection is a mutant allele fraction of about 20% (1,2). Targeted pyrosequencing can detect lower frequency mutations, with some assays reliably detecting a mutant allele fraction as low as 5% (2). The analytical sensitivity of mass spectrometry and next generation sequencing (as currently used for multigene panels) is similar to that of targeted pyrosequencing (1,3). Ultrasensitive technologies such as allele specific PCR or digital droplet PCR can detect even lower frequency mutations. The clinical relevance of low frequency mutations present in minor subpopulations of tumor cells is

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not completely understood, however, even though the importance of tumor heterogeneity is increasingly recognized (4,5). Additionally, spurious low level mutations can occur as a result of the formalin fixation process (6). Regardless of the testing strategy, high tumor content is desirable for molecular testing because the presence of non-tumor cells can hinder the detection of clinically relevant somatic mutations relative to the detection thresholds of various molecular technologies.

Whole sections or scrolls from paraffin blocks are not always acceptable for molecular testing due to variable tumor content. Therefore, some form of tumor enrichment is often necessary when using FFPE tissue. In clinical molecular oncology testing, the most frequently utilized tumor enrichment method is manual tissue dissection from glass slides. Areas of tumor are selected by microscopic examination, manually circling, under microscopic guidance, areas on a hematoxylin and eosin (H&E) stained glass slide with a slide marking pen. Dissection is carried out on stained or unstained serial sections from the same block, using the marked H&E slide as a guide (7). A variety of manual dissection methods have been adopted, including the use of a scalpel, pipette tip, razor blade, or needle manually guided with a micromanipulator (7–13). In a commonly used method called manual macrodissection, a scalpel blade or similar scraping tool is used to remove microscopically marked areas off of the dissection slide without the aid of a microscope. Manual macrodissection is adequate for the majority of cases submitted for clinical testing, but it may be inadequate in cases with low tumor content. Because molecular testing can be an essential part of treatment planning, some cancer patients may have to undergo an additional clinical procedure in order to obtain an adequate sample for molecular testing. In cases where there is sufficient tumor on the slide but it cannot be adequately dissected by hand, a more precise dissection method is desirable. Laser capture microdissection (LCM) was introduced about two decades ago (14), but it has not been widely adopted in the clinical laboratory setting. LCM has been noted in multiple studies to diminish the yield and quality of retrieved material (9,15–17), although others report that sample recovery is not impaired by the LCM process (18). Even so, the precise dissection of single tumor cells from FFPE slides is seldom necessary for clinical molecular testing, whereas the rapid and cost effective retrieval of a sufficient DNA or RNA sample is paramount; therefore, lower resolution microdissection methods can be substituted for LCM.

A new microdissection method utilizing a modified computer numerical control (CNC) milling machine was recently introduced as an intermediate resolution method for dissecting tissue from glass slides. Current milling microdissection technology can dissect regions with a diameter of 200 microns or greater, and early experiments have been promising (19). This new microdissection method can be performed on any glass surface and therefore the method integrates easily into clinical laboratory workflow. When used in combination with digital slide marking ("digitally guided microdissection"), the technology can achieve a much greater resolution than manual dissection methods. We compared digitally guided microdissection to our traditional dissection method, manual macrodissection, on a series of FFPE pancreatic adenocarcinoma specimens. Pancreatic adenocarcinomas usually have an infiltrative growth pattern with small clusters of tumor cells surrounded by non-tumor cells, and so these cancers can be difficult to dissect manually. Pancreatic adenocarcinomas also have a high prevalence of *KRAS* mutations (20,21), and the mutant allele fraction can be used as a surrogate measure of the tumor purity in comparing the same area of a tumor dissected both ways. We used both the estimated neoplastic cellularity and the *KRAS* mutant allele fraction to compare dissection methods on 32 FFPE pancreatic adenocarcinoma samples.

## Materials and methods

### Case selection, slide marking for dissection, and evaluation of tumor content

The University of Utah Institutional Review Board approved the study protocol. Pancreatic adenocarcinoma cases from the University of Utah Department of Pathology archives were evaluated for tumor content. Thirty-two paraffin embedded tissue blocks from eighteen different cases of pancreatic adenocarcinoma were selected for this comparison study. Tumor characteristics are shown in Table 1. Tissue blocks were serially sectioned, deparaffinized, and stained with either hematoxylin and eosin (H&E) or aniline blue, a water soluble collagen stain similar to toluidine blue that permits easy visualization of the tissue on the slide for dissection. For each tissue block, a coverslipped H&E section was used for microscopic evaluation, and additional adjacent aniline blue stained serial sections (5 microns thick) were used for dissection. Regions for manual macrodissection were identified microscopically and marked directly on the H&E slide with a slide marking pen. The marked H&E slides were then digitally scanned using the Aperio ScanScope® XT system (Leica Biosystems, Vista, CA, USA). Regions measuring at least 200 microns in diameter were chosen for digitally guided microdissection within the manually marked areas and were marked digitally using the pen tool in Aperio ImageScope® software (Leica Biosystems). Digital slide annotations were typically performed at 40×–100× resolution in ImageScope software (equivalent to use of the 4× and 10× objective lenses on a standard light microscope with 10× ocular lens magnification). Images exported from ImageScope® software to guide milling microdissection were typically acquired at 10× resolution.

Three pathologists (KG, ED-K, and MB) independently estimated the neoplastic cellularity of the regions designated for manual macrodissection and digitally guided microdissection. Neoplastic cellularity, defined as the percentage of cells that are neoplastic, was estimated on a semi-quantitative scale and stratified into groups of <1%, 1–5%, 5–10%, 10%, 20%, 30%, and so on up to 100%.

### Slide dissection

Serial sections used for manual macrodissection or digitally guided microdissection were distributed equally in distance from the reference slide. For manual macrodissection, the marked H&E reference slide was manually aligned to the corresponding aniline blue stained dissection slide, the marked area was manually traced onto the dissection slide, the area to be scraped was soaked briefly in molecular biology grade water to make it easier to scrape, and the marked area was scraped by hand with a surgical steel scalpel. The MilliSect™