Sequence analysis of glioblastoma found a mutation in isocitrate dehydrogenase 1 (IDH1), which mostly resulted in the replacement of wild-type arginine in position 132 with histidine (R132H). Studies have reported that the IDH1 mutation is frequently detected in diffuse astrocytoma (59%–88%), anaplastic astrocytoma (52%–78%), oligodendroglioma (68%–82%), anaplastic oligodendroglioma (60%–75%), oligoastrocytoma (50%–94%), anaplastic oligoastrocytoma (66.1%–78%), and secondary glioblastomas (50%–88%), but rarely in primary glioblastomas (3%–7%).1,14 IDH1 mutation was also closely associated with young age and better clinical outcome.34

Currently, the detection of IDH1 mutation status can be achieved by DNA-based methods, such as DNA pyrosequencing,10 polymerase chain reaction (PCR),13,24 and immunohistochemistry (IHC). The majority of laboratories use IHC for initial screening for IDH1 mutation and, if results are negative, use DNA sequencing for less common IDH1 mutant types. DNA sequencing provides direct
evidence for \textit{IDH1} gene mutation. However, it is difficult to detect \textit{IDH1} mutation in highly contaminated tumor samples, and enrichment methods are needed.\textsuperscript{32}

Moreover, elaborate laboratory equipment for DNA sequencing or PCR is not available in most neuropathology settings. Anti–\textit{IDH1} R132H immunostaining based on commercially available antibody has been confirmed as a reliable method for the detection of \textit{IDH1} mutation.\textsuperscript{27} For the \textit{IDH1 R132H} mutation type, both its sensitivity and specificity are up to 100\%\textsuperscript{29,30} IHC may be more sensitive than sequencing, especially for tiny biopsy samples.\textsuperscript{27,32} IHC can also be used to identify a single infiltrated tumor cell and discriminate the infiltrating edge of \textit{IDH1}-mutant tumors from reactive gliosis.\textsuperscript{4,5} Above all, a recent study indicated that \textit{IDH1} mutation in malignant astrocytoma could serve as a predictive molecular biomarker to guide extensive resection, allowing for individualized therapy based on tumor genotype.\textsuperscript{3} However, regular IHC takes more than 48 hours and cannot meet the demand of intraoperative analysis.

Microfluidics, also known as lab-on-a-chip, is a technique that manipulates or processes small volumes of fluids at micron dimension.\textsuperscript{2,33} It is attracting growing attention in many fields because it requires less time and less reagent. Other advantages include rapid reaction speed, easy control of reaction conditions, and easy fabrication.\textsuperscript{8,18,31} It can also integrate microvalves with pumps to precisely control the reagent inside the microchips, which is perfect for performing complicated biomarker analysis from a tiny sample within a short period of time.\textsuperscript{5,11,29,30} Herein we describe a new approach for rapid analysis of \textit{IDH1} mutation from microsampling of tissues, which was successfully developed using a microfluidic device. This new approach is capable of rapid analysis of \textit{IDH1} mutation status from tiny amounts of tissue within 30 minutes, using much less sample and smaller reagent amounts compared with conventional methods.\textsuperscript{11,15,35}

\textbf{Methods}

\textbf{Patients and Tissue Specimens}

Forty-seven glioma tumor samples were obtained from the Glioma Tissue Bank of the Glioma Surgery Division, Neurological Surgery Department of Huashan Hospital, Fudan University. All specimens were prospectively collected in the operating room, in accordance with protocols approved by Huashan Institutional Review Board, between 2012 and 2013. Each patient gave informed consent before the operation. For microfluidics, each retrieved frozen sample was around 5 mg. The retrieved samples were used in the experiment without purification. The diagnosis was made according to the histological assessments, following the WHO classification.\textsuperscript{19}

\textbf{Microfluidics}

\textbf{Reagents}

Reagents used included ProteoExtract Complete Mammalian Proteome Extraction Kit (Cat. No. 539779, Calbiochem); anti–human \textit{IDH1} R132H (mouse monoclonal, Histonova); anti–human \textit{IDH1} (rabbit polyclonal, Epitomics); and FITC anti–mouse antibody (Abcam, Ltd). Microspheres (Protein A, 9-micron diameter) were purchased from Bang’s Laboratory. All other reagents (analytical purity) were purchased from Sigma.

\textbf{Microfluidic Device Fabrication}

The microfluidic device used for this study was fabricated from polydimethylsiloxane following standard soft lithography. The device was composed of 2 layers: upper fluidic and bottom control. Two different molds with different patterns were fabricated by standard soft lithographic processes.\textsuperscript{8,18,22,35} The structure of the proposed microfluidic chip is shown in Supplemental Fig. 1 and discussed in Supplemental Methods.

\textbf{Immunohistochemistry of \textit{IDH1} R132H}

The EnVision FLEX detection system (Dako) and anti–IDH1 R132H (Hu) from mouse (Clone: H09) (Dianova) were used. An automated immunostaining procedure was performed according to the Dianova technical note. Immunostaining was interpreted as positive when tumor nuclei showed strong positive staining for \textit{IDH1} R132H.

To precisely quantitate \textit{IDH1}-mutant protein expression in tumor cells, slides were further analyzed with the Vec-
Automated Multispectral Imaging System equipped with inForm software (PerkinElmer, Inc.). Automated acquisition was in a batch run involving ×40 whole-slide scans followed by high-magnification (×200) imaging for regions of interest only. Histological images were digitized and then analyzed with the nuclear algorithm. Scores were generated using the inForm software, which provided the intensity of staining. For each slide, 5 high-magnification (×200) images were obtained and analyzed separately. The average intensity score for nucleic regions of 5 images was considered as immunostaining intensity for each sample.

DNA Sequencing of IDH1 R132H

Formalin-fixed paraffin-embedded (FFPE) tumor samples were used. Two 10-μm-thick slides of each sample were prepared. Tissue was microdissected from 2 FFPE sections to guarantee that tumor content was greater than 70%. The primers of IDH1 R132 hotspot mutation were forward 5′-CGGTCTTCAGAGAAGCCATT-3′ and reverse 5′-CACATTATTGCCAACATGAC-3′. A detailed description of the DNA sequencing process is given in Supplemental Methods.

Statistical Analysis

Statistical analysis was performed using Statistical Product and Service Solutions software (SPSS 20, IBM Corp.) and ROCR package in R (http://www.r-project.org). Fluorescence signals and intensity score difference between IDH1 mutant and wild type was compared using the 2-sample Wilcoxon rank-sum test. Fluorescence intensity and the intensity score between different tumor types were compared using the Kruskal-Wallis test. The cutoff value and receiver operating characteristic (ROC) curve were calculated using the ROCR package in R. All tests were 2-tailed, with a CI of 95%. A p value less than 0.05 was considered statistically significant.

Results

Sample Characteristics

Basic information about the 47 samples is shown in Supplemental Fig. 2. The subjects comprised 10 diffuse astrocytomas, 12 oligodendrogliomas, 8 anaplastic astrocytomas, 7 anaplastic oligodendrogliomas, and 10 primary glioblastomas. Samples of 22 male and 25 female patients were included, and the mean age was 45.70 ± 12.64 years. WHO grading revealed 22 Grade II samples, 15 Grade III, and 10 Grade IV.

Microfluidics

Figure 2 shows examples of fluorescence images of the on-chip microcolumns and corresponding IHC images for various tumor types. Differences between fluorescence images of microcolumns responding to various types of glioma could be easily identified. The shining spots on the images indicated the inhomogeneity of the lysis extract. The average intensity of the fluorescence images was collected and data analyses were executed to evaluate the performance of the proposed microfluidic device.

The fluorescence signal for IDH1-mutant samples was 5.49 ± 1.87 compared with 3.90 ± 1.33 for wild type (p = 0.005). Thus, microfluidics was capable of distinguishing IDH1-mutant samples from wild type. Fluorescent signals for different tumor types are shown in Table 1. Comparison of fluorescence signals between different tumor types revealed statistical significance between primary
glioblastoma and anaplastic astrocytoma (3.15 vs 5.57, \( p = 0.011 \)), oligodendroglioma (3.15 vs 5.14, \( p = 0.004 \)), and diffuse astrocytoma (3.15 vs 6.36, \( p < 0.001 \)). No statistical significance was observed between other tumor types. Each measurement was repeated 3 times and the average number was presented. The SD values were within the acceptable range, suggesting the feasibility of this approach to analyze the IDH1 mutation status from tiny (about 5 mg) samples. The whole process, from injecting samples to fluorescent signal readout, can be completed within 30 minutes, which is fast enough to perform during the surgical procedure. Figure 3 presents the distribution of all fluorescence signals. When the cutoff value was 4.11, it helped to distinguish most of the mutant samples from wild-type ones. Specificity and sensitivity are presented in Fig. 4.

Detection of IDH1 R132H Mutation Status

Mutational hotspot arginine at codon 132 of IDH1 was evaluated by direct sequencing (the gold standard for gene mutation) and IHC (the routinely applied method in our clinical setting). Immunostaining was interpreted as positive when tumor cell nuclei showed strong positive staining for IDH1 R132H. The identical results of these 2 methods, presented in Table 1, confirmed that IHC was a reliable approach. Fluorescent signals of the tumor samples, as well as the IHC and DNA sequencing results, are presented in Table 1. DNA sequencing identified IDH1 R132H mutation in 8 of 10 diffuse astrocytomas, 11 of 12 oligodendrogliomas, 8 of 8 anaplastic astrocytomas, 4 of 7 anaplastic oligodendrogliomas, and 2 of 10 primary glioblastomas. Slide analysis using inForm software revealed that the average intensity score of IHC-mutant samples was 0.151 \( \pm \) 0.069 compared with 0.029 \( \pm \) 0.021 for wild type (\( p < 0.001 \)). The intensity score of IHC for different tumor types is also presented in Table 1. Statistical significance was observed between primary glioblastoma and anaplastic astrocytoma (0.067 vs 0.161, \( p = 0.036 \)), but not between primary glioblastoma and anaplastic oligodendroglioma (0.067 vs 0.091, \( p = 1.000 \)), primary glioblastoma and diffuse astrocytoma (0.067 vs 0.110, \( p = 1.000 \)), primary glioblastoma and oligodendroglioma (0.067 vs 0.139, \( p = 0.337 \)), or between other different tumor types. Conventional IHC could be applied to detect the IDH1 mutation status. However, this semiquantitative method could not be used to compare the protein levels of different tumor types.
Comparison of IHC and Microfluidics

Both IHC and microfluidics showed the potential to detect IDH1 mutation status. For comparison purposes, IHC images of these tumor samples were digitized and are presented in Table 1. The ROC curve was also established and the area under the curve (AUC) for microfluidics was 0.7608 (Fig. 4). When the cutoff value was 4.11, the sensitivity of microfluidics was 87.9% and the specificity was 64.3% (Fig. 4). During the IHC process, the FFPE tumor sample was attached with monoclonal antibody to IDH1 R132H, and stained by the second antibody at visible light range. In this procedure, the polyclonal anti-human IDH1 was not used, as compared with the immunoanalysis procedure using microfluidics. Whereas IHC focused more on the morphological information of the IDH1 distribution, the proposed microfluidics method emphasized the precise measurement of IDH1 expression from the tissue extract, yet could not provide any morphological information. Although a proportional linear relationship between the results from IHC and those from the proposed microfluidics approach was not expected, the comparison revealed that similar amounts of IDH1 were measured.

Discussion
Feasibility of Microfluidics

IDH1 mutation has been identified as a diagnostic and prognostic biomarker in glioma.12,21 Patients with malignant astrocytoma with IDH1 mutations have nearly twice as long, or even 10 times longer, median overall survival compared with those without mutations.3,34 A recent study also revealed that refinement of the classification of the 3 most common types of brain tumors could be achieved by telomerase reverse transcriptase promoter and IDH1 mutation status.16 Mutation in the IDH1 gene mostly affects IDH1 protein, by replacing wild-type arginine in position 132 with histidine (R132H). This makes it feasible to detect IDH1 gene mutation through IHC by using specific antibodies that bind mutant IDH1 protein.25 However, regular IHC analysis can only be applied for postoperative analysis. Preoperative precise detection of 2-hydroxyglutarate
by MR spectroscopy is still under investigation.\(^7,9,17,26\) Our study, which is based on microfluidics and use of specific antibodies for \(\text{IDH1}\)-mutant protein, was also applicable for detection of \(\text{IDH1}\) mutation status.

According to our statistical results, 4.11 was set as a cut-off value to distinguish most of the \(\text{IDH1}\)-mutant samples from wild type, regardless of the tumor type and grade. The microfluidic approach was developed to detect \(\text{IDH1}\) mutation status intraoperatively to guide personalized surgery; in other words, for use when the pathology result was not available or there was only a preliminary result obtained from frozen sections. Setting different cutoff values for different tumor types was not practical when it was applied during surgery, and it could even be confusing. When the cutoff value was 4.11, the sensitivity of microfluidics was 87.9% and the specificity was 64.3%. The low specificity was due to several aspects. First, from Table 1, we can see that different tumor types had different baselines for negative samples. This difference may come from manual operations, such as fluorescence image reading and sample dissection. Tumor inhomogeneity may also contribute to the different negative baselines, even within the same tumor type. We believe that in future experiments, after we modify our sample-input techniques and apply this method to more samples, the baseline deviation can be decreased. After integrating clinical information (e.g., patient age, course of disease, medical images, and frozen-section results), the final specificity will increase.

**Clinical Implications for Individualized Surgery**

Microfluidics is capable of intraoperative analysis of \(\text{IDH1}\) mutation status, and the clinical implication lies in intraoperatively directing the individualized surgery. The chief implications are as follows.

1) For low-grade glioma, due to the lack of macroscopic discriminable tumor margin, the frozen section is the method for intraoperative identification of tumor infiltration. However, this method may not precisely identify the tumor infiltration from reactive gliosis because detectability mostly depends on the morphological experience of the neuropathologists. Thus, the possibility of misdiagnosis always exists. Because \(\text{IDH1}\) mutation has been identified as a Level IIb diagnostic biomarker, microfluidics can complement the conventional intraoperative pathological

---

**TABLE 1. Fluorescence signal and IHC intensity score of different tumor types**

<table>
<thead>
<tr>
<th>Tumor Type</th>
<th>(\text{IDH1 R132H Mutation Status})</th>
<th>Fluorescence Signal</th>
<th>IHC Intensity Score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean   SD</td>
<td>Mean   SD</td>
</tr>
<tr>
<td>Diffuse astrocytoma</td>
<td>Negative (n = 2); wild type (n = 2)</td>
<td>5.35 0.98</td>
<td>0.037 0.015</td>
</tr>
<tr>
<td></td>
<td>Positive (n = 8); mutant type (n = 8)</td>
<td>6.61 2.45</td>
<td>0.128 0.049</td>
</tr>
<tr>
<td></td>
<td>All; all</td>
<td>6.36 2.25</td>
<td>0.110 0.058</td>
</tr>
<tr>
<td>Oligodendrogloma</td>
<td>Negative (n = 1); wild type (n = 1)</td>
<td>5.78 NA</td>
<td>0.026 NA</td>
</tr>
<tr>
<td></td>
<td>Positive (n = 11); mutant type (n = 11)</td>
<td>5.08 1.34</td>
<td>0.150 0.089</td>
</tr>
<tr>
<td></td>
<td>All; all</td>
<td>5.14 1.30</td>
<td>0.139 0.092</td>
</tr>
<tr>
<td>Anaplastic astrocytoma</td>
<td>Negative (n = 0); wild type (n = 0)</td>
<td>NA NA</td>
<td>NA NA</td>
</tr>
<tr>
<td></td>
<td>Positive (n = 8); mutant type (n = 8)</td>
<td>5.57 1.93</td>
<td>0.161 0.057</td>
</tr>
<tr>
<td></td>
<td>All; all</td>
<td>5.57 1.93</td>
<td>0.161 0.057</td>
</tr>
<tr>
<td>Anaplastic oligodendrogloma</td>
<td>Negative (n = 3); wild type (n = 3)</td>
<td>4.69 0.73</td>
<td>0.020 0.008</td>
</tr>
<tr>
<td></td>
<td>Positive (n = 4); mutant type (n = 4)</td>
<td>5.14 1.54</td>
<td>0.145 0.070</td>
</tr>
<tr>
<td></td>
<td>All; all</td>
<td>4.95 1.19</td>
<td>0.091 0.088</td>
</tr>
<tr>
<td>Primary glioblastoma</td>
<td>Negative (n = 8); wild type (n = 8)</td>
<td>3.01 0.85</td>
<td>0.031 0.027</td>
</tr>
<tr>
<td></td>
<td>Positive (n = 2); mutant type (n = 2)</td>
<td>3.74 0.76</td>
<td>0.212 0.064</td>
</tr>
<tr>
<td></td>
<td>All; all</td>
<td>3.15 0.85</td>
<td>0.067 0.083</td>
</tr>
</tbody>
</table>

NA = not applicable.

* \(\text{IDH1}\) mutation status was detected by IHC methods and DNA sequencing. Immunostaining was interpreted as positive when tumor cell nuclei showed strong positive staining for mutant \(\text{IDH1 R132H}\).
diagnosis, and can also be applied for more precise identification of tumor-infiltrated margin.

2) At the same time, the detection of IDH1-mutant protein, which is based on microfluidics, can also be applied for needle biopsy samples. Because diagnostic accuracy of standard imaged-guided needle biopsy is usually hampered by an insufficient amount of sample and the limited availability of IHC,23 microfluidics can compensate for these disadvantages of needle biopsy.

3) A recent study of malignant astrocytomas (i.e., anaplastic astrocytoma and glioblastoma) found that IDH1-mutant lesions are more amenable to resection. Complete resections of enhancing and nonenhancing lesions were associated with better overall outcome, suggesting the benefit of extensive resection for patients with IDH1 mutations.3 The study also revealed that IDH1-mutant lesions displayed lower volumes of preoperative contrast enhancement but higher complete resection rates than wild-type lesions. Additionally, extensive resection in low-grade glioma predicted improved overall survival,26 suggesting a possible survival advantage. Hence, IDH1 mutation may also be a predictive biomarker guiding aggressive resection, in addition to an individualized therapy based on genotype. However, the current pathological technique available during surgery cannot meet the demand of intraoperative analysis. Our study results provide a reliable intraoperative technique for detection of IDH1 mutation.

Advantages

Herein we describe the successful development of a new microfluidics approach for the rapid analysis of IDH1 mutation from tumor tissue. The IDH1 from tiny amounts of tissue obtained by needle sampling can be analyzed within 30 minutes, with much less reagent consumption than conventional methods. Traditional enzyme-linked immunosorbent assay or IHC cannot handle samples at the sub-microgram level, and neither can these methods complete this measurement within such a short time period. Tumor samples from more than 40 patients were used to validate the proposed approach. The results from the proposed approach were also compared with those from IHC. Similar results were obtained between both approaches. The good performance, high sensitivity, and time savings can be attributed to the properties of microfluidics, especially the unique structure of the immunocolumn.

Perspectives

For the further development of microfluidics, technical improvements (such as chips that can be operated in a simpler and more automated way) are still needed. For a more accurate cutoff value between mutant and wild types, a larger-scale study should be performed using the Glioma Tissue Bank. Furthermore, because the feasibility of detecting IDH1-mutant protein has been shown, it could be used in the rapid analysis of other protein biomarkers. Therefore, further investigations should be performed to design and fabricate microfluidics, which are capable of parallel high-throughput analysis for detection of other biomarkers such as MIB-1, P53, GFAP, and so on.

Conclusions

This new approach was capable of analyzing the IDH1 mutation status of tiny amounts of tissue within 30 minutes, using intraoperative microsampling. When the cutoff value was 4.11, the sensitivity of microfluidics was 87.9% and the specificity was 64.3%. In the future, based on larger sample sizes and more advanced industrialized design, it can not only supplement current techniques but also contribute to individualized therapy for improving the clinical outcomes of glioma patients.

Acknowledgments

We would like to thank all of the nurses in operating rooms and wards for their cooperation during the sample collection, Yu-qian Wang for the collection of glioma tissues, and Zhen-xiao Wang and Yan Chen for the data input of clinical information. We would also like to thank Professors Le-ming Shi and Ying Yu for their guidance in statistical analysis. Funding was received from National Key Basic Research Program of China (2013CB932502); National Natural Science Foundation of China (21377026); Shanghai Committee of Science and Technology, China (12DZ2295003); and Shanghai Municipal Health Bureau, China (XBR2011022).

References

12. Guo C, Pirozzi CJ, Lopez GY, Yan H: Isocitrate dehydro-

Disclosure
The authors report no conflict of interest concerning the materials or methods used in this study or the findings specified in this paper.

Author Contributions
Conception and design: Aibaiduala, Zhao, Chen. Acquisition of data: Aibaiduala, Zhao, Chen, Shi, Zheng. Analysis and interpretation of data: Aibaiduala, Zhao, Chen, Shi, Zheng. Drafting the article: Aibaiduala, Zhao. Critically revising the article: Wu. Reviewed submitted version of manuscript: Wu, Aibaiduala, Chen, Mao, Zhou, Sui. Approved the final version of the manuscript on behalf of all authors: Wu. Statistical analysis: Aibaiduala, Shi. Administrative/technical/material support: Wu, Zhao, Mao, Zhou, Sui. Study supervision: Wu, Mao, Zhou, Sui.

Supplemental Information
Online-Only Content
Supplemental material is available with the online version of the article.


Correspondence
Jin-song Wu, Glioma Surgery Division, Neurological Surgery Department, Huashan Hospital, Shanghai Medical College, Fudan University, 12 Middle Wulumuqi Rd., Shanghai 200040, China. email: wjsongc@126.com.