Intratumoral heterogeneity in glioblastoma: don’t forget the peritumoral brain zone

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Glioblastoma (GB) is the most frequent and aggressive primary tumor of the central nervous system. Prognosis remains poor despite ongoing progress. In cases where the gadolinium-enhanced portion of the GB is completely resected, 90% of recurrences occur at the margin of surgical resection in the macroscopically normal peritumoral brain zone (PBZ). Intratumoral heterogeneity in GB is currently a hot topic in neuro-oncology, and the GB PBZ may be involved in this phenomenon. Indeed, this region, which possesses specific properties, has been less studied than the core of the GB tumor. The high rate of local recurrence in the PBZ and the limited success of targeted therapies against GB demonstrate the need for a better understanding of the PBZ. We present here a review of the literature on the PBZ of GB, focusing on its radiological, cellular, and molecular characteristics. We discuss how intraoperative analysis of the PBZ is important for the optimization of surgical resection and the development of targeted therapies against GB.

Keywords: glioblastoma, histology, omics, peritumoral brain zone, radiology, targeted therapies.

Glioblastoma (GB) is the most frequent and aggressive primary tumor of the central nervous system (CNS). Its incidence varies depending on the country and the population, with 4.96 cases per 100,000 inhabitants per year.1 Despite ongoing progress in the therapeutic management of GB, prognosis remains poor, with an overall survival of 14–15 months after complete surgical resection and adjuvant radiochemotherapy.2 Typically, radiological recurrence occurs 6–7 months after surgery, quickly followed by clinical relapse.

The heterogeneity of GB is a hot topic in neuro-oncology. The interindividual heterogeneity of GB has been well known since the pioneering work of Burger and Kleihues,3–7 and several studies have even described intratumoral heterogeneity inside GB at the cellular and molecular level.6,8,9 This intratumoral heterogeneity may lead to a different Verhaak subtype in 2 different samples from the same tumor, or it may even give rise to differences at the cellular level as shown by Patel et al (2014).6,10–12 GB intratumoral heterogeneity may influence tumor aggressiveness or response to chemotherapies such as temozolomide.8,13

Intratumoral heterogeneity is not only limited to the tumor, it also involves the peritumoral brain zone (PBZ), which possesses specific properties that contribute to GB heterogeneity. Few studies have focused on the PBZ and its microenvironment, despite the fact that 90% of recurrences occur in this area.14 We present here a review of the PBZ of GB, focusing on its radiological, cellular, and molecular characteristics based on current literature and our findings obtained during the Grand Ouest Glioma Project, a translational research project on intratumoral heterogeneity that we recently conducted.12,15–24 We also highlight perspectives for future research on the PBZ that may facilitate the development of new targeted therapies against GB and the optimization of surgical resection.

Characteristics of the Glioblastoma Peritumoral Brain Zone

Radiological Aspect

The PBZ is usually defined radiologically as the brain area surrounding the tumor without contrast enhancement in T1 gadolinium-enhanced 3-dimensional (3D) magnetic resonance imaging (MRI). This region is often hyperintense in T2-weighted (especially T2-fluid-attenuated inversion recovery) acquisition, which reflects vasogenic edema in the vicinity of the tumor and suggests tumor infiltration. This definition of the PBZ delimits an area of several centimeters in width around the tumor, which is the site of tumoral invasion and specific molecular,
Further, residual and central GB cells respond differently to drug and irradiation challenges in vitro. Glas et al (2010) and 2 other studies using 5-aminolevulinic acid-assisted surgery found that residual GB cells have a lower capacity for self-renewal than central GB cells. Several genes may distinguish residual GB cells from central GB cells, including genes involved in the stem cell phenotype (CD133, Sox2, nestin, musashi 1), invasion (Galectin-1, Rac1, Rac3, RhoA GTPases, p27, αvβ3 integrin), cell adhesion (CDH20, PCDH19), migration (SNAI2, NANOG, USP6, DISC1), immune or inflammatory responses (TLR4), and blood vessel formation (HEG1, VEGFR2). The identification of these genes has opened new lines of research to improve the understanding of the molecular features promoting the dissemination and progression of GB.

**Reactive Astrocytes**

Astrocytes are star-shaped glial cells in the CNS with several important functions including synaptic transmission and information processing. Many studies have shown that reactive astrocytes are located around GB cells, but their role in GB progression is not completely understood. Reactive astrocytes promote tumor growth and survival and stimulate the metastasis of breast and lung cancer cells to the brain by secreting various cytokines. Astrocytes also enhance the invasive potential of GB cells by producing neurotrophic factors such as TGF-α, CXCL12, S1P, and GDNF. Astrocytes also strongly express IL1β and may be involved in immune reactions against the tumor.

**Inflammatory Cells**

Many studies have identified inflammatory cells in the GB tumor core, particularly tumor-associated macrophages (TAMs) and microglia. More TAMs are present in GB (grade IV) than in grade I or II gliomas, and their numbers are strongly correlated with intratumoral vascular density. Although the abundance of TAMs per se is not associated with the differential survival of GB patients, their activation state (M1 or M2) has some prognostic value. Specifically, TAMs that have differentiated into M2 macrophages act as protumoral macrophages and contribute to disease progression. TAMs are now emerging as a promising target for new adjuvant therapies for GB.

Although many studies have focused on the inflammatory cells present in the GB tumor core, only one study (conducted by Parney et al in 2009) has investigated inflammatory cells in the PBZ. In this study, macrophage-like cells were the most common infiltrating inflammatory cells in the PBZ, followed by microglia-like cells and lymphocytes. These inflammatory cells were also less common in the PBZ than in the GB tumor core. It is not currently known whether macrophage-like cells present in the PBZ share characteristics with the TAMs found in the GB tumor core.

**Other Stromal Cells**

We have isolated, from histologically normal GB PBZ, another population of stromal cells, which we named GB-associated stromal cells (GASCs). GASCs are diploid, do not display the genomic alterations typical of GB cells, and share phenotypic and
functional properties with cancer-associated fibroblasts (CAFs) that have been described in the stroma of carcinomas. In particular, GASCs express markers associated with CAFs such as alpha smooth-muscle actin (α-SMA), platelet-derived growth factor receptor-beta (PDGFRβ), S100A4/FSP1, and CD146. They have angiogenic properties and tumor-promoting effects on GB cells in vitro and in vivo. In a recent study, we showed that 2 extratumoral microenvironments are present in GB patients: an extratumoral microenvironment containing GASCs with procarcinogenic properties and another containing GASCs without such properties. Interestingly, Roman-Perez et al (2012) also identified 2 different subtypes of extratumoral microenvironment influencing the aggressiveness and outcome of human breast cancers. Thus, the subtype of GASCs present after resection may determine the likelihood and timing of GB recurrence. The origin of GASCs is unknown; however, we showed that these cells share several properties with mesenchymal stem cells (MSCs), suggesting that GASCs are derived from these cells.

### Molecular profile

#### Immunohistochemistry

Various immunohistochemical studies of the PBZ suggest that the peritumoral compartment undergoes substantial modifications regarding vascularization and other biomolecular features. For example, markers of neovascularization (e.g., CD105, nestin, phosphorylated extracellular signal-regulated kinases, and c-Jun NH2 terminal kinases [JNKs]) are expressed in the PBZ, irrespective of the presence of tumor cells. In addition, the expression of CD105, JNK, and nestin in the PBZ is associated with poor prognosis. Jensen et al (2014) analyzed molecular markers of hypoxia in the PBZ and found that lower VEGF expression was predictive of longer progression-free survival. The abundance of adenosine A1 receptors and STAT1 expression are also higher in the PBZ than in the GB and normal brain samples. These modifications may have neuroprotective effects against GB progression. Indeed, STAT1 is a well-known tumor suppressor protein that acts via the JAK-STAT pathway, and gliomas grow faster in adenosine A1 receptor-deficient mice than in control mice. The immediate vicinity of the GB tumor core, although we recently conducted such an analysis on 10 PBZs during the Grand Ouest Gioma Project.

Genomic analysis of these 10 PBZs found no genomic alterations in the 6 PBZs considered free of tumor infiltration in histopathological analysis, whereas their corresponding tumor zone presented genomic alterations typical of GB including loss/partial loss of chromosome 10, focal deletion of the CDKN2A/B locus, polysomy of chromosome 7, and focal amplification of EGFR. Genomic alterations were present in the 4 PBZs showing tumor cell infiltration in histopathological analysis, comprising some but not all of the alterations found in their corresponding tumor zones. These PBZs contained classical alterations found in GB, like chromosome 7 polysomy, EGFR amplification, and chromosome 10 deletion, although CDKN2A/B deletion was rare. This result suggests that chromosome 7 and 10 alterations are downstream events in GB tumorigenesis and that, but not all, tumor clones are able to migrate from the tumor core to the PBZ, as described above. These results are consistent with the recent work of Mangiola et al (2013), who identified cells with an EGFR amplification in the PBZ.

The transcriptomic and proteomic analyses that we conducted on these 10 PBZs revealed an intratumoral gradient of gene expression from the tumor core to the PBZ. Gill et al (2014) described similar results and demonstrated (with a larger cohort) that the PBZ in the proneural GB subtype strongly express oligodendrocyte progenitor genes, whereas the PBZ in the mesenchymal GB subtype strongly expresses astrocytic and microglial genes.

We were unable to identify a specific molecular signature of the PBZ with “omic” analyses because of interpatient heterogeneity in these 10 PBZ samples. A large cohort of PBZ samples is thus necessary to identify such a molecular signature, but the constitution of this cohort raises the ethical issue of sampling normal brain tissue around the tumor. Furthermore, the identification of such a signature is complicated by the choice of the control brain sample to be used. Samples from epilepsy patients undergoing brain surgery (EB samples) are commonly used as a control in proteomic or transcriptomic studies. However, we recently showed that EB samples display a tumor-like expression pattern and are not a suitable control for studying the proteomic or transcriptomic profile of the PBZ. Mangiola et al (2013) studied the gene expression profile of the PBZ using control tissue obtained from patients operated on for deep cavernomas with radiological signs of recent bleeding. They identified 57 genes that were significantly differentially expressed between PBZ and control brain samples. Genes associated with growth and proliferation and cell motility/adhesion were upregulated, whereas those involved in neurogenesis were largely underexpressed in the GB PBZ. However, only 4 PBZ samples were included, and more PBZ samples are required to identify a molecular signature of this area able to predict the clinical outcome of GB patients.

Piwecka et al (2015) recently compared global microRNA (miRNA) expression in adult malignant gliomas (mainly GBs), the tumor margin, and nontumor brain tissues. They found that the expression of 97 miRNAs differed significantly between GB and normal brain and that 22 miRNAs were differentially expressed between the border of the tumor and normal brain. Of
note, miR-625 was downregulated in the border of the tumor but not in the GB samples, suggesting that some alterations are specific of the PBZ. Several studies have shown that miRNAs play a role in GB growth by influencing stem cell behavior and that these miRNAs are involved in cellular adaptation to metabolic stress in GB. It will be important to validate PBZ-specific miRNA alterations in order to develop miRNA-based therapies or new diagnostic applications.

The characteristics of the PBZ are summarized in Table 1 and Figure 1.

### Perspectives on the Glioblastoma Peritumoral Brain Zone

#### The Glioblastoma Peritumoral Brain Zone: a Potential Target for Glioblastoma Therapy?

Among the many promising preclinical studies, only a few have been successful in human clinical trials. The most recent example is antiangiogenic therapy, which showed no significant effect on overall survival. The 2 latest trials on bevacizumab,

**Table 1. Summary of the features of the peritumoral brain zone**

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<th>Technique</th>
<th>Characteristics of the PBZ</th>
<th>References</th>
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<tr>
<td>Radiology-MRI</td>
<td>T1</td>
<td>No contrast enhancement</td>
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<td></td>
<td>T2 FLAIR</td>
<td>Hypersignal</td>
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<td></td>
<td>ADC</td>
<td>Intensity of the signal correlated to tumor cell infiltration</td>
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<td></td>
<td>DTI</td>
<td>Inverse correlation between the fractional anisotropy and tumoral infiltration of the PBZ</td>
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<td></td>
<td>DCE</td>
<td>Volume transfer coefficient (ktrans) is correlated with tumoral infiltration and histological grading</td>
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<td></td>
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<td>Extracellular volume (Ve) and capillary transit time (Tc) are correlated with molecular markers of hypoxia and overall patient survival</td>
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<td></td>
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<td>Diffuse response to drug and radiation challenge in vitro</td>
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<td></td>
<td>Migrate along the same route of neural stem cells and share numerous traits with them</td>
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<tr>
<td>Cell biology</td>
<td>Tumor cells</td>
<td>Tumor cells found in the PBZ differ from those in the corresponding tumor mass</td>
<td>25,39–41</td>
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<td></td>
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<td>Some, but not all tumor clones, migrate away from the tumor core into the PBZ</td>
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<td>High proliferation and invasiveness</td>
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<td>Different response to drug and radiation challenge in vitro</td>
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<td>Migrate along the same route of neural stem cells and share numerous traits with them</td>
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<td>Reactive astrocytes</td>
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<td>GASCs</td>
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<td>Angiogenic and tumor-promoting properties in vitro and in vivo, depending on the GASC subtype</td>
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<td>Molecular biology</td>
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<td>Strong expression of adenosine A1 receptor and STAT1, conferring a neuroprotective effect</td>
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<td>High concentrations of copper and zinc</td>
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<td>Expression of CD105, ERKs &amp; JNK, associated with poor prognosis</td>
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<td>Expression of VEGF associated with poor progression-free survival</td>
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<td>Genomics</td>
<td>Presence of some but not all genomic alterations in the nearby tumor zone</td>
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<td>Loss/partial loss of chromosome 10, polysomy of chromosome 7 and focal amplification of EGFR</td>
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<td>Transcriptomics</td>
<td>PBZ has a proneural or neural profile, irrespective of the adjacent GB subtype</td>
<td>10–12</td>
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<td>Interpatient variability, similar to that observed in the corresponding T2</td>
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<td>Intratumoral gradient of gene expression from the tumor core to the PBZ</td>
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<td>Overexpression of genes associated with growth and proliferation and cell motility/ adhesion and underexpression of those involved in neurogenesis</td>
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<td>Large interpatient variability complicates the identification of protein markers of the PBZ</td>
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Abbreviations: ADC, apparent diffusion coefficient; CAFs, cancer-associated fibroblasts; DCE, dynamic contrast enhancement; DTI, diffusion tensor imaging; ERKs, extracellular signal-regulated kinases; FLAIR, fluid-attenuated inversion recovery; GASC, glioblastoma-associated stromal cell; GB, glioblastoma; JNK, c-Jun NH2 terminal kinases; PBZ, peritumoral brain zone.
a humanized monoclonal antibody against VEGF, found that this treatment increased progression-free survival, but not overall survival.73,74 Other GB-targeted therapies focus on the interleukin 13 receptor alpha 2 (IL13Rα2) and EGFR variant III (EGFRvIII).75,76 These targeted therapies showed promising results in preclinical rodent glioma models but demonstrated limited success in human clinical trials due in part to intratumoral heterogeneity in the expression of these targets.

It is important to further characterize the cellular and molecular components of the PBZ to identify new targets and to develop new treatments for GB. Indeed, this region contains the residual disease from which treatment-resistant recurrent disease emerges.

Cuddapah et al (2014)38 indicated in a recent review that invading GB cells retain numerous biological traits inherited from their neural ancestors (eg, Ca2+-AMPA receptors and the Ca2+-activated K+ channel KCa3.1) that should be exploited therapeutically to impair the migration of these cells.38,77,78 Ruiz-Ontanon et al (2013) also found that αVβ3 integrin, low levels of cytoplasmic p27, and their downstream effector proteins Rac and RhoA GTPases are needed for residual tumor cells from the PBZ to acquire an invasive advantage.40 These signaling pathways may...
provide targets for novel anti-invasive therapies for the treatment of GBs. Glycogen synthase kinase-3 (GSK-3) and STAT-3 are other important molecules that modulate GB invasion and proliferation. Preclinical studies involving the inhibition of these molecules with specific drugs have shown promising results. The miRNA, miR-625, was recently reported to be downregulated in the PBZ but not in the tumor. The aberrant downregulation of miR-625 is associated with the local invasion of gastric cancer cells. Therefore, this miRNA may be informative for clinical purposes and for diagnostic and potentially therapeutic applications. We identified 2 subtypes of GASCs in the surgical margins of GB patients: one subtype with tumor-promoting and angiogenic properties and the other without these pro-oncogenic properties. The procarcinogenic GASC subtype overexpressed several markers including CSPG4/NG2, nestin, and CD146. These molecules may be prognostic biomarkers of GB and/or targets for GB treatment, as described for other cancers. Macrophages detected in the PBZ may also be useful targets. Further investigation is needed to determine their phenotype and function in the PBZ.

Technological approaches should also be developed to target the cellular and molecular components of the PBZ. All attempts to target drugs to the brain are faced with the serious challenge of crossing the blood-brain-barrier (BBB) that

![Fig. 2. Nano-biotechnological approaches to target the glioblastoma peritumoral brain zone (GB PBZ). Lipid nanocapsules are promising nanoscale systems for delivering therapeutic molecules to the GB PBZ. They can encapsulate many compounds including drugs, radionuclides, DNA, siRNA, and nuclease-resistant locked nucleic acids. Their association with antibodies against molecular components overexpressed in the PBZ such as aVb3 integrin, KCa3.1, CD146, and CSPG4/NG2 can be used to specifically target the tumor cells and/or GASCs present in the PBZ. Their combination with mesenchymal stem cells, which have endogenous tumor-homing activity and are targeted to the peritumoral region, is another strategy to improve the delivery of therapeutic agents to the PBZ. Abbreviations: Ca-AMPA, Ca\(^{2+}\) permeable AMPA receptor; CSF-1R, colony stimulating factor 1 receptor; CSPG4, chondroitin sulfate proteoglycan; GASC, GB-associated stromal cells; GFAP, glial fibrillary acidic protein; KCa3.1, Ca\(^{2+}\)-activated K\(^{+}\) channel.](http://neuro-oncology.oxfordjournals.org/)

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separates the blood from the cerebral parenchyma.\textsuperscript{87–89} Several strategies have been developed to bypass the BBB such as specific drug formulations able to cross the BBB,\textsuperscript{6} local disruption of the BBB through folicular ultrasound,\textsuperscript{90,91} direct intratumoral drug delivery using convection-enhanced delivery (CED),\textsuperscript{92,93} and cellular vectors with endogenous tumor-homing activity such as MSCs.\textsuperscript{94} To target the delivery of diagnostic and therapeutic drugs to the PBZ of GB, Chekhonin et al (2012) produced immunoliposomal nanocontainers based on antibodies against GFAP and the E2 extracellular fragment of connexin 43\textsuperscript{95} recognizing reactive astrocytes and migrating glioma cells. Our laboratory has developed and patented a novel nanoscale drug encapsulation system, called lipid nanocapsules (LNCs).\textsuperscript{90} These LNCs have the advantage of being prepared without organic solvent using a low-energy process and are able to encapsulate various compounds including drugs,\textsuperscript{96,97} radionuclides,\textsuperscript{98} DNA,\textsuperscript{99} siRNA,\textsuperscript{100} and nuclease-resistant locked nucleic acids.\textsuperscript{101} These LNCs, which showed promising results in glioma models,\textsuperscript{96–98,102} could be used to target the cellular and molecular components of the GB PBZ through the grafting of antibodies onto their surface or the use of MSCs (Figure 2). We recently demonstrated that these cells, which have endogenous tumor-homing activity and are preferentially found throughout the PBZ, can target the delivery of LNCs to the tumor.\textsuperscript{16,94,103–105}

Glioblastoma Peritumoral Brain Zone: Intraoperative Assessment for Optimal GB Resection?

Clearly, the quality of the surgical resection is critical for the overall survival of the patient and the patient’s quality of life and response to adjuvant therapies.\textsuperscript{106–108} The intraoperative assessment of the GB PBZ to detect tumor infiltration is extremely important to optimize surgical resection. The PBZ is typically assessed preoperatively by extemporaneous histopathological examination with a smear test, which provides initial information about the histological nature of the sampled tissue and the presence of tumor cell infiltration. However, this examination lacks sensitivity, and the sample is not representative of the entire resection cavity. Thus, new techniques are required to identify tumor infiltration in the area surrounding the tumor.

Besides examining the PBZ via the preoperative MRI sequences described above, the development of intraoperative MRI in glioma surgery is also a potentially valuable option for optimizing the quality of surgical resection. Intraoperative MRI has shown promising results with a gross total resection rate of 96% in high-grade glioma, which is substantially higher than the classical resection rates of 27%–35% reported in the literature.\textsuperscript{106,108,109} However, intraoperative MRI has less influence on patient survival, with one controlled study reporting an average intraoperative survival of 226 days in the intraoperative MRI group vs 154 days in the control group.\textsuperscript{109} Discussion is ongoing to determine whether intraoperative MRI is useful for neurosurgery because similar results in terms of survival can be obtained using alternative techniques (eg, fluorescence-guided surgery with 5-amino-levulinic acid) at a much lower cost.\textsuperscript{110,111} However, fluorescence-guided surgery is unable to remove the entire tumor because isolated tumor cells may remain outside the fluorescent tumor in the PBZ.\textsuperscript{19} Thus, spectroscopic devices capable of detecting and quantifying fluorescent signals more precisely than the surgeon’s eyes are needed.\textsuperscript{42,112}

A new technique for analyzing the PBZ, optical biopsy, is emerging in different stages of development (Table 2). These optical biopsy techniques are divided into 2 categories: in vivo and ex vivo.

Ex vivo optical biopsies require that brain tissue be sampled during surgery because of technical limitations. However, thanks to recent technological advances, in vivo optical biopsies may hopefully replace ex vivo optical biopsies. Among these techniques, optic coherence tomography imaging uses interference for precise localization of light deep inside tissue. This technique enables noncontact assessment of the brain surface up to 200 \(\mu\)m deep with a spatial resolution of 1 \(\mu\)m, thus allowing discrimination between normal brain, tumor tissue, necrosis, and peripheral infiltrated brain.\textsuperscript{113,114}

Biphotonic microscopy is also currently under evaluation for analysis of the PBZ. This technique allows fast and label-free analysis of brain samples, identifies non-centrosymmetrical structures of the brain (eg, collagen), and provides an overview of the extracellular matrix architecture with a resolution below 1 \(\mu\)m and a depth of 400 \(\mu\)m.

In vivo optical biopsies allow assessment of tumor infiltration during surgery, thus enabling optimal surgical resection. One promising in vivo technique is near-infrared confocal endomicroscopy, which can identify isolated tumors cells in the PBZ during surgery at a precision less than 1 \(\mu\)m and a depth of 250 \(\mu\)m.\textsuperscript{117,119} This technique, however, is not label-free and requires intravenous injection of dyes such as indocyanine green during surgery. Raman spectroscopy is another in vivo optical biopsy technique based on modification of a laser’s wavelength to reflect the brain parenchyma at an axial and depth resolution of 1 \(\mu\)m. This technique has been used in

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<th>Table 2. Description of the different techniques available for intraoperative peritumoral brain zone analysis</th>
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<td>Technique</td>
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<tr>
<td>Optic coherence tomography</td>
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<td>Biphotonic microscopy</td>
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<td>Confocal endomicroscopy</td>
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<td>Raman spectrometry</td>
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<td>Intraoperative MRI</td>
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preliminary studies to assess the histological nature of brain samples and to identify tumor remnants in the surgical resection cavity.119–121 Karabeber et al (2014) have used gold-silica surface–enhanced Raman scattering nanoparticles and a hand-held Raman scanner to perform image-guided surgical resection of GBs in a transgenic murine model.122

**Conclusion**

We have much to learn from the PBZ about the recurrence of GB. The PBZ contains specific tumor and stromal cells that promote GB growth and invasion. Given the interpatient heterogeneity of GB PBZ, large-scale studies are required to further characterize this area in order to identify new molecular targets that could be translated into routine clinical practice. Technical progress is also needed to assess the presence of tumor infiltration in the PBZ during surgery and thus optimize the quality of surgical resection. In our opinion, intraoperative MRI is currently the most reliable, easy-to-use technique for analyzing and delineating brain tumors. This recent progress regarding GB and its peritumoral characteristics will facilitate development of personalized targeted therapy and adjuvant treatments of GB after surgery.

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