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Cancer Immunotherapy Getting Brainy: Visualizing the Distinctive CNS Metastatic Niche to Illuminate Therapeutic Resistance

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Abstract

The advent of cancer immunotherapy (CIT) and its success in treating primary and metastatic cancer may offer substantially improved outcomes for patients. Despite recent advancements, many malignancies remain resistant to CIT, among which are brain metastases, a particularly virulent disease with no apparent cure. The immunologically unique niche of the brain has prompted compelling new questions in immuno-oncology such as the effects of tissue-specific differences in immune response, heterogeneity between primary tumors and distant metastases, and the role of spatiotemporal dynamics in shaping an effective anti-tumor immune response. Current methods to examine the immunobiology of metastases in the brain are constrained by tissue processing methods that limit spatial data collection, omit dynamic information, and cannot recapitulate the heterogeneity of the tumor microenvironment. In the current review, we describe how high-resolution, live imaging tools, particularly intravital microscopy (IVM), are instrumental in answering these questions. IVM of pre-clinical cancer models enables short- and long-term observations of critical immunobiology and metastatic growth phenomena to potentially generate revolutionary insights into the spatiotemporal dynamics of brain metastasis, interactions of CIT with immune elements therein, and influence of chemo- and radiotherapy. We describe the utility of IVM to study brain metastasis in mice by tracking the migration and growth of fluorescently-labeled cells, including cancer cells and immune subsets, while monitoring the physical environment within optical windows using imaging dyes and other signal generation mechanisms to illuminate angiogenesis, hypoxia, and/or CIT drug expression within the metastatic niche. Our review summarizes the current knowledge regarding brain metastases and the immune milieu,

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presents the current status of CIT and its prospects in targeting brain metastases to circumvent therapeutic resistance, and proposes avenues to utilize IVM to study CIT drug delivery and therapeutic efficacy in preclinical models that will ultimately facilitate novel drug discovery and innovative combination therapies.

I. Targeting brain metastasis with cancer immunotherapy

Brain Metastasis and their Unique Microenvironment

Metastasis, the spread of cancer cells from the primary tumor to surrounding tissues and distant organs, is the leading cause of cancer morbidity and mortality (1). Of particular interest are brain metastases, the treatment of which is a critical unmet need in order to successfully combat cancer. It has been estimated that brain metastasis occurs in up to 30% of patients across various solid cancers (2,3). The most common source of brain metastases stems from non-small cell lung cancer (NSCLC), followed by breast, melanoma, renal, and colorectal cancers (2–5).

The composition of the brain's microenvironment, specifically the immune milieu, is distinct from other tissues since it is immune privileged, which is attributable to the presence of a blood brain barrier (BBB) and the lack of conventional lymphatic drainage due to the absence of lymphatic fluid surrounding the brain (6). Traditionally, lymphatic drainage enables circulation of maturing antigen-presenting cells (APCs) to defined lymphatic structures where adaptive immune responses are preferentially mediated. The brain's unconventional lymphatic drainage presents an unclear anatomical route by which APCs traffic from the CNS parenchyma to delineate the role of antigen presentation in neuro-inflammatory diseases. Within the brain, lymphatic fluid drains to the cervical lymph nodes through the subarachnoid space and ventricles by means of cerebrospinal fluid (CSF). In addition, the extracellular space of the brain and spinal cord parenchyma undergo lymphatic drainage by means of interstitial fluid (ISF) (7). Another unique feature within the brain is the neurovascular unit (NVU), which consists of the BBB, endothelial cells and surrounding pericytes, astrocytes, neurons, and extracellular matrix (ECM). Heterogeneous NVU function and inflammation within the brain metastatic site often leads to inconsistent delivery of therapeutics and contrast imaging agents/modalities, a factor that must be considered in immuno-oncology (8).

The immune landscape of the brain during chronic inflammation is predominantly thought to consist of microglia, astrocytes, myeloid-derived suppressor cells (MDSCs), and tumor-associated macrophages (TAMs). Microglia, which are considered the primary APC in the brain microenvironment, serve as tissue-resident macrophages and may adapt to become perivascular macrophages that aid in tumor proliferation, invasion, and angiogenesis to create a more favorable tumor microenvironment (TME) (9,10). It has been shown that microglia have the potential to differentiate into the M1-like or M2-like macrophages to induce a pro-inflammatory (TNFα/IFNγ response) or pro-tumoral (IL-4/TGFβ response) phenotype, respectively (11). Astrocytes, the most abundant glial cell type in the brain microenvironment, induce a pro-inflammatory response through the secretion of TNFα along with other cytokines (12). Exhibiting multiples roles, astrocytes within the brain TME

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reduce survival of newly arriving metastatic cells, while promoting the growth of established brain metastases, highlighting one aspect of the unique tumor-stroma interaction of the brain (13). Although these immune subtypes play various roles in the establishment of brain metastases, the infiltration of lymphocytes within the brain microenvironment is important in immuno-oncology since most approved CITs focus on enhancing T cell anti-tumor immunity. Despite the fact that the exact nature of the heterogeneous immune cell presence and mechanism of infiltration within the TME has thus far been elusive, partly due to the lack of imaging methods to visualize penetrance across the BBB, preclinical data show that depletion of CD4 and CD8 T cells results in increased development of brain metastases and that regulatory T cells (Tregs), a subset of CD4 T cells that suppress anti-tumor immune responses, restrict the expansion and differentiation of T effector cells (14,15). Understanding the dynamic interaction between the unique immune milieu and metastatic tumor cells within the brain TME will underpin which immune subsets are critical for immuno-oncology as therapeutic targets.

Current Diagnosis, Treatment, and Monitoring Options for Brain Metastases: Efficacy, Side Effects, and Challenges

The gold standard for diagnosis and monitoring of CNS metastases in the clinic is contrast-enhanced, high-resolution magnetic resonance imaging (MRI) (8). However, it is challenging to assess tumor response to a therapy and, more importantly, to differentiate between treatment-related changes or disease recurrence. This is due to the unique anatomy of the brain's vasculature, including the microenvironment of the NVU. Current treatment options for brain metastases include whole brain radiation therapy (WBRT), surgical resection, and stereotactic radiosurgery (SRS), all of which are altered depending on the size, number, histology, symptoms and location of metastatic lesions presented at the time of diagnosis (16,17). WBRT remains the standard-of-care for treatment of metastatic central nervous system (CNS) lesions, whereas surgical resection and SRS are considered effective for patients with manageable lesions (5). Typically, WBRT and surgical resection are used for multiple or large lesions, while high-resolution MRI is used to assist with SRS for smaller, emerging lesions, and Tumor Treating Fields (TTFields) are an emerging option for brain metastasis treatment (18–20). However, each of these treatment options for brain metastases displays key limitations and consequences. WBRT can result in cognitive decline and SRS has been shown to be associated with radiation necrosis (RN), cerebral edema, and delayed tumor hemorrhage (19). Focal therapies, such as SRS and surgery, often have limited efficacy because of distant cerebral relapse and lack of treatment of microscopic tumor foci that remain invisible with current imaging technologies (19). TTfields, which disrupt cell division through the physical interactions of oscillating electric fields with key molecules during mitosis, have been applied only to GBM treatment thus far. They hold promise in brain metastasis to specifically address non-resectable regions of the brain and potentially affect stray cells leftover after resection or other interventions in brain metastasis. TTFields in combination with chemotherapy has shown promising early data in a phase 3 clinical trial of GBM in which prolonged progression-free survival and overall survival (OS) were noted in patients with recurrent disease (20). Notably, the non-invasive therapeutic potential of TTFields has been demonstrated by immense improvement of the OS of GBM, reducing tumor growth by up to 50% in preclinical studies alongside early clinical data in

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recurrent GBM demonstrating an increase in progression free survival after 6 months of treatment by 50% (21). Despite the success of TTFields, they have resulted in seizures and nervous system disorders, such as anxiety and insomnia and although TTFields currently present the best quality of life for brain cancer patients, more evidence is needed to validate that other aspects of life quality do not suffer from the continual electric field stimulation required to achieve TTFields cytotoxic effects (20).

Primary challenges when studying brain metastases include (Table 1): first, the lack of imageable preclinical models that accurately recapitulate CNS metastases for both mechanistic and therapeutic studies limits our knowledge of the immune contexture and the ability to accurately study pharmacodynamics parameters. A second challenge involves image standardization of brain metastases to assess response, as well as inclusion of novel imaging models that recapitulate early to late events of brain metastasis (8). Developing intravital microscopy (IVM) methods to effectively image the cellular and molecular determinants of standard-of-care and newly approved CIT drugs could facilitate the development of relevant preclinical models for data-driven combination therapy as well as provide insight into the design of more inclusive clinical trials. IVM offers the spatiotemporal resolution and multi-plexing potential to delineate the dynamic interactions of drugs, immune effectors, and tumor cells, which will lead to improved dosing and delivery of CIT drugs in monotherapy and provide a more rational foundation for testing combination therapies.

Would CIT be effective against brain metastases across various primary cancers?

Emerging systemic immune-modulating treatments have shown prolonged survival for patients with aggressive extra-cerebral disease, specifically brain metastasis from NSCLC and malignant melanoma (22,23). Although it had previously been thought that the brain TME is immune privileged and impervious to lymphocyte infiltration, increasing evidence has shown that tumor infiltrating lymphocytes (TILs) do traffic into the brain, where their presence is associated with increased overall survival and better response to CIT. Indeed T cells can cross the BBB, supporting the use of CIT in treating brain metastasis (24). Moreover, microglia, the resident macrophages of the brain, express PD-1 and activation of either PD-1, its ligand PD-L1, or CTLA-4 result in suppression of the anti-tumor T cell response (25,26). Of specific interest are anti-cancer TILs: CD3+, CD8+, and memory T cells, which have been shown to accumulate around the brain parenchyma along with immune-suppressive lymphocytes such as Tregs and PD-1-positive TILs (27). However, to date there are no published results from clinical studies examining CIT pharmacokinetics or pharmacodynamics into brain lesions, primarily due to challenges in recruiting patients into trials requiring brain biopsies, particularly from patients responding well to therapies (19).

One main challenge currently being addressed by CIT is the study of whether CIT antibodies transit across the BBB despite their large size (23,28). Limited clinical evidence using positron emission tomography scans suggests that monoclonal antibodies are indeed able to cross the BBB, which in turn suggests that CIT drugs may be an effective means by which to target infiltrating TILs and microglial immuno-suppressive cell types (29). Adsorptive transcytosis has also been evaluated as a potential vector for delivering drugs.

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This mechanism facilitates the transport of peptides through the interaction of a ligand with moieties expressed at the luminal surface of cerebral endothelial cells (30,31). However, this nonspecific transport process also occurs in other organs, complicating determination of effective drug doses in the brain, while reducing off-target drug dissemination. CIT drugs for brain metastasis have shown the most clinical promise in patients with melanoma and NSCLC; in particular, antibodies to CTLA-4 and PD-1 cross the BBB and evoke a partial response or in some cases stable disease (32–34). An additional challenge in the field of CIT for brain metastasis is the management of neurological symptoms during or after treatment that potentially arises from perilesional edema, intralesional hemorrhage, necrosis in previously irradiated lesions, or tumor growth due to treatment failure stemming from the inability to target microscopic tumor foci not evident from imaging. Post-immunotherapy side-effects have been addressed through the use of surgery, radiation, steroids, or VEGF inhibitors to control edema, hemorrhage, and/or necrosis (19)

Finally, multidrug resistance (MDR) to anticancer drugs continues to hinder curative therapy of various human malignancies (35–39). MDR may arise from a variety of molecular mechanisms, such as new mutations in key target genes, dysregulation of normal apoptotic controls, or upregulation of chemoresistance due to multidrug efflux pumps of the ATP-binding cassette (ABC) superfamily such as P-glycoprotein (P-gp), which expel a wide spectrum of cytotoxic agents (40). Interestingly, although it has been shown that P-gp is highly expressed within the BBB of humans, patients with brain metastases from malignant melanoma or lung cancer, present with lower P-gp expression than normal brain tissue, suggesting that there are other mechanisms of MDR functioning in metastatic brain tumors (41). With rapid advances in the understanding of MDR and CIT mechanisms, devising novel pre-clinical modalities that could inform on relevant clinical approaches to overcome the frequent emergence of cancer MDR is a major need toward the goal to overcome tumor resistance to therapy.

Current progress in targeting brain metastasis with CIT

Checkpoint inhibition, specifically CTLA-4, PD-1, and its ligand PD-L1, function through the activation of CD4 and CD8 effector T-cells. Typically, CTLA-4 and PD-1 signaling inhibit T-cell activation, and blockade of either modulatory signal can shift the immune system's balance towards activation of T-cells, thereby promoting tumor destruction (42). While CD28 acts as a strong positive stimulatory receptor to CD80 and CD86, two T-cell receptors that initiate and maintain CD4+ T-cell proliferation, CTLA-4 functions as a potent inhibitory molecule against CD80 and CD86 (43). PD-1/PD-L1, expressed by T-cells, B-cells, natural killer T (NKT) cells, activated monocytes, and dendritic cells, can also bind to CD80, which potentially forms a connection between the CD28/CTLA-4 and PD-1/PD-L1 pathways (44–46). More specifically, CTLA-4 is associated with inhibition of Akt signaling and PD-1/PD-L1 is associated with PI3K suppression and Akt signaling (47). PD-L1 is also upregulated on many tumor cells (48). Signaling of PD-1 exerts a pro-tumorigenic effect by inhibiting production of IFNγ, TNFα, and interleukin-2, all of which are established anti-tumor response hallmarks (49). Moreover, chimeric antigen receptor T-cell (CAR-T) therapy is increasingly gaining traction. The FDA's recent approval of Novartis' CAR-T therapy for B-cell acute lymphoblastic leukemia (ALL) demonstrates its promise for hematological

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malignancies. The field of immuno-oncology is heavily investing in CAR-T studies for solid tumors. CAR-T CIT involves the isolation of the patient's own T-cells and genetically engineering them with a tumor-specific CAR onto their surface. This allows these modified T-cells to specifically localize to and eliminate tumor cells by interacting with tumor-associated antigens (TAA) expressed on the tumor cell's surface (50). Although the structure of a CAR is similar to a T-cell receptor, a CAR recognizes TAA independently of presentation via major histocompatibility complex (MHC) molecules, and targets a heterogeneous repertoire of antigens present on the tumor cell surface (51,52). Table 2 details the FDA-approved CITs for specific cancer types and their performance in treating patients with brain metastasis. CIT has made groundbreaking strides particularly in hematological tumors and more recently in solid malignancies.

Preclinical studies with immune-modulating antibodies on primary CNS tumors have shown promising efficacy. Specifically, anti-CTLA-4 antibody was well tolerated in mice with SMA-650 intracranial tumors; a concomitant elevation of CD4+ cells and decrease in Tregs helped increase survival in these animals (19,53). In other animal models, the combination of PD-1 and CTLA-4 inhibitors led to improved survival as compared to either single agent (19,54). These studies indicate that BBB drug penetration might occur in both primary and metastatic CNS tumors, and clinical trials were undertaken to expand the studies on human subjects (19).

One of the first clinical studies evaluating the effect of CIT on brain metastases from melanoma was the phase II trial with ipilimumab (anti-CTLA-4), in which 12 of 115 patients randomized in the parent trial had stable brain metastases at baseline and were evaluated for efficacy. In the study, 2 of the 12 patients partially responded and 3 had stable disease, which were both alive at the last follow-up with a median patient survival of 14 months (range: 2.7–56.4+) (55,56). A different phase II trial with 72 melanoma patients suffering from brain metastases showed that ipilimumab prolonged overall survival (OS) and the OS was particularly significant in asymptomatic brain metastasis patients (57). Based on these promising results, a phase II trial of combination ipilimumab and fotemustine (NIBIT-M1) treatment on 20 asymptomatic patients with brain metastases was initiated. 25% of the patients had stable disease or partial response and another 25% had complete responses, indicating that antibody-based CIT may cross the BBB and in many patients drive durable control of brain metastasis (23,58,59).

The potential for combining CIT following neoadjuvant radiotherapy to overcome therapeutic resistance in the treatment of brain metastasis

The field of CIT is actively shifting from monotherapy to combination treatment. However, whether this entails combining various CIT modalities to increase efficacy or to combine radio- or chemo-therapy with one or multiple CITs is currently debatable due to a lack of understanding of the changing immune milieu during and following therapy. However, it has recently been shown that the efficacy of immune checkpoint inhibitors can be enhanced by combining multiple inhibitors or via combination with chemotherapy or radiation therapy (RT) (65–67). This success can be attributed to a diverse T cell receptor repertoire of TILs following RT and CIT, alleviating the exhausted T-cell phenotype, increasing the CD8/Treg

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ratios for effector function (68–70). In tumors resistant to RT and CIT, a distinct increase in PD-L1 blunts the prevalence of CD8 TILs and interferes with T-cell function (71). However, the absence of an effective innate immune system, including Toll-like receptor signaling (which is involved in antigen presentation), impairs the efficacy of radiotherapy-mediated control of tumorigenesis. Since radiation enhances the up-regulation of MHC class I in a dose-dependent manner to present peptide fragments on the cell surface to cytotoxic T-cells, RT can trigger a systemic or local immune response. This response can elevate the expression of tumor-related antigens above a threshold level required for activation of circulating tumor lymphocytes, ultimately resulting in T cells potentially recognizing and attacking distant tumors (72). Irradiated cells also release a significant amount of high-mobility group box 1 protein, a potent pro-inflammatory mediator that activates dendritic cells and stimulates an immune response following radiation (4). Thus, both the innate and adaptive immune cells in the TME contribute to tumor cell death in the irradiated field.

A number of mechanisms has been elucidated that help explain the success of combination checkpoint inhibitors with radiation in a neoadjuvant setting. For instance, radiation upregulates inflammatory cytokines (e.g. TNFα, IFNγ, and CXCL16), which promote tumor detection and facilitate T-cell infiltration (19). In some cases, CIT and RT can have a combined synergistic effect that leads to a systemic abscopal effect, or distant bystander effect, which refers to localized immune stimulation inciting a systemic immune response that results in tumor shrinkage at both treated and untreated sites. The effect thereby supports the use of radiation combined with immune-modulating agents. Studies on an orthotopic glioma mouse model showed that focal radiation therapy and CIT using anti-CTLA-4 improved survival via a CD4-dependent mechanism and generated antigen-specific memory that are likely valuable for long-term surveillance. Interestingly, although increases were seen in both CD8 and CD4 TILs, depletion of CD8 T-cells had no effect on treatment outcomes while depletion of CD4 T-cells abrogated the antitumor effect of RT with CIT (73). Another study using an intracranial implant of GL261, a mouse glioma cell line, showed a ~50% increase in survival when using RT with PD-1 where an immunogenic response including elevated CD8/IFNg/TNFα was observed (74). Collectively, recent work reveals that local RT can increase tumor-specific cytotoxic T-lymphocytes, induce the presentation of previously occult cancer antigens to T-cells, and increase the permeability of the BBB to CIT agents.

What are the open questions in the field that could be addressed by IVM?

The major open questions involved in developing better therapies to treat and monitor brain metastases focus on understanding: 1) the efficiency of drug delivery through the BBB, 2) the mechanism of action of CIT within the brain, and 3) interactions with brain-specific stromal and immune cells, such as microglia or astrocytes. The primary limitation in imaging the brain is a lack of spatiotemporal resolution, which is compounded by: 1) heterogeneous NVU function and inflammation within the tumor, resulting in inconsistent imaging (8), 2), imaging modalities' inability to differentiate the enlargement of lesions due to inflammation, necrosis, or tumor growth (19), and 3) in both RT alone and in combination with CIT, it is difficult to differentiate RN from tumor progression by current imaging techniques (75,76).

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In this review we address how IVM can provide greatly improved imaging-based methodologies for diagnosis and monitoring of response to CIT in preclinical models. We describe how IVM on preclinical models can facilitate the development of new clinical imaging techniques that are pertinent for distinguishing the etiology of CNS lesions and provide insights into CIT's mechanism of action and efficiency and mechanism of therapeutic delivery across the BBB to identify targets to improve therapeutic strategies.

II. Examining how intravital microscopy is used to study immune cells in brain metastasis pertinent to CIT drug delivery and action

Intravital Microscopy

Methods including histological examination, biochemical assays, primary cell culture, and *ex vivo* microscopic approaches have been used for many years to study tumor growth and progression (77–81). While valuable, each of the above methods is limited to terminal endpoints and *ex vivo* data, and therefore cannot fully recapitulate the dynamics or complexity of cellular and subcellular interactions involved in tumor progression and metastasis at high resolutions. Such biases may lead to overlooking or even misinterpreting phenomena critical to the development of better cancer treatments (82,83). We summarize the current IVM imaging techniques in Table 3 to provide a more thorough understanding of the dynamic, high-resolution capabilities of IVM.

Advances in IVM offer high-resolution measurement of cell fate and behavior in living animal models, enabling long-term, dynamic studies of tumor growth and treatment effects. These advances include combination single- and multiphoton microscopy with advanced genetic models, improved surgical techniques, and fluorescent tracking of cell fate for weeks to many months. Notably, compared with MRI and nuclear imaging modalities, as an optical modality IVM does not penetrate tissue deeply; moreover, the penetration depth of IVM is dependent upon the excitation and emission wavelengths as well as tissue type and size (84–86). Yet the advantages of IVM help compensate for its deficiencies as depicted in Figure 1. Many groups are further pushing the boundaries of what is possible in IVM, for example, developments that enable *in vivo* super-resolution—better than diffraction-limited spatial resolutions (87). Continued refinement of these techniques is revealing remarkable and previously unknown phenomena underlying the molecular, cellular, and functional biology of tumor growth, regenerative medicine, and immune cell trafficking (88). The ability to understand the interactions between tumor and immune cells within the tumor/metastatic microenvironments using preclinical models is key to effectively target and treat cancer with immunotherapies.

Intravital Imaging of Tumor

Two different IVM imaging approaches are primarily used—chronic and acute (single-session) imaging—which tends to dictate the surgical techniques employed to access target tumor sites. The approach is selected based on the question to be answered (88,97). The surgical manipulation required to expose the area of interest for IVM can lead to inflammatory or other confounding effects. Thus, the architecture of the tissue of interest is a key constraint for the design of successful IVM experiments, in which minimally-perturbed

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tissue provides the most biologically-relevant results (98). Acute preparations are not only limited in the duration and frequency of the observation, which may include irrevocable exteriorization of anatomy or surgery (88,99–101), but the preparation procedures might also impact the physiological parameters.

Chronic IVM often employs a clear, biocompatible window that is surgically implanted over the target site to enable long-term and repetitive microscopic access that may include the ability to stabilize motion (102,103). This method has been used at a number of sites, including the cranium. Cranial windows, an optically transparent glass placed over the brain, are appropriate for studying neural activity, brain blood flow, or brain metastasis, and may endure for several months up to a year as compared to, for example, dorsal and abdominal windows, which typically last 3–6 weeks (104,105). Use of fluorescent probes, transgenic mouse models, and repeated imaging with IVM enables long-term, high-spatiotemporal resolution (down to ~0.1 μm and up to hundreds of frames per second) observation of tumor progression at the cellular to subcellular level. Prudent choice of which cells and molecules to label with exogenous probes or to engineer as transgenic reporters can enable significant insight into cell identity, state, movement, and interaction. For example, fluorescent labeling of adoptively transferred CAR-T cells means that cell migration, infiltration, proliferation, and activation may be monitored and quantified dynamically, potentially in combination with a transgenic reporter model. This approach permits direct examination of T cell-tumor interactions so that the influence of cancer proximity on each CAR-T parameter may be directly measured. Moreover, microendoscopic methods combined with IVM provide access to deeper tissues for chronic imaging, such as the hippocampus or striatum, which cannot be directly observed via conventional means (106). Therefore, with IVM, a deep-lying glioma may be tracked at high-resolution over months to measure, for example, dynamic three-dimensional angiogenesis progression, changing microcirculatory velocities, infiltration, and proliferation, and health of fluorescently-labeled immune cells interacting with the tumor (106–110).

IVM to Investigate Tumor Immunobiology

Many features of the TME, elaborated upon below, are amenable to interrogation via IVM. Apart from malignant cells, the TME contains various components that play a major role in influencing the outcome of the malignancy. These can be broadly classified into three main groups: cells of haematopoietic origin (cells of the immune system), cells of mesenchymal origin (including fibroblasts, myofibroblasts, mesenchymal stem cells), and the non-cellular components of the ECM, which consists of complex interacting proteins, glycoproteins and proteoglycans (112,113). The TME bidirectionally and strongly influences the effects of cancer immuno- and other therapies, making it an important parameter to study in concert with therapeutic delivery.

Tumor Growth—Use of transgenic mice and stably-transfected fluorophore-expressing tumor cell lines enables long-term observation of the dynamic growth of tumors. By observing the cellular morphology and subcellular processes involved in tumor growth in various tissue sites under treatment, IVM offers rapid insights into not only the proliferation and apoptosis of tumor cells, angiogenesis, and trafficking of immune cells, but also the

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effects of treatment. For example, tumor-focused IVM allows visualization of the efficacy of new chemo- or immuno-therapeutics as well as their multifactorial mechanisms of action (114,115). Notably, tumors do not necessarily need to express fluorophores, as label-free OFDI or CARS may also be used to measure parameters such as the rate and extent of tumor growth through tissue derangement (116).

Extracellular Matrix (ECM)—Aggressive lesions actively degrade and subvert the surrounding ECM to facilitate tumor growth, tissue invasion, and metastasis. MPM and SHG are well-suited for the *in vivo* study of tumor-mediated derangement of the ECM. As displayed in Table 3, SHG microscopy is highly sensitive to changes in collagen fibril/fiber structure that occur during cancer and can provide important biological information about ECM alterations that accompany cancer progression and metastasis. Hence, SHG can be used to quantify collagen fiber formation, direction, and remodeling that may be affected by cell signaling or mechanical/biomechanical interaction between cells and collagen fibers (117). Moreover, rapidly growing tumor cells directly and indirectly, through recruited immune cells, degrade the ECM through the secretion of matrix metalloproteinases (118). Detachment of tumor cells from the surrounding tissue structure precedes invasion and metastasis, and the establishment of new metastases requires degradation of ECM to establish a hospitable growth niche (119), making it a critical tool to study the formation of the brain metastatic niche. Thus, IVM-based observation of tumor-mediated changes in ECM and tumor and angiogenic development offers the potential for profound insights into the impact of therapeutics.

Tumor Microenvironment (TME)—Tumor growth is fueled by both intrinsic mutational and transcriptional drivers within the tumor itself as well as by extrinsic environmental factors that arise through the interaction of tumor, stromal, and immune cells (120). Collectively, these extrinsic factors comprise the tumor microenvironment (TME). Rapid tumor proliferation results in the secretion of glycolytic metabolism byproducts, depletion of local normoxia, cytokine signaling, and accumulation of damaged cancer and stromal cells (121–123). These features drive localized inflammation that, in turn, results in the infiltration of diverse innate and adaptive immune cell subpopulations. Infiltrating anti-cancer immune effectors such as CD8+ and CD4+ T-cells, M1 macrophages, and NK T-cells coordinate tumor lysis and adaptive immune memory to onco-antigens. However, the infiltration/maturation of immunosuppressive cells, such as myeloid-derived suppressor cells and Tregs, into the TME results in potent inhibition of anti-cancer immune effectors (120,124). Increased immunosuppressive cellular infiltration significantly correlates with rapid disease progression, treatment resistance, and greater metastatic dissemination in a number of tumor types, including breast and skin cancer (125,126). These phenomena are further complicated by their interactions with a) tissue-specific stromal factors, such as the astrocytes and microglia of NVU in brain metastases, b) ongoing angiogenesis, c) lysyl oxidase- and matrix metalloproteinase-mediated extracellular matrix alterations, and d) the profound effects of chemo-, radio-, and immune-therapy upon the TME itself (127,128). Furthermore, growing tumors are not governed by the architecture of their surrounding tissues and may create dense desmoplastic networks that result in widely varied circulation, drug infiltration, and interstitial pressure. Not only does the nature of stromal activation and

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immune infiltration vary by primary tumor tissue site, but metastases also form their own distinct TME upon arrival in distant tissues, which likely differ from the primary TME in remarkable and as-yet-undefined manners (129,130). The influences of brain-specific factors, such as the BBB, microglia, or reduced lymphatic drainage, on the formation and evolution of brain metastasis TMEs are currently unknown and the results of their enhanced understanding may represent compelling targets for therapy development.

Transgenic animal studies, in which tumors or immune cells have been selectively edited, have been critical to define the mechanisms and interactions underlying the relationship of the tumor to its TME in previous literature (131–133). Combination of transgenic animal models, fluorescently-tagged antibodies, gene fusion reporter cell lines, and the spatiotemporal capabilities of IVM will yield revolutionary insight into the underlying immunobiology of tumor-rejecting and tumor-promoting immune responses to brain metastases, the mechanism of CIT in the brain, and the impact of NVU-specific microglial or astrocytic activity on the therapeutic response. TME physico-chemical properties may also be imaged via exogenously-administered contrast agents, e.g., molecular probes with optical properties that vary upon change in pO_2 or pH, which are well-suited for *in vivo* study of the dynamics of tumor-mediated hypoxia and the downstream metabolic reactions of tumor stroma and infiltrating immune cells (134,135). IVM of the immune component of the TME is detailed below.

Blood Supply—OFDI and MPM may be used to measure the growth of new endothelial venules and capillaries associated with tumor growth, making them well-suited to characterize the mechanisms by which metastases establish initial blood supplies. IVM has also been used to directly measure hemodynamics, vascular permeability, vessel pore cutoff size, leukocyte-endothelial cell interactions, lymphangiogenesis, and circulation of investigational drugs into the tumor site (83,136–140). For example, IVM has helped demonstrate that chemotherapeutic efficacy in primary brain tumors and metastasis is extremely limited due to poor transport of the fluorescently-labeled drug across the BBB and blood brain tumor barrier (141–143). This suggests that IVM could similarly be used to iteratively and directly test newly designed drugs for improved transport kinetics.

Immune Cell Infiltration—Tumor progression is intimately shaped by several layers of feedback with the tumor-responsive immune system, mandating examination of the infiltrating and *in situ* proliferating immune system in the study of the mechanisms underlying tumor growth. *In vivo* discrimination between unlabeled immune and non-immune cells in tissues is complex, so the best-suited approaches for study of immune infiltration are exogenously-administered (adoptively-transferred and/or fluorescently-labeled) or endogenously-expressed (transgenic-fluorescent immune cells) reporters (82,144–148). Strategic use of Cre recombination to generate lineage- and function-specific conditional fluorescent reporters enables the detailed tracking of cell fate and migration through living tissue by cell type, such as tracking the chemotaxis of labeled stem cells through developing lung tissue (149). IVM may be used to examine the infiltration of immune cells into the TME, their interactions with the growing tumor, interactions between immune cell types, and immune influence on stromal cells and migratory cancer cells.

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Extension of these capabilities of IVM together with advanced exogenously-administered and endogenously-expressed probes for the study of immune function in the TME will likely yield significant insight into the underlying immunobiology of tumor-rejecting and tumor-promoting immune responses and could help guide the development of new immunomodulatory drugs.

IVM In Brain Immuno-oncology (IO)

IVM is well-adapted to study tumor immunobiology and has been used in preclinical models of glioblastoma to measure the synergistic effects of anti-angiogenic and cytotoxic therapy (150,151). Many features of the brain TME including, but not limited to, immune cell infiltration and interaction with CIT drugs are depicted in Figure 2. IVM techniques promise to surpass alternative terminal, static immune response characterization methods by applying the capabilities to chronically study immune dynamics in response to tumor growth and treatment. The nature and interplay of anti- and pro-tumor immune effectors are critical determinants of treatment success, so a more detailed understanding of their underlying mechanisms, and how those mechanisms interact with the unique microenvironment of brain metastases, is necessary. The traditional static methods may alter the behavior or expression of immune or cancer cells upon sample collection and processing. Complementing these methods, or potentially in their stead, IVM offers dynamic, long-term, and high-resolution imaging of cell-cell interactions, tissue structure, and treatment effects in a minimally-perturbed (e.g., after the post-window implantation/surgical rest period) living animal, where the influences of distal tissue sites upon, and cell interactions within, the measured site remain intact.

Specifically, IVM can facilitate exploration of the following:

Fundamental Tumor Immunobiology—Use of cranial windows enables direct, dynamic imaging of cell-cell interactions, cell proliferation, and cell migration in and around metastatic and primary brain tumors (111). Fluorescent transgenic reporters and/or exogenously-administered, labeled cells may be applied to study several properties fundamental to tumor growth and immune response, such as T-cell activation, the effects of hypoxia on myeloid cell development in the tumor margin, or the spatial organization and migration rates of stromal and myeloid cells involved in tumor-promoting angiogenesis (152).

Checkpoint Inhibitor Development—As discussed above, monoclonal antibody-mediated neutralization of immune checkpoints has shown clinical benefit in various solid tumors, such as melanoma, colorectal, lung, and urothelial bladder cancers (153). However, the development of new checkpoint inhibiting antibodies is hampered by the heterogeneity of the immune response and sample processing-induced changes in immune function in most currently-employed preclinical models of cancer. Intravenous and intratumoral injection of fluorescently-labeled monoclonal antibodies for proteins of interest enables intravital imaging of: 1) therapeutic target and immune effector distribution, 2) target dynamics, 3) cell type expression of target antigens, 4) function of labeled antigens in cell-cell interactions, and 5) infiltration of antibodies, such as anti-PD-1L into the TME (154).

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Cell Therapy Development—Cancer-targeted, adoptive cell therapy, such as CAR-T, is most effective when transferred cells survive and proliferate in the TME (155,156). Adoptive cell therapies rely upon isolation, modification, and expansion of isolated patient-derived immune cells to increase their affinity for TAAs. The *ex vivo* modification of immune cells presents an opportunity to fluorescently label cells to track their fate and activity *in vivo*. IVM may also be used to track the migration paths and antitumor activity of individual immune cell subtypes.

Combination Therapy—Combination therapy is a rapidly advancing sub-field of clinical oncology as described in Section I. Accumulating evidence, including a clinical trial targeting Glioblastoma Multiform (GBM), shows promise in RT with CIT drugs targeting PD-1/PD-L1 such as pembrolizumab, nivolumab, atezolizumab, and, durvalumab lead to enduring responses (157). While combination therapy shows immense promise in NSCLC and melanoma, careful therapeutic design consideration must be applied to the brain, because it is a sensitive organ, in order to leave healthy tissue intact during therapy. Immune checkpoint blockers such as CTLA-4 and PD-1/PD-L1 enhance the antitumor response induced by radiation therapy (71,74,158–163); thus, the interplay between the biological effects of radiation therapy and the immune system may be a critical mechanism for inhibiting tumor growth and extending overall survival in brain metastasis patients. However, the ability to successfully design combination therapeutic strategies is limited due to a lack of understanding of the molecular and cellular mechanisms in radio-oncoimmunology (164). IVM's spatiotemporal resolution overcomes limitations that constrain other techniques, enabling understanding of immune milieu changes following local or systemic treatment. Since tissue-resident immune cells cannot easily be studied *ex vivo* due to their short lifespan and inadequate recapitulation of the microenvironment, dynamic microscopic imaging of the immune and tumor milieus can critically contribute to uncover effective therapeutic combinations to target brain metastases.

III. Perspective: Utilizing IVM of brain metastasis to advance drug discovery and facilitate effective targeting with approved CIT

Characterization of Brain Metastatic Tumor Cells—Circulating tumor cells often accumulate at vascular branch points, penetrate through vascular walls, and remain in close proximity to microvessels in the process of remote niche formation (190). Recently, the fates of metastasizing cancer cells were tracked in relation to blood vessels in living mouse brains using real-time MPM, a common form of IVM, which revealed the single steps of metastasis formation *in vivo* over minutes to months (111). Exploiting this strategy to observe metastasis formation under treatment conditions has key implications for the design of improved therapies, specifically by examining immunosuppression patterns and immune cell interactions. Multiple steps of metastasis depend on myeloid cells, such as escorting of circulating tumor cells out of the vasculature and into their niche in the brain—this rare event can now be visualized, and interventions against it can be studied. The use of stably-transfected fluorescent tumor cell lines and IVM techniques such as OFDI, MPM, or CARS, can also allow visualization of the distinct characteristics of brain metastasis vs. primary brain tumors, most notably GBM, and their differential immune milieus. Through the use of

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fluorescent labels, IVM could be further extended to study the infiltration (migration tracking, ECM disruption, and angiogenesis) and survival (tumor proliferation, immune cell capture, and tissue invasion) of incipient brain metastases from other origin tissues, where illumination of origin tissue-specific differences may yield actionable insight into tailored clinical treatment. The almost limitless potential to characterize various poorly understood aspects of brain metastases, such as the high propensity of melanomas to form brain metastases compared to other primary cancer types or the rapid development of brain metastases in individuals with lung carcinoma, can provide novel insights and opportunities to effectively treat, and even possibly cure, previously untreatable metastatic malignancies.

Interactions and Changes in TME—Although current preclinical and preliminary clinical data suggest that CIT and RT synergize in the treatment of brain metastases, several remaining open questions must be addressed to guide future clinical practice. These questions include whether, and how, infiltration of immune cells depends upon extravasation through the BBB, where brain endothelium, pericytes, and microglia may all exert brain-specific effects on infiltrating immune cells—particularly under the influence of a malignancy. It is also not well-understood how RT affects the brain microenvironment and increases the efficacy of CIT; evidence suggests that RT induces inflammatory responses in tissue, to which non-tumor-suppressed immune cells respond with greater vigor—an effect yet to be shown directly (191). In-depth understanding of how and why tumor-immune cell interactions and the vasculature are altered is crucial for improved prognosis and treatment decisions in the clinical setting. Furthermore, there are several other poorly-understood phenomena that may underlie the behavior and/or success of metastases in the TME, including, but not limited to: a) the interactions of pericytes/podocytes with invasive tumor margins, b) interactions of microglia with infiltrating lymphocytes and innate immune cells, c) the role of lymphangiogenesis, and d) immune cell-immune cell interactions. By exploiting the multiplexing capabilities of intravital imaging (i.e., using multiple fluorescence channels in parallel or in series) and its spatiotemporal resolution, the changes in the TME and immune cell infiltration resulting from treatment with different combinations of RT and CIT can be tracked, quantified, and understood. For example, MPM could readily be used to track the localization and trafficking of fluorescently-labeled myeloid cells in a metastatic brain tumor-bearing mouse model to examine the movement and co-localization of myeloid effectors in relation to tumor cells. This system could be further extended with an inducible Cre recombinase model to knockout putative immuno-suppressive tumor factors or immune-activating stromal factors to confirm the role of such factors in metastatic growth. In another instance, transient antibody labeling of checkpoint sites could be used to assess changes in the CIT “druggable state” after treatment with chemo- or radiotherapy. Potentially more interesting is dynamic observation of CIT within the brain TME, including whether immunotherapeutics interact more with the classical CD4/CD8 T-cells or with myeloid cells (e.g., perivascular macrophages) within the brain by employing OFDI or MPM with a fluorescently tagged CIT drug. Identification of the particular cells interacting, their lengths of interaction, and the tissue sites of such interactions may pave the way toward the development of new blocking agents to inhibit specific cell-cell interactions or provide hints toward a promising combination therapy. This

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will help address current challenges in the field of CITs such as the efficiency of drug delivery and mechanism and efficacy of action and dependence on drug size.

Tracking Immune Cells—Cognitive function, especially memory, is severely affected by brain metastases and the complications of treatment. For example, WBRT causes damage within the hippocampus (192). IVM can be used to study these effects because of its high spatiotemporal resolution, allowing tracking of the changes in immune cell interactions in models of cognitive dysregulation. This will provide novel insights into changes within the brain's immune milieu. This technique can be further combined with optogenetic switches, which enables the activation or repression of transgenic loci with specific wavelengths of light to directly study the effects of WBRT or other treatment on normal or diseased neural activity and whether CIT affects these functions.

Additionally, MPM performed on fluorescently labeled CAR-T cells in the brain (see Section II) could help uncover the multi-dimensional processes by which CAR-T cells function in the brain. Similarly, MPM could allow study of mechanisms of CAR-T exhaustion in solid tumors. Direct examination of adoptively transferred, fluorescently-labeled CAR-T cell-tumor interactions will thus yield insight on the major immunosuppressive molecules with the brain TME.

Drug delivery and mechanism of action—Current challenges in drug development for the treatment of brain metastases include the following:

- Accurate study of drug penetrance across the BBB as well as drug uptake and effect by cell type
- Differences in the mechanism of action of CIT drugs in the specific context of the brain TME (e.g., compared with primary brain tumors or with other metastatic sites)
- Altered expression patterns of immune checkpoints in the brain
- Brain-specific patterns of response to radio-, chemo-, and immuno-therapies

IVM should be used to explore BBB integrity and diffusion or transport of small molecules across the endothelial-pericyte barrier using fluorophore-tagged drugs or proxy molecules, such as fluorescent high molecular-weight dextrans or nanoparticles of varied sizes and shapes (193–195). Using super-resolution techniques, IVM might support, for example, the direct identification of drug entry mechanism, i.e., whether therapeutics enter via transcytosis, interendothelial gaps, or other processes in the brain vasculature. This knowledge is critical to therapeutic design considerations. Drug uptake as a function of cell type may be assessed through combination of exploration of trans-BBB transport and diffusion with fluorescently-labeled cells and may be further extended to assess the drug's effect on cell proliferation, survival, hypoxia, localization, or functional activity. Data gathered from such studies may inform whether brain-specific differences in the action of immunomodulatory drugs exist and affect the target therapeutic window. As noted above, microglia may express PD-1 in inflammatory conditions, but the effects of that expression on the function of infiltrating lymphocytes, pericyte, neuron, and astrocyte components of

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the NVU remain unknown. Better elucidation of the effect of PD-1 inhibition thereof in the unique context of the NVU and brain TME will enable design of more incisive CIT therapies in brain metastasis. Furthermore, transient labeling of proteins/receptors using fluorophore-bound antibodies may be employed to image the spatiotemporal distribution of checkpoints targeted by checkpoint inhibitor therapy in brain metastases before, during, and/or after treatment. Mapping checkpoint expression may then guide the choice of combination therapies, dose levels, and dose scheduling in subsequent studies to combat the spread of brain metastases. By employing the above methods alone, or in combination with fluorescent protein-expressing tumors, the long-term effects of chemo-, radio-, and immunotherapy on brain metastasis growth patterns, survival, and spread can be integratively studied. The use of IVM in these studies provides a unique advantage through long-term dynamic imaging with high spatiotemporal resolution in which endogenous tissue architecture and behavior are maintained in far greater detail than is possible with terminal endpoints and assays that derange tumor or immune behavior.

Continued progress in application of CIT to brain metastases will demand improved study design, for which IVM techniques are well-suited. Previously unanswerable questions, such as the migration dynamics of PD-1-postive microglia or the influence of CTLA-4 inhibition upon T-cells transiting the BBB, may begin to be addressed through the long-term studies enabled by IVM. Such studies will provide pivotal proof for CIT mechanism of action and therapeutic efficacy in the unique tumor microenvironment of brain metastases. Furthermore, correlative studies wedding dynamic IVM use with traditional study endpoints, such as flow cytometry and immunohistochemistry, offers enhanced understanding of the cell-cell interactions that underlie brain metastasis progression and effective treatment. Combination of IVM with emerging terminal endpoints, such as high-dimensional cytometry or immunohistochemistry capable of surveying dozens of antigens (196–199), is also a compelling line of investigation that may provide revolutionary—and clinically actionable—insights into the spatiotemporal dynamics of immune and brain metastasis cell interactions that lead to better therapies for metastatic brain cancer patients.

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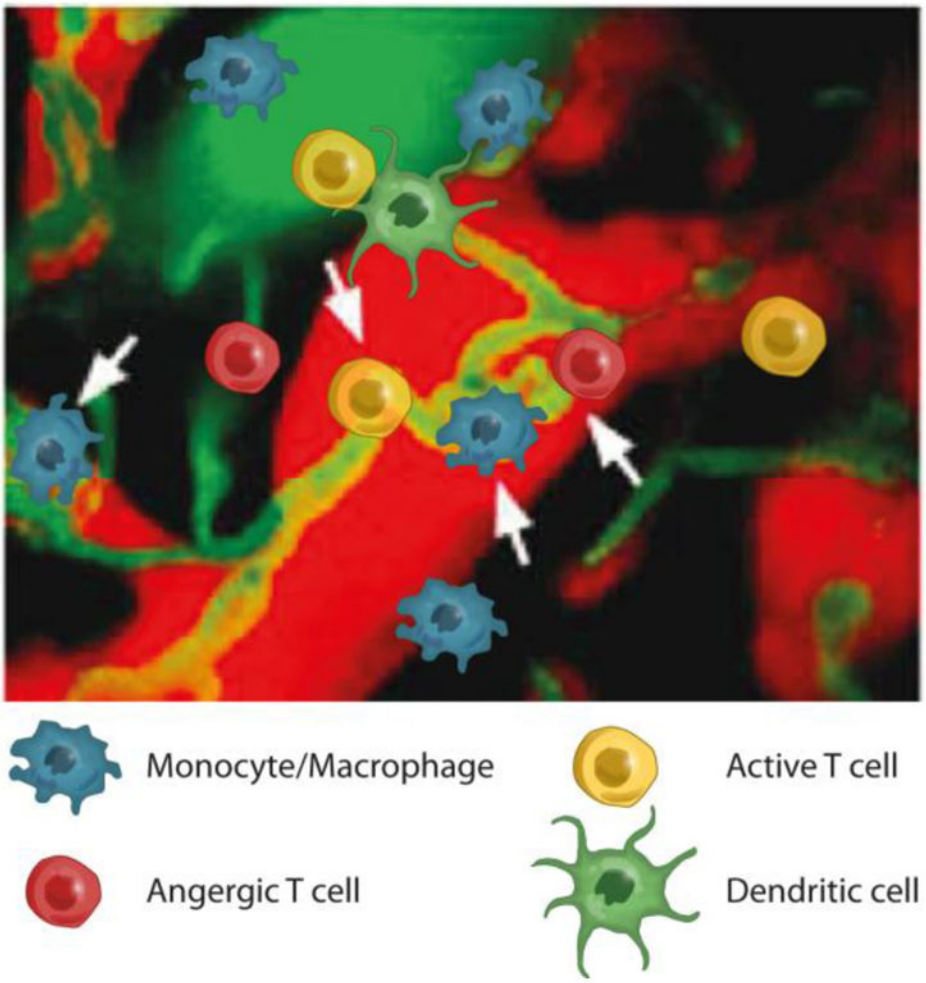

Figure 1.

In-vivo fluorescent multiphoton imaging of brain metastatic melanoma cells (red) interacting with brain vasculature (Tie2-GFP (green fluorescent protein) endothelial cells, green), creating a metastatic niche. The image was obtained from a depth of 200–210μm. White arrows included in the cartoon overlay depict potential immune-tumor interactions that can be visualized using IVM, such as immune cell extravasation across the BBB, vascular permeability, and cell type distributions at endothelial and tumor borders. Image adapted from Kienast et al. (111).

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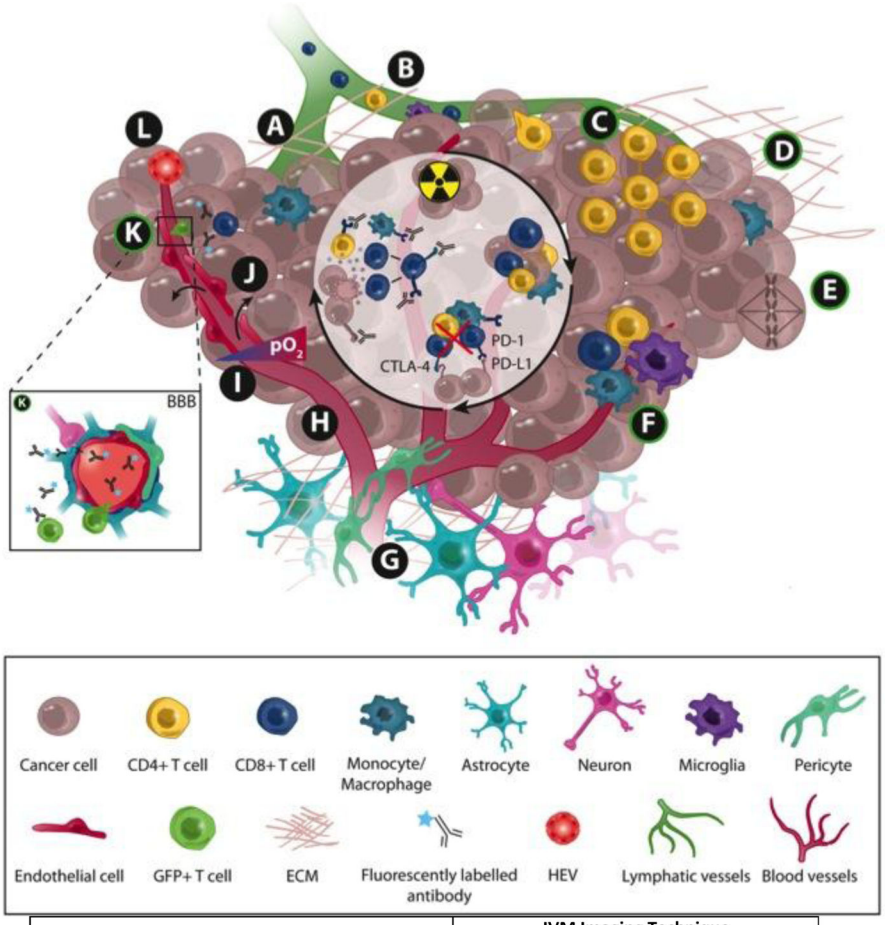

		IVM Imaging Technique						
	Tumor Microenvironmental Processes	**1P**	**MPM**	**OFDI**	**FLIM**	**SHG**	**THG**	**CARS**
A	Lymphangiogenesis		■	■				
B	Cell Migration Through Lymph Vessels	■	■					
C	Immune Infiltrate Proliferation and Organization	■	■	■	■		■	
D	Extracellular Matrix Changes				■	■	■	
E	Tumor Growth and Migration	■	■	■	■	■	■	■
F	Immune and Microglial Infiltration	■	■				■	
G	Neurovascular Unit-Specific Features		■	■	■	■	■	
H	Angiogenesis	■	■	■	■		■	
I	Blood Flow, pO2	■	■		■			
J	Blood Vessel Permeability	■	■		■	■	■	
K	Blood-Brain Barrier	■	■	■	■	■	■	■
L	High Endothelial Venule Formation	■	■	■	■	■	■	■

Figure 2.

The brain metastatic microenvironment is shaped by tumor growth, immune infiltration, and stromal cell interactions. Several features, described clockwise, can be assayed via IVM (88,107,108,116,145,165–189).

A) Lymphangiogenesis reshapes lymphatic drainage and immune infiltration and may be assayed via MPM or OFDI

B) Cell migration through lymphatic vessels can be observed by fluorescently labeling the cell types of interest to image via PMP

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C) The organization and proliferation of immune cells in the tumor microenvironment shapes host response and tumor growth, these processes have been observed with 1P and MPM but may be amenable to further investigation via OFDI, FLIM, and THG

D) Changes in the extracellular matrix dictate tumor growth and cell interactions and may be imaged via FLIM, SHG, or potentially even THG

E) Tumor growth and migration can easily be tracked with fluorescent reporter tumor cell lines and any IVM imaging modality

F) Immune and microglial infiltration from endothelial vessels, particularly the special architecture of the BBB, can be imaged via 1P or MPM and may be detectable via THG

G) Neurovascular unit-specific features, such as neurons, astrocytes, and pericytes, may be observed via fluorescent labeling suggested above

H) Angiogenesis is a critical process for tumors to continue growing, where blood vessel change can be imaged via contrast dyes and fluorescent antibodies against endothelial markers

I) The rate of blood flow and pO2 levels can be imaged directly via 1P, MPM, and may be amenable to imaging via FLIM, SHG, or THG

J) Immature blood vessels frequently present with enhanced permeability, which may be imaged via fluorescent conjugates of dextran to separate active extra/intravasation from passive diffusion in immune or cancer cell trafficking

K) The blood brain barrier (BBB) is a unique feature of the brain and may be imaged via fluorescent reagents and 1P, MPM, or CARS and may be suitable for imaging via OFDI, FLIM, SHG, or THG

L) High endothelial venules are mature, selectively permeable blood vessel structures that may regulate the trafficking of immune and cancer cells around tumors, tracking their distribution and function via 1P and MPM, or other imaging techniques, will reveal critical trafficking parameters of metastasis initiation, survival, and dissemination.

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Table 1

Features of Brain Metastasis Treatment[5,8,16–19]

Current Treatment Modalities	Modality Side Effects	Associated Therapy Challenges
Whole-body radiotherapy (WBRT)	Radiation toxicity	Differentiation of primary and secondary tumor responses to treatment
Stereotactic radiosurgery (SRS)	Neurocognitive decline	Definition of tumor borders and micrometastases
Brachytherapy	Brain tissue damage	Heterogeneous inflammatory response of neurovascular unit
Surgical resection	Surgical trauma Repeated	Detection of minimal residual disease margins
Monitoring disease progression or treatment response via high-resolution CT or MRI	exposure to CT radiotoxicity and/or MRI contrast agent toxicity	Detection of quiescent micrometastases, differentiation of necrotic vs. inflamed cerebral edema, patient compliance

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Table 2

FDA Approved Immunotheraptics and Current Progress on Brain Metastasis[32,34,60–64]

Immunotherapeutic	Target	Indicated Disease States	Current Progress on Brain Metastasis
Ipilimulab	CTLA-4	Unresectable or metastatic melanoma	Phase II clinical trials shows 10–25% disease control in metastatic brain cancer
Nivolumab	PD-1	Locally advanced or metastatic urothelial carcinoma	Phase III clinical trial extended survival of melanoma brain metastases patients by 6.9 months, while combination Nivolumab and Ipilimulab extended survival by 11.5 months
Atezolizumab	PD-L1	Metastatic non-small cell lung cancer (NSCLC) and locally advanced or metastatic urothelial carcinoma	Phase III clinical trial is currently on-going and shows promising preliminary results in patients with NSCLC and brain metastasis
Avelumab	PD-L1	Metastatic Merkel cell carcinoma (MCC)	Patients with brain metastasis are excluded from clinical trials using Avelumab
Durvalumab	PD-L1	Locally advanced or metastatic urothelial carcinoma	Phase II clinical trial currently recruiting participants with brain metastases from epithelial-derived tumors
Pembrolizumab	PD-1	Microsatellite instability- high (MSI-h) or mismatch repair deficient (dMMR) unresectable or metastatic solid tumors	Phase II clinical trial of brain metastases in melanoma and NSCLC currently on-going with early responses reported in 20–30% of patients
Chimeric Antigen Receptor T-Cell Therapy (CAR-T)	Antigen on targetted tumor cells	B-cell acute lymphoblastic leukemia (ALL)	Phase I clinical trial for brain tumors currently under-going recruitment

CD4 and CD8 T-cell infiltration within brain microenvironment not well described, but preclinical data on brain metastases patients show promise in efficacy of checkpoint inhibition.

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Table 3

Intravital Microscopy Techniques[87,89–96]

IVM Imaging Technique	Description	Penetration Depth	Resolution	
			Spatial	Temporal
Optical Frequency Domain Imaging (OFDI)	Advanced optical coherence tomography (OCT) tech that can rapidly examine tissue structure dynamics	**3.8mm**	~5 μm	~msec
Multi-Photon Fluorescent Microscopy (MPM)	Multiple photon fluorescence excitation enables higher penetration depths and novel imaging modes	**300–600μm**	~200n m	~msec
Second-Harmonic Generation (SHG)	Non-linear photonic imaging, label- free and discriminates highly ordered subcellular structures, e.g. Collagen	**~500μm**	~0.5 μm	~msec
Third-Harmonic Generation (THG)	Non-linear photonic imaging that is label-free and discriminates disordered structures, e.g. Lipid bodies	**~300μm**	~0.5 μm	~msec
Coherent Anti-Stokes Raman Scattering (CARS)	High-contrast, stimulated Raman-based spectroscopic imaging method that is label-free	**200μm**	~200 nm	~10 msec
1-Photon Fluorescent Microscopy (1P)	Typically using a confocal approach (pinhole) for fluorescence imaging	**80–150μm**	~200 nm	~msec
Fluorescent Lifetime Imaging Microscopy (FILM)	Detection of molecular interactions based on differences in fluorescence decay rates	**N/A**	~nm	~500 fs