



Face/on: the future of craniofacial medicine through cell-based biofabrication

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Abstract

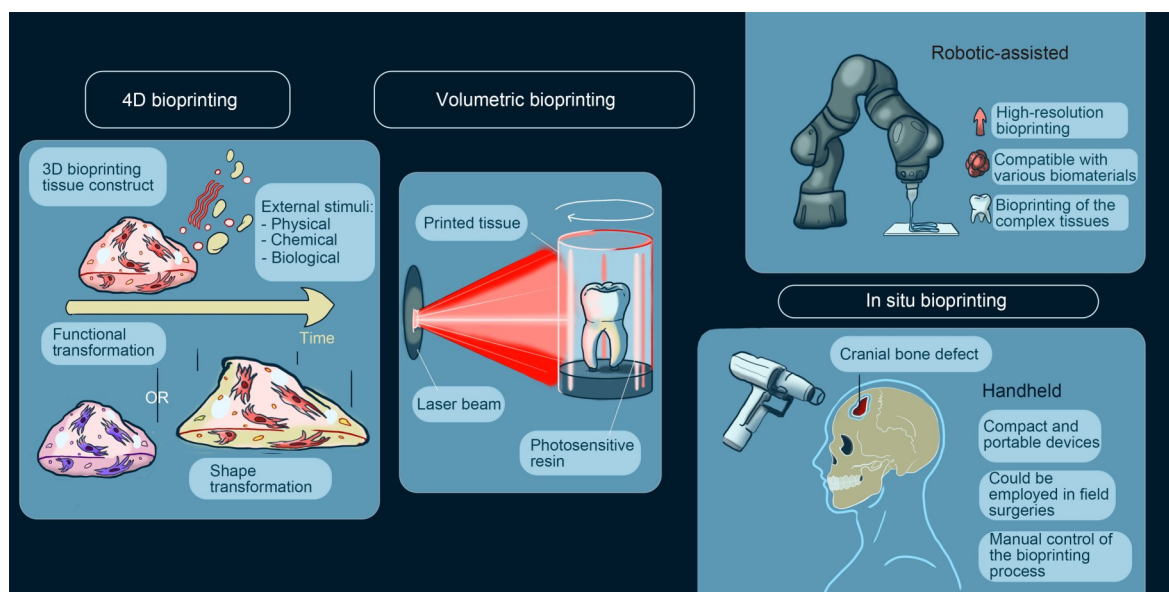
Traumatic injuries to the craniofacial skeleton, along with diseases and structural defects affecting oral tissues—including teeth, gums, and the periodontal complex—are among the most prevalent pathological conditions in humans. Over the course of a lifetime, nearly everyone requires some form of dental or maxillofacial care. While current routine clinical procedures—such as surgical reconstructions, tissue grafting, and prosthetic restoration—provide varying degrees of therapeutic benefit, they often fail to fully restore the complex architecture and functions of craniofacial tissues. This limitation primarily reflects the intricate organization of the maxillofacial region, in which soft and hard tissues are tightly coordinated within a precisely regulated anatomical framework. Emerging cell-based biofabrication technologies, such as organoids, three-dimensional bioprinting, and microfluidic organ-on-chip systems, offer promising strategies for improving tissue equivalents and in vitro tissue models, bringing their architecture and functional properties closer to native tissues. In this review, we examine recent advances in craniofacial cell-based biofabrication, focusing on the development of oral tissue organoids, advanced bioprinting techniques—including in situ bioprinting, four-dimensional bioprinting, and volumetric bioprinting—and the implementation of craniofacial organ-on-chip systems. Furthermore, we discuss the principal challenges currently limiting the field and outline prospective directions that may guide the next generation of cranial and oral tissue engineering.

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Graphical abstract



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1 Introduction

Craniofacial and oral tissue defects are currently the most common injuries in contemporary human populations. Their primary causes include congenital malformations, industrial and sports-related traumas, motor vehicle accidents, and tumor lesion resection. Additionally, inflammatory diseases of the oral cavity, such as gingivitis, peri-implantitis, periodontitis, and dental caries [1, 2], have a destructive effect on tissues, leading to defects that significantly reduce patients' quality of life. Of these pathologies, periodontitis is particularly severe, arising from the accumulation of bacteria and their metabolic products in the oral cavity. This disease leads to the destruction of the junctional epithelium and resorption of the alveolar bone, which ultimately results in tooth loss [3].

Successful reconstruction of craniofacial hard and soft tissues remains a significant clinical challenge due to the presence of vital sensory organs, a high density of blood vessels, and regional differences in skeletal tissue characteristics [4]. Despite the diversity and accessibility of existing methods for replacing defects in the craniofacial region, most have failed to fully restore the architecture and functionality of native tissues. Classical tissue engineering strategies, including polymer scaffolds, cellular constructs, and decellularized matrices, have been increasingly incorporated into routine dental and maxillofacial practice [5]. However, these approaches often show limited efficacy, primarily due to insufficient biomimicry. To overcome these limitations and improve the sophistication

of tissue-engineered constructs, various biofabrication strategies are currently being explored.

Biofabrication is a rapidly evolving, multidisciplinary field in which advances in volumetric (third-order unit processes), hybrid (multimodal and assisted), and scaffold-free bioassembly strategies hold promise for the precise spatial organization of cells, extracellular matrix (ECM) components, and other materials across multiple length scales [6]. Biofabrication strategies are broadly categorized into cell-free and cell-based. Cell-free biofabrication involves creating biomimetic scaffolds using additive manufacturing technologies, such as extrusion-based three-dimensional (3D) printing [7], melt electrowriting [8], and direct ink writing (DIW) [9]. Cell-based biofabrication approaches include the production of highly organized cellular structures, such as organoids and microtissues, the application of bioprinting technologies, and the use of microfluidic systems to develop advanced in vitro models and organ-on-chip systems [10]. Organoids are self-organizing cellular aggregates that serve as simplified models of whole organs or specific organ regions, in which cells undergo a multistage differentiation process [11]. They are used to examine physiological processes, model diseases, and evaluate drug responses, and they are also considered foundational building blocks for constructing full-scale tissue equivalents [12, 13]. Notably, 3D bioprinting is among the most rapidly evolving biofabrication techniques, enabling the creation of 3D tissue analogs through precise spatial deposition of living cells and biomaterials [14]. In addition to traditional approaches, such as extrusion-based bioprinting (EBB), inkjet printing,

and laser-assisted printing [14], more advanced methods, such as four-dimensional (4D) bioprinting [15], volumetric bioprinting (VBP) [16], and in situ bioprinting [17], are being actively explored. Finally, microfluidic systems incorporating cells, organoids, or tissue constructs, commonly referred to as organ-on-chip platforms, provide state-of-the-art tools for modeling tissue functions and facilitating the maturation of printed tissue constructs [18].

In this review, we provide a systematic overview of the latest cell-based biofabrication approaches for the reconstruction and replacement of craniofacial and oral cavity tissues. We begin by examining the structural organization of tissues and organs within the craniofacial region, alongside current clinical needs in maxillofacial surgery and dentistry. Next, we evaluate key biofabrication strategies, including

organoids, advanced bioprinting technologies, and microfluidic systems. Finally, we explore prospects for craniofacial bio-fabrication and highlight the challenges that must be addressed to enable continued progress in this field. Table 1 lists the main abbreviations and their corresponding full names.

2 Clinical needs in craniofacial medicine

2.1 Craniofacial tissues and organs

The craniofacial region consists of a diverse array of tissues, including cranial, facial, and mandibular bones, as well as hard and soft tissues of the oral cavity [4] (Fig. 1). In the adult human cranium, 9 cranial bones and 17 facial

Table 1 Main abbreviations and their full names

Abbreviation	Full name	Abbreviation	Full name
ALI	Air–liquid interface	ICC	Immunocytochemistry
ALK	Activin receptor-like kinase	IHC	Immunohistochemistry
ALP	Alkaline phosphatase	IOB	Intraoperative bioprinting
BMP	Bone morphogenetic protein	iPSCs	Induced pluripotent stem cells
CAD	Computer-aided design	LAB	Laser-assisted bioprinting
CLP	Cleft lip and palate	LIFT	Laser-induced forward transfer
CSCs	Calcium silicate cements	LPS	Lipopolysaccharides
DBB	Droplet-based bioprinting	(micro-)CT	(Micro-)computed tomography
(d)ECM	(Decellularized) extracellular matrix	MNPs	Magnetic nanoparticles
DIW	Direct ink writing	(n)HAp	(Nano)hydroxyapatite
DLP	Digital light processing	OCN	Osteocalcin
DMP-1	Dental matrix protein-1	OSP	Osteopontin
DSPP	Dentin sialophosphoprotein	PCL	Polycaprolactone
EBB	Extrusion-based bioprinting	PDGF	Platelet-derived growth factor
ELISA	Enzyme-linked immunosorbent assay	PDL	Periodontal ligament
FDA	Food and Drug Administration	PG	Parotid gland
GelMA	Gelatin methacryloyl	PTFE	Polytetrafluoroethylene
GFP	Green fluorescent protein	RT-qPCR	Reverse transcription quantitative real-time polymerase chain reaction
(h)DFCs	(Human) dental follicle cells	SCAPs	Stem cells from the apical papilla
(h)DPCs	(Human) dental pulp cells	SCs	Stem cells
(h)DPSCs	(Human) dental pulp stem cells	SEM	Scanning electron microscopy
H&E	Hematoxylin and eosin	SGs	Salivary glands
HEMA	2-Hydroxyethyl methacrylate	SLA	Stereolithography
HES	Hematoxylin/eosin/saffron	SLG	Sublingual gland
(h)GFs	(Human) gingival fibroblasts	SMG	Submandibular gland
(h)MSCs	(Human) mesenchymal stromal cells	TMJ	Temporomandibular joint
(h)OBs	(Human) osteoblasts	TNF- α	Tumor necrosis factor alpha
(h)PDLFs	(Human) periodontal ligament fibroblasts	VBP	Volumetric bioprinting
(h)PDLSCs	(Human) periodontal ligament stem cells	VEGF	Vascular endothelial growth factor
(h)(r)BMSCs	(Human) (rat) bone marrow-derived mesenchymal stromal cells	VP	Vat polymerization
HUVECs	Human umbilical vein endothelial cells		

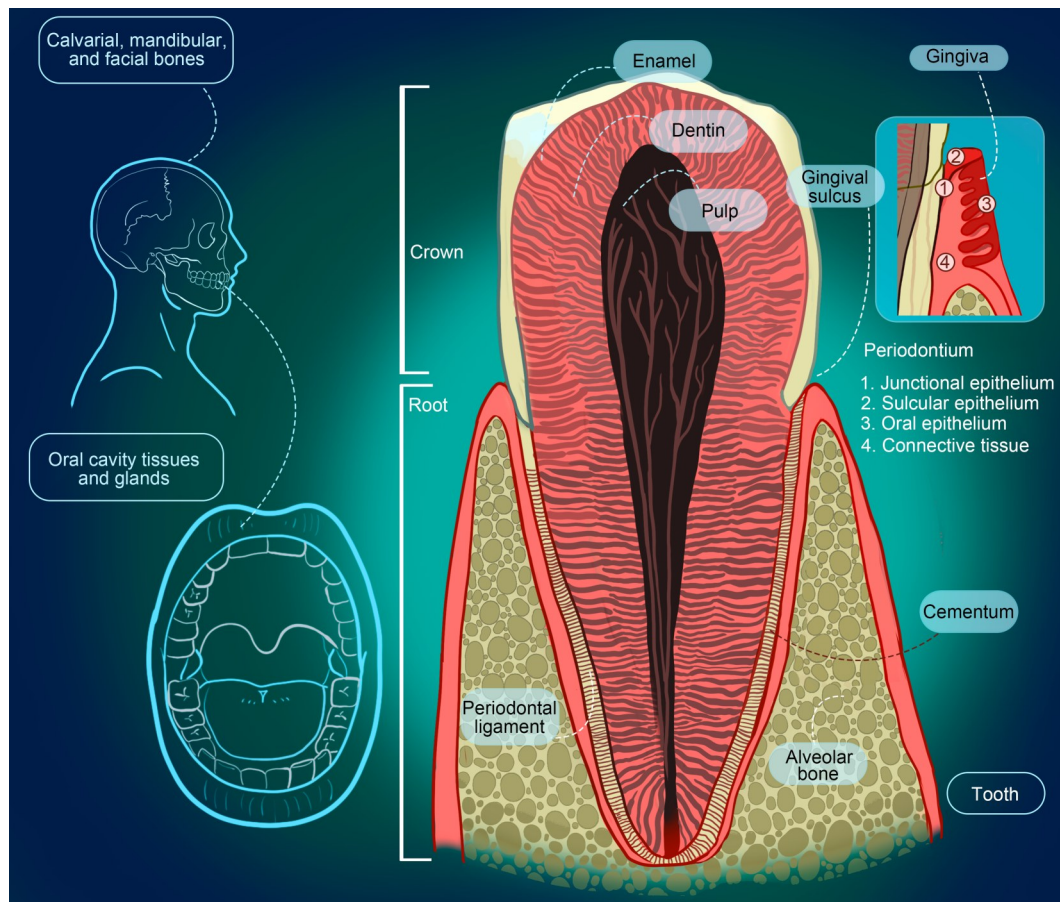


Fig. 1 Overview of craniofacial tissues and organs

bones are interconnected through a complex system of approximately 40 sutures [19]. These oral and craniofacial tissues support the human face and play a crucial role in shaping our individual and collective identities [20]. The bones of the hard palate, maxilla, and palatine region form the anterior roof of the oral cavity [21]. The palate provides essential structural support for oral–nasal functions, such as breathing, speaking, and swallowing, while acting as a barrier between the oral and nasal cavities [22]. The temporomandibular joint (TMJ), commonly known as the jaw joint, is a synovial articulation essential for everyday functions, such as speaking and chewing [23]. Each TMJ is located on one side of the face and is composed of the mandibular fossa, joint tubercle, and condylar process of the mandible, which are separated by an articular disk. Ligaments and muscles attach to these structures, providing stability and facilitating controlled movement [24]. Salivary glands (SGs) secrete saliva into the oral cavity, which moistens the mouth, initiates digestion, and provides antimicrobial and buffering functions to help prevent oral infections. There are three major pairs of SGs—the parotid gland (PG), submandibular gland (SMG), and sublingual gland (SLG)—along with numerous minor SGs located within the submucosal layer of the oral cavity [25, 26].

Teeth are unique organs composed of enamel, dentin, and soft dental pulp tissue, which contain blood vessels and nerves [27]. Enamel, the hardest substance in the human body, is highly durable and adapted to withstand lifelong mechanical, chemical, and thermal stresses while protecting the underlying tooth structure. Its formation, known as amelogenesis, occurs within the jawbone before tooth eruption and involves complex signaling pathways that regulate cellular activity. During this process, ameloblasts secrete ECM containing specific proteins and proteases that guide enamel shape, arrangement, and mineralization [28]. Dentin is the primary structural framework of the tooth, protecting the pulp and preventing enamel fractures under occlusal loading. It comprises approximately 10% water, 20% organic compounds—primarily type I collagen—and 70% inorganic hydroxyapatite crystals. Dentinal tubules, formed by odontoblast extensions, contribute to dentin structure and protection, with tertiary dentin produced in response to pathological or physiological stimuli [29]. Dental pulp tissue occupies the pulp cavity within the tooth and comprises nerves, blood vessels, and lymphatic vessels. The pulp cavity is larger in the crown region, forming the pulp chamber, and narrows within the root to create the root canal [30].

Periodontal tissues surround and support the teeth, maintaining stability by distributing and absorbing masticatory forces while serving as a protective barrier against heterogeneous invasion by pathogenic microorganisms [31]. The intact periodontium is highly organized and consists of four primary components: the gingiva, root cementum, periodontal ligament (PDL), and alveolar bone. The PDL is a network of oriented fibers that provides cushioning, barriers, and sensory functions [32]. Histologically, it is a highly cellular structure rich in blood vessels and sensory nerve endings and is located between the cementum and alveolar bone [33, 34]. The PDL not only supports the teeth but also transmits and disperses occlusal forces. Additionally, it plays a critical role in maintaining proper mandibular positioning and facilitating tissue repair [35].

Primary teeth erupt through the mucous membrane from the inner alveolar bone, forming unique structures that interface between the exposed tooth surface and surrounding tissues. These interface structures include the gingival sulcus and the junctional epithelium located beneath the gingival sulcus (Fig. 1). The junctional epithelium is the epithelial component of the gingival tissue that is in direct contact with the tooth surface [36]. Most intracellular cells of the connective epithelium form and maintain tight adherence to the mineralized tooth surface, referred to as the epithelial attachment. This specialized architecture, involving both epithelial and nonepithelial cells, provides powerful antimicrobial defense mechanisms [37, 38]. The architecture of both the oral mucosa and cutaneous epithelium is primarily composed of a superficial epithelium resting on an underlying basement membrane that serves as a barrier against pathogens and mechanical stress. The palatal and gingival regions of the oral cavity routinely sustain greater mechanical forces and associated physical trauma during eating and chewing; therefore, they have increased keratinized epithelium. Conversely, more elastic regions of the oral mucosa exposed to lower mechanical stress, such as the buccal tissue, are typically composed of nonkeratinized epithelium with loose ECM [39].

2.2 Pathologies of the craniofacial region

Craniofacial deformities can develop after oncological resection, traumatic injuries, or congenital disorders. These conditions often result in nonhealing composite tissue defects characterized by the degeneration of both soft and hard tissues [40].

Discal pathology is an antecedent to a series of degenerative changes that can affect the entire TMJ. These conditions include internal derangement (disk displacement), disk thinning, and disk perforation. The early stages of TMJ disorders—such as disk thinning—are inadequately addressed by current clinical interventions, and effective treatment at

these initial stages remains an unmet clinical need. The complex and unique anatomy of the TMJ poses challenges for the development of new surgical strategies, regardless of disease severity [23, 41].

Cleft lip and palate (CLP) is among the most common congenital defects. These defects arise from the failure or incomplete fusion of the maxillary processes and are associated with multiple risk factors, including genetic predispositions, pharmacological exposures, physical insults, maternal infections, injuries, and metabolic and nutritional imbalances. CLP may occur as part of a broader syndrome or independently, as observed in nonsyndromic cleft cases [42].

Among pathologies of the craniomaxillofacial tissues, diseases affecting the teeth and tooth loss are particularly significant. For example, dental caries is a complex, multifactorial, biofilm-mediated disease that involves sequential processes—including demineralization, neutralization, and remineralization—that affect the hard tissues of both primary and permanent teeth [43]. Inflammation of the dental pulp often occurs early in the carious process, even before pulp exposure. As caries advances toward the pulp, the severity of inflammation tends to increase. If left untreated, this inflammation can progress to infection and ultimately lead to pulp necrosis [44].

In addition to dental pathologies, periodontal diseases represent a major clinical concern. Under pathological conditions, exposure to various pathogenic factors causes progressive and often irreversible changes in periodontal tissues, such as gingival recession, PDL rupture, periodontal pocket formation, and alveolar bone resorption, ultimately leading to tooth mobility or loss [45]. Periodontitis is the most common periodontal disorder and is characterized by inflammation, destruction of the epithelial attachment, and loss of alveolar bone height [46]. Although disease onset is initiated by bacterial accumulation and bacterial lipopolysaccharides (LPS), the subsequent tissue destruction supporting the teeth primarily reflects an exaggerated host immune response in susceptible individuals [47]. Gingival recession is another common periodontal condition, defined as the apical displacement of the gingival margin relative to the cemento-enamel junction. It is associated with attachment loss and exposure to the root surface, which can lead to both carious and noncarious lesions in the vestibular region, hypersensitivity, further attachment loss, and increased susceptibility to local inflammatory processes [48].

2.3 Options for craniofacial reconstruction

For much of the history of craniomaxillofacial surgery, defective bones or missing teeth have been replaced with artificial prostheses; however, such replacements cannot fully replicate the physiological functions of native tissues [49]. Advances in regenerative medicine and tissue engineering

have expanded reconstructive options, leading to the clinical adoption of bone substitutes derived from autologous, allogeneic, and xenogeneic sources, as well as various osteoplastic materials of both natural and synthetic origin. These materials include hydroxyapatite, tricalcium phosphates, hydroxyethyl methacrylate, and bioactive glass, all of which have been incorporated into routine clinical practice [50].

The management of nonsyndromic CLP requires a multidisciplinary approach, with surgical intervention serving as the primary treatment modality. In recent years, passive pre-surgical infant orthopedics have gained attention as a potential adjunct to surgical therapy in patients with CLP [51]. Surgical treatment typically involves palatal reconstruction, and in some cases, orthodontic preparation is performed before surgical intervention [52].

The surgical management of advanced intracranial and extracranial communicating skull base tumors is challenging, particularly regarding the reconstruction of large composite defects following tumor resection. Endoscopic repair of large anterior skull base defects has yielded excellent results, especially when multilayer reconstruction techniques incorporating a nasoseptal flap are employed. The nasoseptal flap is a reliable and commonly used reconstructive option for anterior skull base defects during endonasal surgery. Additionally, free flap transfer is a safe and effective reconstructive strategy, with acceptable complication rates, and is useful for reconstructing large composite skull base defects following salvage resection of extensive intracranial and extracranial tumors [53–55].

Dental pulp capping is a widely used therapeutic approach to preserve pulp vitality when pulp tissue is partially compromised by caries, trauma, or mechanical exposure. The procedure involves placing a protective material directly on the exposed or nearly exposed pulp to promote healing and stimulate the formation of reparative dentin, thereby sealing the pulp and reducing the risk of further infection or degeneration. The most commonly used materials for direct pulp capping are calcium hydroxide and mineral trioxide aggregate, both of which facilitate the biological processes necessary for pulp repair and regeneration; however, they differ in their physicochemical properties, handling characteristics, and long-term clinical outcomes [27, 56].

Periodontitis treatment includes the elimination of periodontal pathogens, the control of inflammation, and the restoration of the structure and function of periodontal tissues [57]. Among these goals, PDL regeneration is significant. Ideally, newly formed, highly organized collagen fibers should be reoriented perpendicular to the tooth root and firmly attached to the regenerated cementum and newly formed alveolar bone [50]. However, the complex filamentous architecture of the PDL, which must withstand high mechanical forces, makes its reconstruction challenging. Elastic hydrogels are optimal for filling the space between the tooth

and alveolar bone, as they can contain cells and growth factors, promote tissue regeneration, and allow self-optimization by adapting to local stresses [32]. A barrier membrane—either resorbable or nonresorbable—is used to preserve the space between the defect site and the root surface. The most commonly used nonresorbable membranes are polytetrafluoroethylene (PTFE), which is available in high-density PTFE and titanium-reinforced PTFE forms. Although nonresorbable membranes are biocompatible and have exhibited clinical effectiveness, they are biologically inert and primarily serve as physical barriers [34]. Composite membranes composed of chitosan, polyvinyl alcohol, and hydroxyapatite incorporating meloxicam, a nonsteroidal anti-inflammatory drug, have shown significant anti-inflammatory effects, suggesting their potential use in periodontal therapy [34].

Conventional periodontal treatment can achieve limited new connective tissue attachment and alveolar bone regeneration; however, full regeneration of the complex periodontal architecture is rarely achieved. The placement of osteoplastic materials within periodontal defects commonly results in the formation of a long connective epithelium rather than the proper regeneration of structures identical to a healthy natural periodontium. Therefore, the functional quality of the newly formed attachment remains uncertain. The newly formed cementum typically consists of cellular internal fiber cementum and acellular external fiber cementum, with fewer fibers and poor tissue connectivity [50].

The craniofacial region encompasses a wide range of structures, including bones, teeth, the periodontal complex, oral mucosa, and salivary glands, each characterized by specialized architecture and functions. Common pathologies affecting this region include trauma of the cranial and maxillofacial bones, as well as oral diseases, such as dental caries, pulp inflammation, and periodontitis. Conventional reconstructive methods predominantly rely on prostheses and bone substitutes, whereas regenerative approaches use graft-based systems, such as hydrogels and barrier membranes, to facilitate tissue repair. Nevertheless, complete restoration of the intricate native architecture of tissues, such as the PDL, remains a significant clinical challenge. Consequently, cell-based biofabrication approaches are increasingly regarded as promising solutions to address this limitation.

3 Novel cell-based biofabrication approaches for recreating oral and craniofacial tissues

3.1 Oral organoids

Organoids are 3D cellular structures generated through the self-organization of stem or somatic cells and exhibit

architectural and functional features similar to native organs [58]. Craniofacial organoids are applied primarily in fundamental research to study organogenesis during embryonic development, to serve as disease models for drug screening, and to provide a platform for the development of personalized cancer therapies. Additionally, organoids may be applied in regenerative medicine as modular building blocks or as cell sources for craniofacial biofabrication. Craniofacial organoids can be derived from pluripotent stem cells (SCs), including induced pluripotent SCs (iPSCs) and embryonic SCs, as well as from adult SCs (e.g., mesenchymal stromal cells (MSCs)) and, less often, from somatic cells [59, 60]. Examples of various organoid types are discussed in the following subsections and presented in Fig. 2.

3.1.1 Tooth organoids

Tooth development arises from reciprocal interactions between the oral ectoderm and the mesenchyme, which is derived from the neural crest [61]. Thus, the formation of the tooth germ organoid requires the recreation of ectodermal–mesenchymal interactions. This, in turn, necessitates the use of appropriate cell populations and carefully controlled culture conditions.

Dental pulp stem cells (DPSCs) are commonly used to fabricate tooth germ organoids because they are the most extensively characterized among dental SCs and can be readily obtained from accessible sources, such as the extracted third molar [62]. DPSCs are classified as MSCs due to their specific phenotypic and functional characteristics [63]. Specifically, they express characteristic mesenchymal lineage markers, including CD29, CD90, CD105, CD73, and CD44 [64]. DPSCs exhibit sufficient differentiation potential to produce organoids resembling native tooth germ structures. For example, human DPSCs encapsulated in MatrigelTM have been induced to differentiate into odontoblast-like cells, resulting in the formation of dentin-pulp organoids with a calcified matrix. The organoids showed appropriate gene expression and underwent further differentiation following treatment with Biodentine[®] [65]. Additional dental SCs applied for organoid production include periodontal ligament SCs (PDLSCs) and dental follicle cells (DFCs) [66]. For instance, Hemeryck et al. [67] derived mesenchymal–epithelial composite organoids by combining DPSCs with epithelial SCs derived from the dental follicle. These organoids exhibited the differentiation of epithelial SCs into ameloblasts in the presence of DPSCs, thereby replicating the essential mesenchyme–epithelium interactions that govern tooth development and amelogenesis. Recently, a research group generated tooth root organoids with complex structures by combining DPSCs and PDLSCs. These organoids exhibited a striated mineralization pattern with

distinct dentin-like, cementum-like, and rudimentary PDL-like structures, indicating that DPSCs and PDLSCs can effectively orchestrate the formation of a tooth root [68]. In an earlier study, the same research group developed cementum–PDL-like complex organoids by culturing PDLSCs on pin-patterned dishes, resulting in cylindrical tissues containing only cells and their matrices. The central tissue was mineralized and expressed markers similar to cementum, whereas the peripheral tissue was uncalcified and expressed PDL-associated proteins. This tissue organization was maintained *in vivo* after implantation in immunocompromised mice [69]. To produce organoids with more complex structures, DPSCs can be combined with somatic cells, such as keratinocytes and endothelial cells. In one study, DPSC-derived cells were co-cultured with keratinocytes from gingiva under nonadherent conditions, producing organoids comparable in size to a human tooth germ and exhibiting odontogenic marker expression [70]. Furthermore, DPSCs combined with human oral epithelial cells were seeded onto MatrigelTM or collagen gels to obtain an early tooth epithelium invagination-like model [71].

iPSCs are a promising tool for biofabrication, as they can be readily obtained and differentiated *in vitro* into diverse cell types. They are commonly used in organoid production, including gastrointestinal, lung, liver, kidney, and brain organoids [58]. Alghadeer et al. [72] generated organoids from iPSC-derived induced early ameloblasts (ieAM). To produce mature induced secretory ameloblast (isAM) organoids, ieAM organoids were further co-cultured with odontoblast progenitors. The resulting isAM organoids exhibited mineralization and expressed key enamel proteins, such as ameloblastin, amelogenin, and enamelin.

The development and growth of tissues depend on an adequate nutrient and oxygen supply. Dental pulp is a soft connective tissue containing blood vessels and nerves and therefore plays an essential role in tooth nutrition [73]. Dental pulp organoids may serve as structural units for *in vivo* pulp regeneration or as *in vitro* models for recapitulating tooth vascularization. To this end, a mixture of DPSCs and human umbilical vein endothelial cells (HUVECs) has been used to produce prevascularized, scaffold-free microtissue spheroids for tooth regeneration. These microtissues promoted angiogenesis *in vivo* when engrafted into immunodeficient mice, resulting in the formation of pulp-like tissue [74]. In a related approach, organoids from DPSCs and HUVECs, encapsulated in gelatin methacryloyl hydrogel, were injected into tooth root segments, which were subsequently transplanted to model dental pulp regeneration [75]. Additionally, Xu et al. [76] derived dental pulp organoids composed of endothelial cells, dental pulp cells (DPCs), and dental pulp-derived ECM, which exhibited upregulated expression of odontogenic markers.

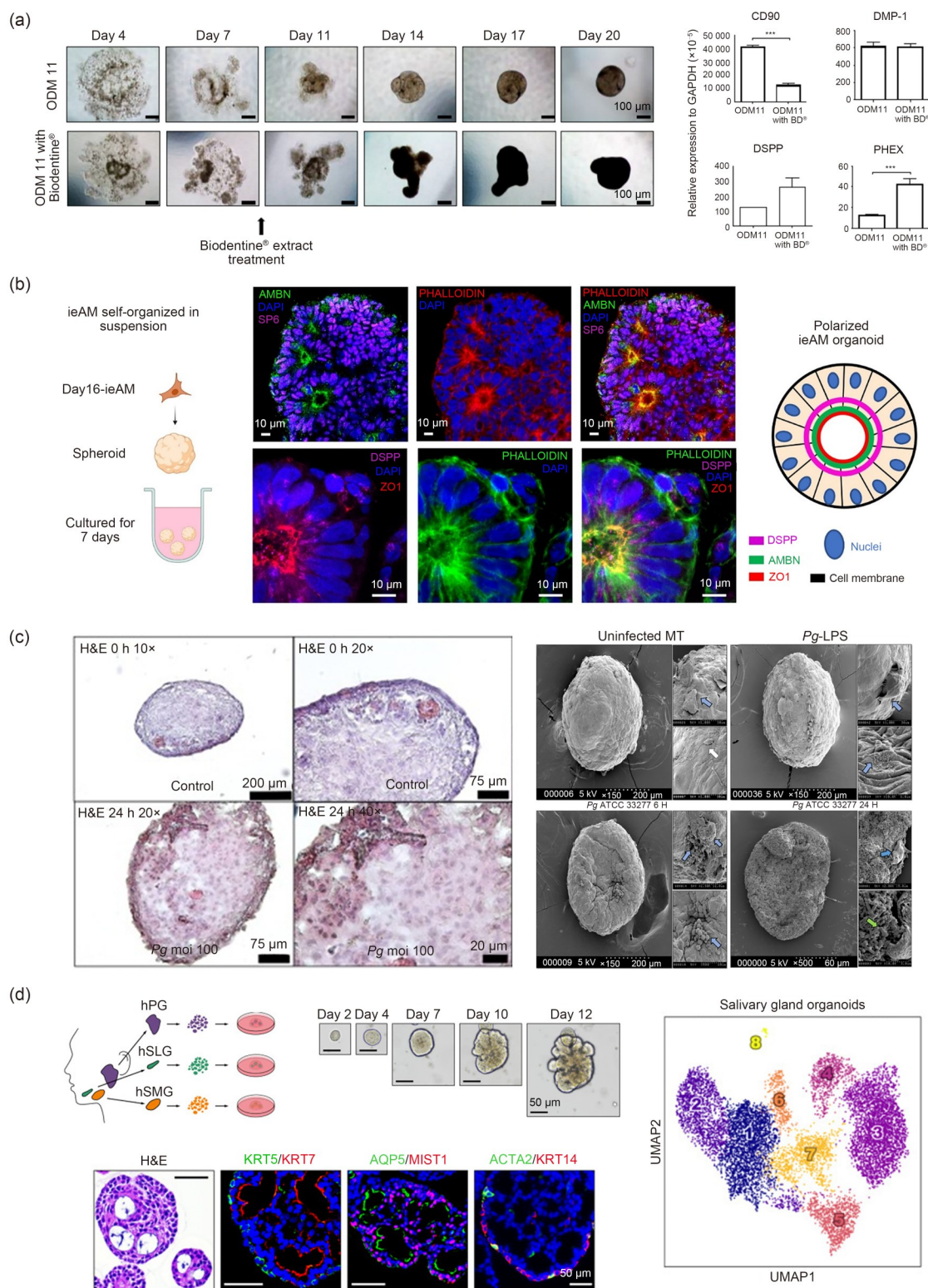


Fig. 2 Types of oral organoids, their formation, and analysis. (a) Dentin-pulp organoids derived from dental pulp stem cells (DPSCs) cultured with the dentin substitute biomaterial Biodentine®. Biodentine® accelerated cell aggregation and upregulated the expression of odontogenic and osteogenic differentiation genes. Adapted from [65], licensed under CC BY 4.0. (b) Induced early ameloblast (ieAM) organoids. Adapted from [72], with permission from Elsevier Inc. (c) Gingiva microtissue spheroids generated from human oral fibroblasts and gingival epithelial cells and subsequently infected with *Porphyromonas gingivalis*. Infection disrupted the organoid structure and compromised the epithelial barrier. Adapted from [82], licensed under CC BY 4.0. (d) Salivary gland organoids derived from dissociated tissues of human parotid gland (PG), sublingual gland (SLG), and submandibular gland (SMG). Adapted from [95], licensed under CC BY 4.0

3.1.2 Oral mucosa tissue equivalents and organoids

Artificial oral mucosa is most commonly generated using two principal cell types: oral keratinocytes and gingival fibroblasts. These cells are usually seeded onto a collagen-based scaffold in two distinct layers to recreate dermal and epithelial layers that resemble those of native oral mucosa. One of the earliest standardized protocols comprises three main steps: preparation of a fibroblast-containing collagen gel, addition of the epithelial cell component, and subsequent culture at an air–liquid interface (ALI) to induce cell differentiation [77]. With minor modifications, this protocol remains widely used to generate organotypic cultures of the oral mucosa [78–81]. In addition to layered tissue equivalents, spherical oral mucosa organoids have been reported. Bugueno et al. [82] generated oral mucosa microtissues using the hanging-drop culture method, combining human gingival epithelial cells derived from the TERT-2 OKF-6 cell line with primary human oral fibroblasts. The resulting microtissues formed 3D spheroidal structures, which were characterized using histological analysis, immunofluorescence staining, and scanning and transmission electron microscopy. Similarly, Driehuis et al. [83] derived oral mucosa organoids from donor epithelial cells. Their protocol involved microdissecting the epithelial layer of the tissue sample to obtain a cell suspension, which was then seeded onto a basement membrane extract. Organoids emerged from single cells or cell clusters within a few days of seeding and progressively developed dense structures with keratinized cores. Immunohistochemical staining for the basal cell marker TP63 and the proliferation marker Ki-67, together with electron microscopy examination, revealed that these organoids effectively recapitulated oral mucosa tissue.

Three-dimensional oral mucosa cultures are used in fundamental research to study host–pathogen interactions [82, 84] and assess tissue responses to dental materials and abutments [80]. Additionally, oral mucosa organoids have been employed in cancer research. Cancer oral mucosa organoids, derived from either tumor tissue or healthy oral mucosal cells, serve as models to study the development of oral neoplasms and to assess the efficacy of antitumor drugs [83, 85].

3.1.3 Salivary gland organoids

The formation of SGs, like that of teeth, strongly depends on ectodermal–mesenchymal interactions. During embryogenesis, SGs undergo morphogenesis, beginning as branched ductal structures that occur from an epithelial placode and subsequently grow into the mesenchyme [86]. Impairments in the structure or function of SGs can lead to hyposalivation, commonly known as xerostomia or “dry mouth” syndrome. This condition may arise from various systemic

diseases, such as Sjögren’s syndrome, uncontrolled diabetes, or granulomatous disorders, but it is most often a consequence of radiotherapy for head and neck cancer. Patients with xerostomia frequently experience difficulties swallowing and speaking and are at increased risk of oral and dental infections. Current treatments primarily involve artificial saliva substitutes and systemic sialogogues, which offer only short-term relief [86, 87]. SG organoids are primarily used to simulate pathological conditions of SGs [88, 89], but they also show promise as tissue grafts for restoring functions in patients with xerostomia [90].

SCs isolated from SGs are commonly used for SG organoid production. For instance, human single-clonal SG SCs were cultured in nanofibrous microwells, where they underwent spheroid preformation and cell differentiation. The resulting organoids exhibited elevated expression of specific markers, as well as higher α -amylase secretion and intracellular calcium levels in response to adrenergic and cholinergic agonists, respectively [91]. Human SMG epithelial stem/progenitor cells differentiated under fibroblast growth factor 10 (FGF10) induction formed organoids exhibiting the expression of differentiation markers, regular structures, and enhanced responsiveness to neurotransmitters in vitro [92]. In another study, Pringle et al. [93] derived cells from human submandibular SG tissue biopsies and generated salispheres containing c-Kit-positive cells. These salispheres were encapsulated in type I collagen hydrogel combined with growth factor-reduced Matrigel to produce organoids. Human primary salivary stem/progenitor cells derived from tissue biopsies formed spheres in suspension culture and can be maintained long term in a 3D hyaluronate-based hydrogel culture. Notably, cells cultured in a 3D hydrogel system modified with bioactive, basement membrane-derived peptides exhibited enhanced expression of progenitor markers, increased proliferation, and improved viability. Furthermore, stimulation of these 3D hydrogel cultures with β -adrenergic and cholinergic agonists promoted the differentiation of epithelial stem/progenitor cells toward an acinar-like cell lineage, which is responsible for saliva production [94].

Although established cell cultures are commonly employed, cell mixtures isolated from SG tissue can also serve as starting materials for functional organoid production. Yoon et al. [95] generated SG organoids from dissociated tissue samples that expressed gland-specific genes and proteins and exhibited appropriate biological responses upon stimulation with neurotransmitters. Single-cell RNA sequencing further revealed that the organoids contained heterogeneous populations of distinct cell types, effectively recapitulating the cellular diversity of native SG tissue. A similar approach was employed to generate SG organoids used in experiments examining the inhibition of activin receptor-like kinase signaling [96]. Additionally, cells isolated from human PG tissue formed acini-like spheroid assemblies in

both 2.5-dimensional (2.5D) and 3D hyaluronic acid-based hydrogels. Treatment with β -adrenergic agonists, including norepinephrine and isoproterenol, enhanced granule formation and α -amylase expression within these structures. When transplanted into an athymic rat model, acini-like spheroids in 3D hydrogel preserve their structural integrity, functional activity, and viability for over three weeks in vivo [97].

To reconstruct the natural complexity of gland tissue, SCs can be combined with somatic cells. SG microtissues, which retained the structural and secretory characteristics of natural gland acini, were established from human salivary myoepithelial cells and gland stem/progenitor cells seeded on a hyaluronic acid-based hydrogel [98]. Furthermore, studies have shown that DPSC-based spheroids can be differentiated into SG organoids with the addition of specific signal molecules, such as retinoic acid and FGF10 [99, 100].

3.2 Advanced bioprinting of craniofacial tissues

3.2.1 Conventional bioprinting technologies

Bioprinting involves the use of computer-aided transfer processes to precisely pattern and assemble living and nonliving materials into specified two-dimensional (2D) or 3D structures, aiming to create functional bioengineered tissues [101, 102]. A bioink consists of a natural or synthetic hydrogel with an encapsulated living cellular component. The hydrogel provides mechanical support and protects cells from external stresses, and its composition is crucial for enhancing tissue-specific ECM production. Achieving an optimal formulation of a bioink with suitable mechanical and structural properties is essential for ensuring both printability and biocompatibility [103]. Bioprinting technologies form the basis of cell-based biofabrication, enabling the production of complete tissue constructs with specified sizes, shapes, and complex architectures. Currently, four primary 3D bioprinting approaches are commonly employed: EBB, droplet-based bioprinting (DBB), laser-assisted bioprinting (LAB), and vat polymerization (VP)-based bioprinting [104]. The current advancements in conventional craniofacial bioprinting are summarized in Table 2 and illustrated in Fig. 3.

EBB is the most commonly used bioprinting technology and operates by forcing a viscous mass of bioink through a dispensing needle. In this process, the bioink is deposited layer by layer using computer-aided design (CAD), allowing the precise placement of cells encapsulated within cylindrical filaments to fabricate 3D tissue constructs with specific shapes. The bioink dispensing system can be powered by pneumatic pressure, mechanical actuation (piston or screw), or solenoid-based mechanisms [105]. The simplicity, availability, and reproducibility of EBB have promoted its widespread

application in craniofacial tissue engineering, especially for reconstructing oral cavity tissues.

In DBB, liquid bioinks in the printhead are divided into discrete droplets by an activator, which can be thermal, acoustic, microwave, or piezoelectric [104]. LAB also uses a droplet-formation strategy but through a different mechanism. This bioprinting approach employs laser-induced forward transfer (LIFT), in which the bioink is precisely transferred from a donor substrate to a collector surface using a pulsed laser [106]. Due to its high spatial resolution, LIFT technology enables the fabrication of complex craniofacial structures, such as the mandible and calvarial bones. For example, Keriquel et al. [107] developed an in situ LIFT-based bioprinting system to repair skull bone defects. In this approach, a cell-laden bioink composed of collagen hydrogel supplemented with hydroxyapatite was printed onto a biopaper substrate. In vitro studies have shown that cells maintain metabolic activity after laser exposure, with activity more than doubling by Day 8 of cultivation. Animal experiments further revealed complete restoration of bone tissue in the area of injury when using a disk-shaped construct.

VP-based bioprinting utilizes patterned light for the high-resolution photopolymerization of bioinks formulated from photosensitive resins, enabling the fabrication of tissue constructs. It includes stereolithography and digital light processing (DLP). In stereolithography (SLA), an ultraviolet (UV) laser induces the polymerization of photopolymer resins, such as elastomers and acrylates, with galvanoscanners controlling laser movement for speed and accuracy. Printing speed depends on light penetration and curing depth, which can be adjusted by modifying resin viscosity, incorporating light absorbers, or using accelerators. However, UV curing can damage cellular DNA, thereby reducing viability; hence, visible light sources are increasingly being used as alternatives [108, 109]. DLP also uses UV light to induce resin polymerization, but unlike SLA, it polymerizes entire layers simultaneously using digital micromirror devices, enabling higher printing speeds. Additionally, light sources in both techniques have evolved from traditional lamps to light-emitting diodes, offering a broader wavelength range at a lower cost [109, 110]. VP-based bioprinting allows for the fabrication of constructs with controlled topography of structural elements, a feature particularly important for reconstructing the PDL, where fibers must be organized in a specific orientation. For example, a biomimetic patch for periodontal tissue regeneration was successfully fabricated using the microscale continuous DLP method [111].

Bioprinting technology continues to evolve rapidly, with new approaches emerging beyond the four conventional methods. Prominent among these advancements are 4D bioprinting, where time functions as a designed parameter driving controlled, dynamic transformations of the printed structure; VBP, which allows for the instant fabrication of

Table 2 Representative applications of common bioprinting technologies for fabricating craniofacial and oral tissue equivalents

Bioprinting type	Construct design	Tissue	Bioprinting parameters	Cells	Hydrogel/Scaffold composition	Analysis of the constructs	Outcomes	Ref.
EBB	Cuboidal, dual-components comprising PDL and alveolar bone, cultured in a microfluidic chip	PDL and alveolar bone	27G conical nozzle	hPDLFs; hOBs (4×10^6 cells/mL for each cell type)	PDL: 12.5% GelMA+0.5% Irigacure 2959; Alveolar bone: 12.5% GelMA+0.1% HAp—magnetic iron oxide nanoparticles (GelMA/HAp-MNPs)	In vitro: AlamarBlue assay (Days 1, 3, and 7); live/dead staining (12 h, Days 3 and 7), ICC (STRO-1 for hPDLFs and OCN for hOBs; Days 1, 3, and 7); tetracycline testing (periodic acid Schiff histology staining and ELISA to measure drug absorption)	Constructs maintained structural integrity under continuous flow for over 10 d. ICC revealed spatial cohesion between STRO-1 ⁺ hPDLFs in the PDL layer and OCN ⁺ hOBs in the bone layer, indicating partial permeability to cell migration. Initial drug testing showed that tetracycline (0–1.0 µg/mL) had no significant toxic effects on the constructs.	[112]
EBB	Grit-lattice cuboidal constructs (10 mm×10 mm×0.3 mm) printed in multiwell plates or on tooth roots for in vivo studies	Periodontal tissues	25G nozzle (250 µm inner diameter); printing speed of 6–8 kPa/12–15 mm/s (10 mg/mL gels) and 10–15 kPa/12–15 mm/s (15 mg/mL gels) with 10 ms postflow; 23 °C (printed)	hPDLSCs (GFP-labeled; 1×10^6 cells/mL and 2×10^6 cells/mL)	Type I collagen (10 and 15 mg/mL)	In vitro: live/dead staining (Days 0 and 3); osteogenic differentiation and further analysis (21 d); Alizarin Red S staining; RT-qPCR (<i>RUNX2</i> , <i>ALP</i> , <i>OCN</i> , <i>COL1A1</i>) In vivo (mice transplantation of tooth root fragments with printed construct); H&E, Gomori's trichrome staining, and IHC (anti-GFP)	Constructs maintained high cell viability (>95% at 0 and 3 d), promoted hPDLSCs differentiation, and exhibited biocompatibility with proper cell organization and attachment to the root surface both in vitro and in vivo.	[113]
EBB	Layered cuboidal constructs (10 mm×10 mm×5 mm) with the upper five layers made of collagen/alginate (periodontal soft tissue) and the lower 10 layers made of collagen/alginate/HA (alveolar bone), whose upper three layers had a denser structure, whereas the lower seven layers were more sparse, thus forming a density-varying printed structure	Periodontal tissues	27G or 30G nozzles; platform temperature of 4 °C; pressure of 0.15 MPa; speed of 8 mm/s	hPDLSCs (1×10^6 cells/mL)	(1) Type I collagen+10% alginate (2) Type I collagen+nHA+10% alginate	In vitro: live/dead staining (Days 1, 4, and 7); CCK-8 assay (Days 1, 3, 5, and 7); fluorescent staining; osteogenic differentiation and further analysis (ALP staining; 7 and 14 d); Picrosirius red staining In vivo in mice (transplantation into the dorsal region): H&E staining, Masson's trichrome, IHC	Constructs showed high viability and heterotopic cell differentiation in vitro; in vivo experiments revealed their biocompatibility and improved regeneration of both oral hard and soft tissues.	[114]

To be continued

Table 2 (continued)

Bioprinting type	Construct design	Tissue	Bioprinting parameters	Cells	Hydrogel/Scaffold composition	Analysis of the constructs	Outcomes	Ref.
EBB	Tooth-shaped construct	Dental tissue	300- μ m nozzle; printing speed of 0.5735 μ L/s and 50 mm/min; 18 °C (enclosure temperature)	hDPSCs (3×10^6 cells/mL)	10% human DDMp+(fibrinogen+gelatin+HAp+glycerol)+polycaprolactone mold+aprotinin (10 μ m/mL)	In vitro: live/dead staining (7 d), osteogenic differentiation and further analysis (15 d; Alizarin Red S staining, RT-qPCR (<i>DSPP</i> and <i>DMP-1</i>)), ICC (<i>DSPP</i>)	The DDMp bioink maintained high cell viability and significantly enhanced in vitro odontogenic differentiation of DPSCs. Using a CAD model, a 3D cellular tooth-shaped construct was created with this bioink, showing its potential for bioprinting patient-specific, clinically relevant dental shapes and sizes.	[115]
EBB	Solid cuboidal constructs (5 mm $\times$ 5 mm $\times$ 2 mm)	Dental tissue	330 μ m nozzle; 130–160 kPa pressure; 18 °C (dispensing syringe with bioinks); 22 °C (bioprinting chamber)	hDPSCs	Gelatin (37.5 mg/mL), GelMA (30 mg/mL), and saline. HA (3.75 mg/mL), 10% glycerol+Irgacure 2959+BMP-mimetic peptide (300 μ g/mL)	In vitro: live/dead staining (1, 4, 7, and 14 d), Alamar-Blue assay, osteogenic differentiation and further analysis (7 d; Alizarin Red S staining), H&E staining (2 and 4 weeks), IHC (2 and 4 weeks; DSPP and OCN)	BMP-mimetic peptide-tethered bioink exhibited suitable printability and provided a dental-specific microenvironment that supported hDPSCs differentiation. Bioink maintained high hDPSC viability (90% after 1, 4, 7, and 14 d) and proliferation while also accelerating their odontogenic differentiation within the 3D bioprinted constructs.	[116]
EBB	Cuboidal construct (10 mm $\times$ 5 mm $\times$ 2 mm) with dense and porous components	Oral soft tissues	0.26-mm nozzle; pressure of 0.2–0.3 bar; printing speed of 30 mm/s; layer height of 0.3 mm; 4–10 °C	hGFs (1 $\times$ 10 ⁶ cells/mL)	ADM:A:G=6%: 2%:2%	In vitro: CCK-8 assay (Days 1, 3, 5, 7, and 9); live/dead staining (Days 1 and 7); F-actin staining (7 d); RT-qPCR (Days 1, 7, and 14; <i>COL1A1</i> , <i>PECAM1</i> , and <i>VEGF-A</i>)	ADM/A/G scaffolds significantly enhanced cell proliferation and viability (>80% after 1 d, >90% after 7 d). <i>COL1A1</i> expression in cells within constructs increased markedly over time compared to the control group, and on Day 14, the expression levels of <i>PECAM1</i> and <i>VEGF-A</i> were slightly higher in the ADM/A/G scaffolds.	[117]

To be continued

Table 2 (continued)

Bioprinting type	Construct design	Tissue	Bioprinting parameters	Cells	Hydrogel/Scaffold composition	Analysis of the constructs	Outcomes	Ref.
EBB	Cuboidal construct (10 mm×10 mm×5 mm)	Oral soft tissues	260 µm nozzle; ambient temperature of 10 °C; platform temperature of 10 °C	hGFs (1×10 ⁶ cells/mL)	Hydrogel groups: 4% alginate+8% gelatin (AG), 10% i-PRF+90% AG (10% iPAG), 30% i-PRF+70% AG (30% iPAG), and 50% i-PRF+50% AG (50% iPAG)	In vitro: live/dead staining (Days 1, 4, and 7); CCK-8 assay (Days 1, 7, and 14); RT-qPCR (type I collagen, type III collagen, and fibronectin) In vivo in nude mice (subcutaneous transplantation): H&E, Masson's trichrome, IHC (CD31, F4/80)	The bioink was effectively used in 3D bioprinting, demonstrating its capacity to be tailored to individual patient needs. It significantly extended the release duration of multiple growth factors. The enhanced bioavailability of these growth factors was further supported by increased cell proliferation and viability (≥96% after 7 d). The bioink also proved biocompatible and promoted enhanced angiogenic activity when utilized in vivo.	[118]
EBB	Cuboidal construct	Calvarial bone	27G dispensing nozzle; pressure of 0.1 bar	hOBs	GelMA	In vitro: RT-qPCR, osteogenic differentiation with further analysis In vivo in mice (transplantation into the calvarial defect): micro-CT, histology	The constructs showed increased osteogenic differentiation. In vivo studies have revealed effective bone regeneration.	[119]
EBB	Cylindrical construct	Calvarial bone	27G nozzle	rBMSCs (GFP-labeled), rat aortic endothelial cell line (RAOEC, mCherry-labeled)	5% GelMA (BM-SCs), 10% synthesized thermosensitive hydrogel (3P, RAOEC)	In vitro: biomechanical tests; fluorescence microscopy (3, 5, and 7 d); osteogenic differentiation and further analysis (7 and 14 d; ALP activity, ALP staining, Alizarin Red S staining), angiogenesis analysis (3, 7, and 12 d), RNA sequencing analysis (7 d), RT-qPCR (7 and 14 d; <i>RUNX2</i> , <i>OSP</i> , <i>osterix</i> , <i>ALP</i> , <i>COL1A1</i> , <i>CD31</i> , <i>VEGF</i> , <i>PDGF</i> , <i>HIF1α</i> , <i>GAPDH</i>), and western blot (<i>RUNX2</i> , <i>HIF1</i>) were performed In vivo in rats (transplantation into the calvarial defect): micro-CT, histology (H&E, Masson staining, IHC)	The constructs exhibited a coupling effect between angiogenesis and osteogenesis. In vitro, bioprinted endothelial cells formed a vascular network through proliferation and migration. RNA sequencing revealed that 3D bioprinting enhanced osteogenic and angiogenic gene expression in cells. In vivo studies have shown that the constructs effectively promoted new bone growth in rat calvarial critical-sized defects.	[120]

To be continued

Table 2 (continued)

Bioprinting type	Construct design	Tissue	Bioprinting parameters	Cells	Hydrogel/Scaffold composition	Analysis of the constructs	Outcomes	Ref.
EBB	Grit-lattice cuboidal constructs (8 mm×8 mm×1.5 mm) with two layers containing different types of cells (each layer of HERS cells followed by two layers of dental papilla cells)	Alveolar bone	27G nozzle; pressure of 0.8–1.1 bar; printing speed of 9–11 mm/s; temperature of print head: 25–27 °C; temperature of platform: 15 °C; distance between lines: 0.8 mm	Rat HERS cells Rat dental papilla cells (4×10 ⁶ cells/mL, both cell types)	10% GelMA + 0.25% LAP	In vitro: live/dead staining (1, 4, and 7 d); DAPI and DAPI cell labeling (0, 3, and 8 d); microscopic observation In vivo in rats (alveolar socket transplantation): H&E, Masson staining, IHC (COL1, OCN, RUNX2, DSPP)	The engineered 3D constructs had viable cells and appeared to mimic the in vivo tooth development microenvironment, facilitating interactions between two cell types. Sufficient alveolar bone regeneration was revealed in a rat model.	[121]
DLP and DIW	Cuboidal construct consisting of PDL module (DLP-fabricated, lattice-type) and alveolar bone module (DIW-fabricated, spatial grid structure)	PDL and alveolar bone	DLP: 405-nm light; 10 s exposure DIW: 90–150 kPa printing pressure; 12 mm/s printing speed	hDFCs (2×10 ⁶ cells/mL for the PDL part and 5×10 ⁶ cells/mL for the alveolar bone part)	GelMA+porcine dental-follicle-derived dECM (10% GelMA + 2.5 mg/mL dECM, 10% GelMA + 5 mg/mL dECM, and 10% GelMA + 10 mg/mL dECM)	In vitro: PDL module—live/dead staining (3, 7, and 14 d); ICC (7 d; fibronectin, laminin); alveolar bone module—live/dead staining (1, 3, and 7 d); ICC (7 and 14 d; ALP, RUNX2, OCN) In vivo in rats (subcutaneous transplantation) and beagles (transplantation into the periodontal defect): micro-CT, H&E, Masson's trichrome, IHC (periostin, OCN, OPG), Sirius Red, and Movat–Russell modified pentachrome staining	The constructs closely replicated natural anatomical and biochemical features of periodontium. They exhibited high cell viability (PDL module: 85% at Day 3 and reached 92% at 14 d; alveolar bone module: 90% after 7 d) and osteogenic cell differentiation in vitro. They also showed robust biological induction by suppressing M1 macrophage activation and promoting collagen fiber alignment and osteogenesis in vivo.	[122]
Microscale continuous DLP bioprinting	Patterned cuboidal construct	Periodontal tissues	–	hDFCs	GelMA	In vitro: live/dead staining (3 and 14 d); F-actin and DAPI staining (14, 21, and 60 d) In vivo in rats and beagles (transplantation into the periodontal defect): H&E and Masson's trichrome staining, IHC (CD31, nestin, fibronectin, periostin, osteonectin, and collagen III), and Sirius Red staining	DFCs within the constructs showed high viability (70%–80% after 3 d, 80%–90% after 14 d). Orthotopic transplantation of the mandibles exhibited effective periodontal regeneration. The constructs closely resembled the natural periodontium, particularly in the formation of “sandwich” structures with correctly oriented fibers that varied regionally along the tooth root. The regenerated periodontium could support stable tooth movement under orthodontic force without any adverse effects.	[111]

To be continued

Table 2 (continued)

Bioprinting type	Construct design	Tissue	Bioprinting parameters	Cells	Hydrogel/Scaffold composition	Analysis of the constructs	Outcomes	Ref.
DLP	Cylindrical porous construct (DPGC)	Dental pulp	405-nm light; exposure time of 10 s	hDPSCs	GelMA–dextran emulsion: 15% GelMA+0.5% LAP+dextran solution (GelMA: dextran volume ratio=2:1)	In vitro: live/dead staining (Days 1, 5, and 7); CCK-8 assay (Days 1, 5, and 7); ALP activity; RT-qPCR analysis of pluripotent genes (<i>OCT4</i> , <i>NANOG</i> , and <i>SOX2</i> at Days 3 and 5); osteogenic genes (<i>ALP</i> , <i>RUNX2</i> , <i>OCN</i> , <i>OSP</i> , and <i>DSPP</i> at Days 7 and 14); ICC staining for YAP; migration test using a transwell assay In vivo: subcutaneous transplantation in nude mice followed by H&E staining	The DPGC promoted nuclear translocation of the YAP protein in encapsulated DPSCs, indicating enhanced stemness. Additional experiments confirmed improved cell viability, migration, spreading, and angiogenic potential. In vivo studies have shown that micro porous DPGC facilitated effective dental pulp tissue regeneration, characterized by organized dentin formation and vascular-like structures that closely resemble native teeth.	[123]
DLP	Cell-loaded microspheres	Dental pulp	–	hDPSCs	GelMA	In vitro: live/dead staining; ICC staining (F-actin, Ki-67, SOX2, OCT4, DSPP, CD31, GAP43); single-cell sequencing; RT-qPCR (<i>DMP-1</i> , <i>VEGF-α</i> , <i>GAP43</i> , <i>DSPP1</i> , <i>ANGPT1</i> , <i>MAP2</i>) In vivo: subcutaneous transplantation in nude mice followed by H&E staining, IHC (CD31, MAP2, DSPP), and RT-qPCR (<i>DMP-1</i> , <i>VEGF-α</i> , <i>CD31</i> , <i>vWF</i> , <i>GAP43</i> , <i>MAP2</i>); orthotopic transplantation into the root canal of minipigs followed by H&E staining	The microspheres exhibited enhanced stemness and increased potential for multidirectional differentiation, including angiogenic, neurogenic, and odontogenic lineages. In heterotopic implantation tests on nude mice, ICC staining for CD31, MAP2, and DSPP indicated the formation of vascular, neural, and odontogenic tissues. In minipig models, the constructs supported the regeneration of highly vascularized dental pulp tissue with uniformly organized odontoblast-like cells within the root canals of incisors.	[124]

STRO-1: stromal cell surface marker-1; RUNX2: runt-related transcription factor 2; COL1A1: collagen type I alpha 1 chain; HA: hyaluronic acid; CCK-8: cell counting kit-8; DDMp: demineralized dentin matrix particle; ADM: A: G: acellular dermal matrix/alginate/gelatin sodium; VEGF-A(α): vascular endothelial growth factor A(α); AG: alginate/gelatin; i-PRF: injectable platelet-rich fibrin; iPAG: i-PRF/alginate/gelatin; CD31 (PECAM1): cluster of differentiation 31 (platelet/endothelial cell adhesion molecule 1); RAOEC: rat aortic endothelial cell; HERS: Hertwig's epithelial root sheath; COL1: collagen I; OPG: osteoprotegerin; HIF1 α : hypoxia-inducible factor 1 alpha; GAPDH: glyceraldehyde-3-phosphate dehydrogenase; HIF1: hypoxia-inducible factor 1; LAP: lithium phenyl-2,4,6-trimethylbenzoylphosphonate; DAPI: 4',6-diamidino-2-phenylindole; DPGC: dental pulp guidance construct; OCT4: octamer-binding transcription factor 4; SOX2: SRY-box transcription factor 2; YAP: yes-associated protein; GAP43: growth-associated protein 43; ANGPT-1: angiopoietin-1; DSPP1: dentin sialophosphoprotein 1; MAP2: microtubule-associated protein 2; vWF: von Willebrand factor. Other full names for abbreviations can be found in Table 1. 1 bar=100 kPa

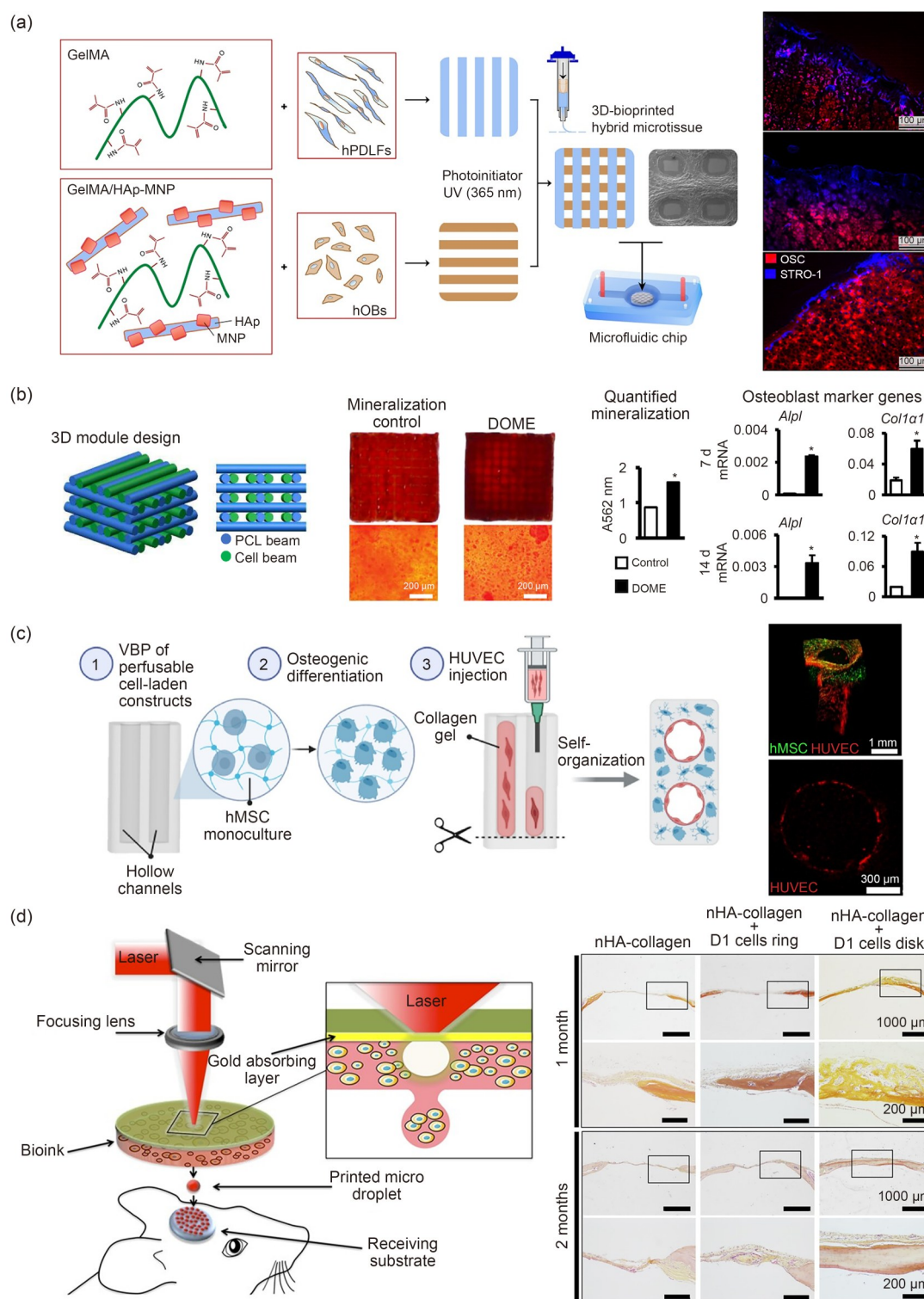


Fig. 3 Application of cutting-edge bioprinting technologies for craniofacial tissue reconstruction. (a) Extrusion-based 3D bioprinting of a periodontal ligament (PDL)–alveolar bone biointerface tissue construct. The upper PDL layer is composed of gelatin methacryloyl (GelMA)-encapsulating human periodontal ligament fibroblasts (hPDLFs), while the lower alveolar bone layer is fabricated using GelMA/hydroxyapatite-magnetic nanoparticle (HAP-MNP) bioinks containing human osteoblasts (hOBs). The construct was cultured within a microfluidic chip system. Adapted from [112], with permission from Chongqing Medical University. (b) A 4D-bioprinted bone construct with functional transformation through developmental osteogenic microenvironment (DOME). Adapted from [155], licensed under CC BY 4.0. (c) Mesenchymal stromal cell (MSC)-laden bone constructs with perfusable vessel-like channels seeded with human umbilical vein endothelial cells (HUVECs) fabricated using volumetric bioprinting (VBP). Adapted from [162], licensed under CC BY 4.0. (d) Laser-assisted in situ bioprinting applied to calvarial bone regeneration. Adapted from [107], licensed under CC BY 4.0

complex tissue structures within a bulk of bioink; and in situ bioprinting, which enables tissue constructs to be printed directly at the site of injury.

3.2.2 Four-dimensional bioprinting

The emergence of a fourth dimension in the fabrication of 3D scaffolds dates back to 2013, when Skylar Tibbitts showed that the properties of scaffolds formed using 3D printing could undergo programmed changes over time under the influence of external factors [125]. Accordingly, 4D printing is defined as the time-dependent evolution of the shape, properties, and functionality of a 3D-printed construct when exposed to specific stimuli. This technology relies on the use of so-called smart or stimuli-responsive materials designed to change their properties in response to external stimuli, which can be physical, chemical, or biological [126, 127]. Physical stimuli include UV light [128, 129], humidity [130], magnetic fields [131, 132], electric fields [133], and temperature changes [134, 135]. Chemical stimuli encompass substances that induce variations in pH levels [136], whereas biological stimuli primarily involve enzymes [137].

In maxillofacial surgery, 4D printing is widely used to fabricate stimulus-sensitive bone grafts, which can adapt to irregular defect geometries [138–141]. For example, Wang et al. [142] developed a 4D printing method to generate photothermally responsive scaffolds for the regeneration of critically sized, irregularly shaped bone defects. Upon exposure to near-infrared irradiation, the scaffold was heated to approximately 45 °C, increasing its shape pliability. After implantation, cooling to 37 °C enabled the scaffold to retain a stable shape. This approach was successfully applied to the regeneration of calvarial bone defects in a rat model. Using a similar 4D printing strategy, subsequent studies reported the effective regeneration of cranial bone defects in rabbits in vivo, and its potential was also shown in ex vivo models of rabbit tibia, mandible, and teeth [143]. Recently, researchers have developed a self-bending scaffold for the regeneration of calvarial bone defects. The bending resulted from a three-stage UV crosslinking process of the hydrogel, which served as the basis for the structure. In vivo studies in a rat cranial defect model revealed successful bone regeneration accompanied by new bone formation [144]. Moreover, 4D printing technology can be used to regenerate bones and the tissue surrounding them—the periosteum. Liu et al. [145] fabricated a bilayer periosteum-mimicking construct that can adapt to patient-specific bone geometry. This construct consisted of an MSC sheet and a programmed hydrogel produced using DLP-based 4D printing. Its regenerative potential was validated in a murine calvarial bone defect model.

Notably, 4D bioprinting uses the same fundamental principles as 4D printing, with the key difference being the

addition of a cellular component. Generally, 4D bioprinting relies on two strategies: shape transformation and functional transformation. Shape transformation involves the use of smart, stimuli-responsive materials that deform when exposed to external stimuli, thereby altering the overall shape of the construct. Conversely, functional transformation focuses on creating a dynamic microenvironment that promotes tissue maturation, including cell differentiation and patterning [146, 147]. Current research efforts in 4D bioprinting have focused on the fabrication of muscle [148, 149], cartilage [150, 151], and vascularized tissues [152, 153]. While 4D bioprinting remains an emerging technology, several pioneering studies have shown its potential for craniofacial biofabrication. Notably, Betsch et al. [154] reported a drop-on-demand 4D bioprinting method for cartilage regeneration that allowed for the specific alignment of collagen fibers within the engineered tissue. In the study, bioinks composed of agarose and type I collagen were used to encapsulate human knee articular chondrocytes. Additionally, iron nanoparticles were incorporated into the bioinks. Under the influence of a magnetic field, the nanoparticles embedded within the hydrogel were mobilized, leading to the parallel alignment of collagen fibers. This process exemplifies the shape transformation strategy in 4D bioprinting. Although this technology was initially employed to regenerate cartilage, the researchers emphasized its potential application in recreating dental pulp, where interodontoblastic collagen fibers are oriented perpendicular to the dentin. In another study on the 4D bioprinting of bone tissue equivalents, a functional transformation strategy was employed. The researchers fabricated two-component constructs using polycaprolactone (PCL) ink, along with bioinks containing 20% gelatin methacryloyl (GelMA)-encapsulated MLO-Y4 (osteoblasts (OBs)) and ST2 (bone marrow stromal cells). Notably, Wnt signaling was activated in MLO-Y4 cells using the GSK-3 β S33 inhibitor. The study reported that the activated MLO-Y4 cells stimulated the proliferation and osteogenic differentiation of ST2 cells within the construct, thereby creating a developmental osteogenic microenvironment (DOME), as described by the authors. This indicates that external stimulation can induce functional transformation within a bioprinted tissue construct [155].

3.2.3 Volumetric bioprinting

Bernal et al. [156] first introduced VBP as an innovative bioprinting approach in 2019. Unlike conventional layer-by-layer bioprinting methods, which deposit materials sequentially, VBP employs spatially modulated light patterns to solidify an entire volume of photosensitive bioink simultaneously within seconds. This technique allows for higher printing speeds and greater precision in fabricating complex tissue constructs, achieving resolutions ranging from

approximately 40 to 50 μm , with printing speeds sufficient to generate centimeter-scale objects within 10–120 s [156, 157]. The VBP process generally involves rotating and exposing a container filled with a photopolymerizable gel to a series of 2D light projections. These projections, generated using a DLP modulator or a laser source, represent object views from multiple orientations and collectively establish a 3D light-dose distribution. Upon exposure to light, the photosensitive molecules in the bioink undergo a chemical reaction, causing the material to solidify at specified locations. By precisely controlling the intensity and distribution of light, intricate 3D structures can be formed within the gel matrix. After fabrication, the unpolymerized liquid resin surrounding the solidified part was removed through washing, thereby revealing the final printed structure [156, 158]. As with other bioprinting techniques, VBP requires biocompatible materials that support cell adhesion and viability. To achieve high printing resolution, materials used in volumetric additive manufacturing must also exhibit viscosities exceeding 10 Pa·s. Additionally, photoresin transparency is a crucial requirement, as projected light patterns must penetrate the entire volume to ensure uniform polymerization. This transparency is primarily determined by the concentration of the photoinitiator, given that the most commonly used synthetic and natural polymers exhibit minimal light absorption within the 365–405 nm wavelength range typically employed in light-based printing [156].

VBP is a cutting-edge biofabrication technology that is only beginning to be applied to generate tissue and organ substitutes. For example, it has been used to fabricate liver tissue equivalents using organoids as building blocks [159] and to model interactions between pancreatic β -cells and adipocytes [160]. Falandt et al. [161] pioneered 4D VBP by generating intricate 3D patterns of thiol-functionalized dyes and biomolecules within centimeter-scale hydrogels. Through the covalent linking of vascular endothelial growth factor (VEGF), the study reported controlled endothelial cell growth and adhesion within biofunctionalized regions of the hydrogel construct. Efforts are ongoing to generate bone tissue equivalents using VBP, an approach of particular relevance to cranio–maxillofacial surgical applications. For instance, Gehlen et al. [162] developed a bone construct using tomographic VBP, employing 5% GelMA bioresin as the bioink and incorporating 3D co-cultures of human MSCs and HUVECs. The resulting construct exhibited osteogenic differentiation of MSCs, as evidenced by the expression of osteoblastic and osteocytic markers, including the human pro- α 2(I) collagen gene (*COL1A2*), osteocalcin (*OCN*), podoplanin (*PDPN*), and dental matrix protein-1 (*DMP-1*). Additionally, the authors fabricated a perfusable MSC-laden bone construct containing vessel-like channels, which were subsequently seeded with HUVECs. Collectively, these findings suggest that the applied VBP strategy holds promise

for generating vascularized bone tissue substitutes. In a recent study aimed at recreating the osteoid niche using VBP, researchers utilized bioinks composed of norbornene–norbornene functionalized gelatin in combination with thiolated gelatin, exploiting a step-growth mechanism. The printed constructs exhibited high cell viability and effective osteogenic differentiation of DPSCs [163].

3.2.4 In situ bioprinting

In situ bioprinting, or intraoperative bioprinting (IOB), is a technique that enables the direct deposition of bioinks onto an injured site within the body. First introduced in 2007 as a translational bioprinting approach, in situ bioprinting offers several advantages over other bioprinting methods. Eliminating the need for an in vitro culturing stage reduces the risk of contamination of printed tissue. The technology also allows real-time parameter adjustments during printing, enabling modifications to the shape and size of the constructs based on the characteristics of the defect. Additionally, maturation of the printed structure occurs in vivo under physiological conditions, resulting in constructs with properties similar to native tissue [17, 164].

There are two primary types of in situ bioprinting systems: robotic-assisted and handheld. Robotic-assisted in situ bioprinting utilizes robotic devices, such as robotic arms, to deposit bioinks. This technology enables the simultaneous printing of multiple high-resolution biomaterials and supports the fabrication of tissues with complex organization or large-scale constructs. Despite these advantages, robotic-assisted systems are limited by low mobility and high equipment costs [165, 166]. Conversely, handheld bioprinting employs portable, compact devices that can be deployed in a variety of settings, from conventional operating rooms to field surgeries and even space missions. Although these systems typically provide lower printing resolution and support a narrower range of biomaterials, their manual control allows for real-time adjustments tailored to the specific features of the damaged tissue [167].

In situ bioprinting is currently employed to repair various tissues and organs, including skin [168, 169], muscle [170, 171], and cartilage [172, 173]. In maxillofacial surgery, its application is still in its early stages (Table 3); however, several studies have focused on bone reconstruction using in situ bioprinting, which is particularly relevant for craniofacial biofabrication. For example, Mostafavi et al. [174] utilized a partially automated handheld melt-spun 3D printing pen along with bioinks composed of PCL, zinc oxide (ZnO), and hydroxyapatite (HAp) microparticles for in situ printing scaffolds aimed at repairing bone tissue, including calvarial bone defects. In vitro analysis of the printed scaffolds populated with MSCs exhibited sustained cell viability and effective osteogenic differentiation, as indicated by alkaline

Table 3 In situ bioprinting of craniofacial tissues

In situ bioprinting	Construct design	Tissue	Bioprinting parameters	Cells	Hydrogel/Scaffold composition	Construct analysis	Outcomes	Ref.
DBB (drop-on-demand)	Cell-laden hydrogel deposited within the root canal	Dental pulp	Valve orifice: 300 µm; pressure: 0.25–2.5 bar	hDPCs (3×10 ⁶ cells/mL) and HUVECs (3×10 ⁶ cells/mL)	0.2% Type I collagen and 0.5% agarose	Ex vivo: F-actin staining (14 d); IHC for CD31 (14 d)	Evidence of successful vasculogenesis within bioprintable bioinks confined to the root canal was observed.	[175]
EBB	Cuboidal construct (10 mm×10 mm×3 mm) with two grid patterns. The foundation of the construct is SSAM bioinks with encapsulated bone marrow-derived hMSCs. Cell-only bioinks, consisting of human adipose tissue-derived stromal cells and HUVECs, are printed onto this base to promote vascularization of the construct.	Calvarial bone	SSAM bioinks: 22G or 20G nozzles Cell-only bioinks: 25G nozzles	hBMSCs; human adipose tissue-derived stromal cells; HUVECs	SSAM: 2% oxidized alginate in DMEM, 0.05% photoinitiator, RGD peptides+200 µL supersaturated CaSO ₄ (0.21 g/mL)	In vitro: osteogenic differentiation and further analysis (28 d), RT-qPCR (<i>CDH5</i> , <i>OCN</i>), ALP activity, Alizarin Red S staining, CD31 immunostaining on mouse cadaver In situ bioprinting on (calvarial bone defect)	SSAM bioinks supported cell viability and differentiation and facilitated in situ bioprinting of vascularized tissue constructs within a mouse calvarial bone defect, providing mechanical stability and ensuring that the bioprinted constructs stay securely in place within the defect site.	[176]
EBB+DBB	Composite construct consisting of two cylindrical layers: a lower layer imitating bone and an upper layer replacing skin	Hard and soft tissues of the cranio-maxillofacial region (bones and skin)	EBB (bone): 23G nozzle; set-up temperature of 23 °C; robot arm speed of 400 mm/min; pressure of 80–140 kPa; layer thickness of 0.2 mm Dropjet-based (skin): 250 µm valve orifice; pressure of 126 kPa; 6 mm layers	rBMSCs (5×10 ⁶ cells/mL); rat dermal fibroblasts (1.33×10 ⁵ cells/mL)	HT-ink: 2% stocked CS+56% β-GP (9:1 =CS:β-GP)+9 mg/mL type I collagen (CS-β-GP: Coll=4:1)+freeze-dried collagen+HAP ST-ink: 2 mg/mL type I collagen+20 mg/mL fibrinogen (1:1)	In vitro: bone constructs—morphological characterization, mechanical characterization, live/dead staining (Days 1 and 7), SEM (Days 7, 14, and 21), F-actin staining, IHC (BSP, RUNX2), degradation study; skin constructs—mechanical characterization, live/dead staining, SEM (Days 1, 3, and 5), and F-actin staining In vivo in rats (in situ bioprinting): micro-CT, histology (H&E, Masson's trichrome), IHC (BSP, RUNX2, vimentin, loricrin), and vascularization analysis	Osteogenic HT construct achieved significant bone regeneration with approximately 80% coverage of calvarial bone defects within 6 weeks. ST construct accelerated full-thickness skin defect reconstruction, resulting in up to 60% wound closure within 6 d. The IOB approach was used to reconstruct hard and soft composite tissues in a stratified arrangement. IOB achieved approximately 80% skin wound closure in 10 d and 50% bone coverage area after 6 weeks.	[177]
EBB	Defect-shaped constructs (5 mm diameter×1 mm thickness)	Cranio-maxillofacial bones	Nozzle: 410 µm; set-up temperature: 23 °C; printing speed: 60 mm/min; pressure: 80 kPa	Ex situ delivery of the plasmid (PEI-pBMP-2); hMSCs (5×10 ⁶ cells/mL)	2% CS+56% β-GP (9:1=CS:β-GP)+9 mg/mL type I collagen (CS-β-GP:Coll=4:1)+freeze-dried collagen sponges+nHAP	In vitro: live/dead staining (Days 1 and 7); osteogenic differentiation and further analysis (Days 7 and 14); IHC (BSP, RUNX2); RT-qPCR (BSP, RUNX2, <i>OCN</i> , <i>COL1A1</i>) In vivo in rats (in situ bioprinting): micro-CT, histology (H&E, Masson's trichrome staining)	The plasmid (PEI-pBMP-2) loaded into a collagen-based bioink was delivered into critical-sized calvarial bone defects with IOB technology, allowing direct bioink deposition into the defects. The application of PEI-pBMP-2 loaded bioink significantly enhanced bone regeneration compared to both ex situ delivery and untreated control groups.	[178]

To be continued

Table 3 (continued)

In situ bioprinting	Construct design	Tissue	Bioprinting parameters	Cells	Hydrogel/Scaffold composition	Construct analysis	Outcomes	Ref.
EBB	Defect-shaped constructs (7 mm diameter×2 mm thickness)	Craniofacial bones	Nozzle: 410 µm; set-up temperature: 23 °C; printing speed: 400 mm/min; pressure: 80–140 kPa	rBMSCs (8×10 ⁵ cells/construct)	2% CS+56% β-GP (9:1=CS:β-GP)+9 mg/mL type I collagen (CS-β-GP:Coll=4:1)+freeze-dried collagen sponges+nHAp+CS nanoparticles with pBMP-2+aqueous form of pPDGF-B	In vitro: CCK-8 (Days 1, 4, 7, 10, and 14), calcium content assay, ELISA (BMP-2), and RT-qPCR (Days 7 and 14; <i>RUNX2</i> , <i>BMP-2</i> , <i>ALP</i> , and <i>OCN</i>) In vivo: in situ bioprinting of rats, micro-CT, histology (H&E, Masson's trichrome staining), IHC (<i>RUNX2</i>)	The intraoperatively bioprinted bone constructs containing controlled-release pDNAs achieved approximately 40% new bone tissue formation and around 90% bone coverage after 6 weeks, compared to about 10% new bone tissue and 25% over-all bone coverage in untreated empty defects.	[179]
EBB	Accellular scaffold in situ bioprinted using the PCL filament	Calvarial bone	–	hMSCs (30,000 cells/cm ² , for in vitro biocompatibility studies)	Filament composed of PCL, HAp, and ZnO	In vitro: live/dead staining, osteogenic differentiation and further analysis (Days 21, 28, and 35; ICC (OSP, BSP, <i>RUNX2</i> ; type I collagen), ALP activity, xylenol orange staining), in situ bioprinting on rat cadaver In vivo in mice (in situ bioprinting in a subcutaneous pocket): histology (H&E, Masson's trichrome staining), IHC (CD31, CD68)	In situ printing of the filaments achieved good adhesion of the scaffolds to surrounding tissues without raising the local temperature above 40 °C. The scaffolds supported cell growth, proliferation, and osteogenic differentiation. The incorporated ZnO particles effectively inhibited bacterial growth on the scaffold surfaces.	[174]
LAB	The constructs composed of discrete, dot-based deposits arranged on biopaper and fabricated in two geometries: a disk with 2 mm diameter and a ring with external and internal diameters of 3 mm and 2.1 mm, respectively	Calvarial bone	Printing speed: 300 µm/s; frequency: 1 kHz; laser energy: 27.5 µJ; gap: 1000 µm	Mouse bone marrow stromal precursor D1 cell line (120×10 ⁶ cells/mL)	Type I collagen (2 mg/mL)+1.2% nHAp	In vitro: resazurin-based assay (Days 1 and 8) In vivo in rats (in situ bioprinting): in vivo bioluminescence imaging, micro-CT, histological analysis - HES	The construct promoted cell viability and bone regeneration in a calvarial defect model in mice; spatial cell arrangements within the construct influenced the extent of bone tissue regeneration.	[107]

CD31: cluster of differentiation 31; SSAM: shear-thinning and self-healing alginate microgel; DMEM: Dulbecco's modified Eagle medium; RGD: arginylglycylaspartic acid; CDH5: cadherin 5; HT: hard tissue; CS: chitosan; β-GP: β-glycerophosphate disodium salt hydrate; Coll: type I collagen; ST: soft tissue; BSP: bone sialoprotein; *RUNX2*: runt-related transcription factor 2; PEI-pBMP-2: polyethylenimine-plasmid of bone morphogenetic protein 2; pPDGF-B: plasmid encoding platelet-derived growth factor-B; COL1A1: collagen type I alpha 1 chain; CCK-8: cell counting kit-8; CD68: cluster of differentiation 68. Other full names for abbreviations can be found in Table 1. 1 bar=100 kPa

phosphatase (ALP) activity and osteopontin (OSP) expression levels. Subcutaneous transplantation of these scaffolds into mice showed a normal immune response and induced vascularization, indicating the potential of this technology for restoring bone defects. In dentistry, *in situ* bioprinting has been applied to regenerate dental pulp using bioinks composed of agarose and type I collagen hydrogel combined with cells, including DPCs and HUVECs. A handheld drop-on-demand device was employed to deposit acellular bioinks into human tooth root canals *ex vivo*. Subsequently, cell-laden bioinks were cast into bovine tooth root canals, and after prolonged cultivation, effective vascularization within the root canals was observed. Future research will continue to explore these approaches using *in vivo* models [175]. Furthermore, Moncal et al. [177] developed a sophisticated IOB system for hard, soft, and composite tissues in the craniomaxillofacial region, integrating two bioprinting methods—EBB and DBB—with two types of bioinks: an osteogenic hard tissue bioink and a soft tissue bioink for skin regeneration. In a murine model of calvarial bone defect, the system achieved 80% bone tissue restoration within 6 weeks and 60% skin wound closure within 6 d. Additionally, the simultaneous use of both bioinks resulted in 50% closure of bone defects within 6 weeks and 80% wound healing within 10 d. This study presents an integrated approach to the regeneration of craniofacial and maxillofacial defects, as bone damage is almost always associated with compromised skin integrity. This *in situ* bioprinting system appears promising for future clinical translation. Recently, researchers have developed an *in situ* bioprinting protocol that promotes the immediate prevascularization of printed constructs. This two-stage approach first involved printing bioinks composed of alginate microgels containing human bone marrow-derived mesenchymal stromal cells (hBMSCs). In the second stage, cell-only vascular bioinks, comprising HUVECs and human adipose tissue-derived stromal cells, were printed on top. Incorporating cell-only bioinks has been shown to enhance the osteogenic differentiation of constructs *in vitro*. *In situ* bioprinting into a mouse calvarial bone defect showed the formation of a mechanically stable structure [176].

Advanced bioprinting technologies, such as 4D bioprinting, VBP, and *in situ* bioprinting, are revolutionizing regenerative medicine. Four-dimensional bioprinting introduces dynamic, stimuli-responsive constructs designed to undergo controlled changes in shape and function over time, offering promising applications in craniofacial bone regeneration and patient-specific tissue customization. VBP enables the rapid fabrication of complex centimeter-scale constructs with high resolution, facilitating the generation of organoids and tissue models relevant to maxillofacial research and reconstruction. *In situ* bioprinting allows for direct, real-time deposition of bioinks at defect sites, enabling personalized

repair of tissues such as bone, cartilage, and dental pulp. Collectively, these innovative approaches have significant potential to enhance clinical outcomes by providing more precise, functional, and adaptable solutions for craniofacial tissue reconstruction.

3.3 Microfluidic technologies for cell-based craniofacial biofabrication

Over the past decade, the interdisciplinary technology known as “organ-on-chip” has rapidly advanced, bringing researchers significantly closer to the development of physiological models of tissues and organs *in vitro* [180]. This technology combines achievements in tissue engineering and microfluidics to recreate key aspects of the native microenvironment in which organs function *in vivo*. An organ-on-chip system includes a microfluidic chip integrated with cells or tissues. External forces, such as shear stress and tension compression, can also be applied to cellular components within the system [181]. The channels of a microfluidic chip act as artificial blood vessels, delivering a culture medium enriched with nutrients and oxygen to the cells while removing metabolites and debris. Microfluidic chips designed for organ-on-chip manufacturing represent complex systems with different compartments, such as reservoirs, chambers, and microchannels. These systems may also incorporate functional components, including valves, mixers, and pumps, which can move the liquid phase in a fixed mode [182]. One of the remarkable features of organ-on-chip technology is its ability to fine-tune system parameters—such as concentration gradients, biomechanical forces and stimuli, and spatial cell distribution—thereby regulating biological processes. Furthermore, the compact design of the system allows for the integration of biosensors, enabling the real-time monitoring of processes [181]. This capability is particularly valuable for examining tissue and organ responses to therapeutic compounds and for modeling disease mechanisms. Currently, organ-on-chip technology allows for *in vitro* modeling of a wide range of organs, including the lung [183], gut [184], kidney [185], and skin [186]. In dentistry and maxillofacial surgery, organ-on-chip platforms—such as oral mucosa-on-chip, PDL-on-chip, and tooth-on-chip—are used to model relevant structures. These models enable the study of tissue physiology and host–microbe interactions, as well as the evaluation of therapeutic agents and oral care formulations, such as mouthwash and toothpaste. Microfluidic devices designed for craniofacial organs-on-chips are applications that are most commonly fabricated from polydimethylsiloxane (PDMS), although their structural designs may vary. The simplest configuration is the one-chamber chip, which consists of a single culture chamber connected to microfluidic channels. Parallel chamber chips consist of two or more culture

chambers separated by semipermeable membranes and are particularly useful for modeling multilayered structures and tissue interfaces. Conversely, serial chamber chips contain several culture chambers connected in series through microchannels and are used to recreate organ systems [182]. Perfusion of the culture medium through the microchannels of a microfluidic chip can be performed using various approaches or, in some cases, may be absent entirely. External pumps are often used; however, the circuit through which the medium flows can be either closed or open. In specialized systems, mass transfer may be driven by gravitational forces, as observed when installing the chip on a rocket platform. In static systems, the transport of nutrients, oxygen, and metabolites relies solely on diffusion [187]. Figure 4 presents examples of various craniofacial organ-on-chip systems, highlighting their designs and applications.

3.3.1 Oral mucosa-on-chip and gingiva-on-chip

Oral mucosa-on-chip models are typically generated using oral mucosa tissue equivalents, as described above. Although microfluidic device designs may vary, the overall architecture of the mucosa equivalent is generally consistent. A layer of fibroblasts encapsulated in a collagen matrix represents the dermal component of the mucosa, whereas an overlying keratinocyte layer recreates the epidermis [188]. An ALI is employed to induce stratification of keratinocytes in the oral mucosa-on-chip system [189]. This system serves as a convenient platform for assessing the toxicity of dental biomaterials and oral care products. To test the dental monomer 2-hydroxyethyl methacrylate (HEMA), an oral mucosa-on-chip system was developed comprising a microfluidic chip with a single culture chamber connected through pores to parallel channels. Gingival fibroblasts encapsulated in a collagen matrix were cultured in the culture chamber, and a layer of keratinocytes was positioned in the pores on the apical side of the chip. Studies have shown that this microfluidic system exhibits greater sensitivity than conventional cell monolayers for assessing cell viability after exposure to HEMA [190]. In another study, a mucosa-on-chip system composed of bilayer tissue equivalents—gingival fibroblasts and keratinocytes cultured across four culture chambers—was used for irritation testing with commercial mouthwashes and for permeation studies using dental anesthetics lidocaine and articaine. The resulting microphysiological system showed its effectiveness for downstream applications [191].

Oral mucosa-on-chip is also a valuable model for studying the biology of the oral microbiome and its interactions with oral tissues. Microorganisms are essential inhabitants of the oral cavity; most maintain commensal relationships with the host, while some act as opportunistic pathogens. Disruption of the balance between normal flora and

pathogens can trigger disease progression [192]. Bacteria species such as *Porphyromonas gingivalis* (*P. gingivalis*) [193], *Akkermansia muciniphila* (*A. muciniphila*) [193], *Streptococcus mutans* [188], *Streptococcus oralis* [194], and *Fusobacterium nucleatum* [194] are commonly used to examine host–microbe interactions in mucosa-on-chip systems. Hu et al. [193] developed an oral mucosa-on-chip model to study periodontitis induced by the pathogenic bacterium *P. gingivalis*. Their system consisted of a four-chamber microfluidic chip comprising compartments for the gingival sulcus, epithelium, connective tissue, and blood vessels. The addition of *P. gingivalis* inhibited immune cell migration and decreased cluster of differentiation 14 (CD14) expression in THP-1 cells, whereas adding the probiotic *A. muciniphila* restored immune response.

Microfluidic devices are widely used to model tissue barriers, including the gingival epithelium–capillary interface present in oral tissues. This interface is critical for oral health, contributing to barrier function, homeostasis maintenance, and immune response regulation. However, in periodontitis, the integrity of this interface can be compromised. To examine these mechanisms, Jin et al. [195] developed an organ-on-chip model simulating the gingival epithelium–capillary interface under periodontitis conditions. Their microfluidic chip consisted of two parallel chambers separated by a porous membrane, with human gingival epithelial cells seeded on the upper surface and HUVECs on the lower surface, forming an epithelial–endothelial bilayer. Subsequent analysis using microscopy, viability assays, transepithelial electrical resistance measurements, and permeability tests revealed the functionality, integrity, and suitability of the system for drug testing. Using LPS and tumor necrosis factor alpha (TNF- α), researchers have modeled inflammatory conditions to recreate a periodontitis microenvironment. In a similar study, Gard et al. [196] seeded gingival fibroblasts and keratinocytes on the upper membrane surface and microvascular endothelial cells on the lower surface to construct three-layered mucosa equivalents within a multiarray chip system. Furthermore, they induced inflammation using interleukin-1 beta (IL-1 β) and TNF- α , resulting in increased secretion of proinflammatory cytokines and disruption of barrier function, indicating that the system can be used to screen new therapeutic targets for periodontitis. Another study employed an oral epithelium barrier-on-chip to identify the role of mechanical forces in maintaining epithelial barrier integrity [197].

3.3.2 Tooth-on-chip

Tooth-on-chip systems integrate key anatomical structures, such as dentin, pulp, and the entire tooth. Similar to oral mucosa-on-chip models, tooth-on-chip systems are valuable for evaluating dental materials and drugs. For example, one

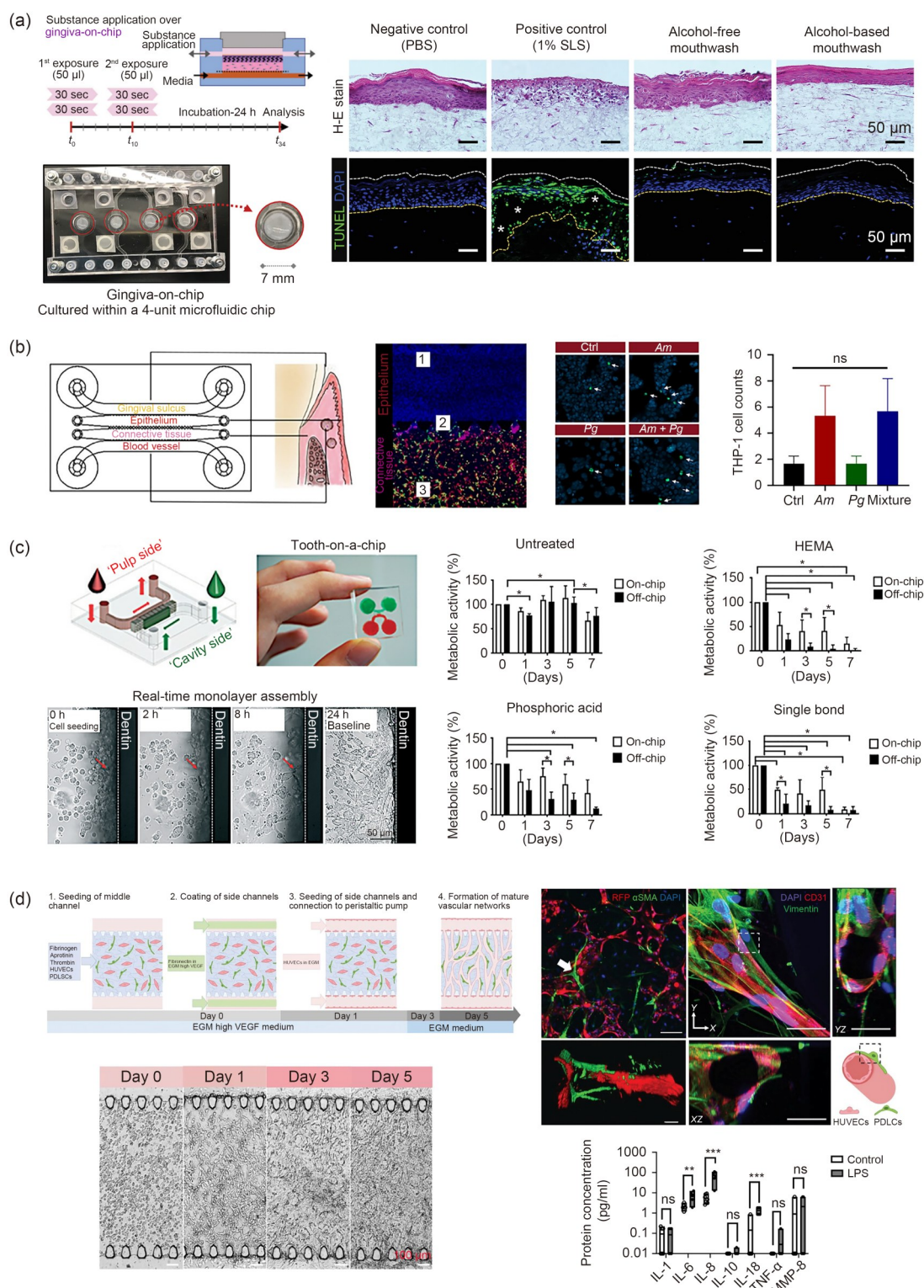


Fig. 4 Organ-on-chip approaches to recreate oral tissues. (a) Gingiva-on-chip used for testing oral care products and drugs. Adapted from [191], licensed under CC BY-NC. (b) Gum-on-chip for studying host–microbe interactions. The interface between epithelial (blue) and connective (red) tissues is recreated, showing that *Porphyromonas gingivalis* (Pg) inhibits THP-1 cell (green) migration, whereas *Akkermansia muciniphila* (Am) restores it. Adapted from [193], licensed under CC BY-NC. (c) Tooth-on-chip system for evaluating dental biomaterials. Two parallel chambers are separated by a dentin fragment covered with stem cells from the apical papilla (SCAPs). Adapted from [198], with permission from The Royal Society of Chemistry. (d) Periodontal ligament (PDL)-on-chip for modeling and studying periodontal diseases. The central chamber contains cells embedded in fibrin gel; side channels are coated with fibronectin and seeded with human umbilical vein endothelial cells (HUVECs). The system is connected to a peristaltic pump, and a mature vascular network forms by Day 5. Adapted from [204], licensed under CC BY 4.0

study developed a tooth-on-chip to test dental materials, such as HEMA, phosphoric acid, and Adper-Scotchbond™. The microfluidic chip consisted of a culture chamber with two parallel channels separated by a dentin fragment, which divided the chamber into two isolated sections. Stem cells from the apical papilla (SCAPs) predifferentiated into odontoblasts were seeded on one side of the dentin, and the culture medium flowed through a parallel channel on the same side. A medium containing the test materials was supplied through the channel on the opposite side. This configuration allowed the side with cells and culture medium flow to replicate the dental pulp, with a layer of dentin with odontoblasts positioned above it, whereas the opposite side mimicked oral cavity conditions. The authors concluded that the resulting model was physiologically close to the pulp–dentin interface of a native tooth and can be used as a relevant test system [198]. Subsequently, the same group developed a similar system using DPSCs instead of SCAPs to evaluate calcium silicate cements (CSCs)—biomaterials used for tooth pulp and dentin regeneration. The study assessed the effects of three different CSCs on the viability and proliferation of DPSCs, the pH of the surrounding microenvironment, and transforming growth factor beta (TGF- β) secretion within the pulp chamber. The resulting system exhibited functionality and efficiency for the intended applications [199].

Transplantation of SCs and tissue constructs into the root canal is a novel approach to regenerate dental pulp. However, the conical shape of the root canal may limit vascularization due to the limited space. To explore vascularization under these constraints, a research group developed a microfluidic model of the root canal. GelMA-based scaffolds with conical microchannels of different sizes were placed in culture chambers, and HUVECs and SCAPs were seeded into the microchannels at different ratios. The authors found that optimal conditions for angiogenesis—defined by the highest average sprout length—occurred in tapering microchannels using a 1:1 ratio of HUVECs to SCAPs, combined with a 0.04 conical channel and a 5% GelMA scaffold [200].

Dentin is a mineralized hard tissue that provides the teeth with mechanical strength. Dentin formation occurs through the deposition of mineralized ECM by specialized cells known as odontoblasts. Odontoblasts are composed of two main parts: the body and the process. The cell body is located at the periphery of the dental pulp, while the process extends into the dentin, within structures known as dentin tubules [201]. Niu et al. [202] developed a microfluidic chip that recreated key *in vivo* features of the odontoblast microenvironment. Their microfluidic device included two parallel culture chambers interconnected by several perpendicular microchannels. Odontoblasts were seeded into one chamber, from which their cellular processes extended into microchannels, imitating the architecture of dentin

tubules. The authors revealed that the processes formed on the chip were morphologically similar to those observed in native tissues and expressed the marker protein Aquaporin-4 (AQP4). This system was the first *in vitro* platform to successfully recreate odontoblast processes. In a separate study, another dentin-on-chip system was developed using a GelMA-based hydrogel scaffold with encapsulated SCAPs. The researchers reported that the cells maintained high viability and uniform distribution within the hydrogel matrix. Additionally, the cells were able to differentiate into odontoblasts, with differentiation efficiency peaking at a GelMA concentration of 5% [203].

3.3.3 Periodontal ligament-on-chip

PDL is an important component of the periodontal complex, as it anchors the tooth to the alveolar bone. In periodontitis, progressive destruction of the PDL compromises tooth support and subsequently leads to tooth loss. Svanberg et al. [204] were the first to develop a PDL-on-chip model to study its inflammatory pathology under microfluidic conditions. Their microfluidic device included three parallel microchannels separated by micropillars. The middle channel was filled with fibrin hydrogel containing PDLSCs and HUVECs, whereas HUVECs were seeded along the walls of the lateral channels. After 5 d of culture, a branched vascular network formed, imitating the vessels of the native PDL. Inflammatory conditions were induced through the introduction of LPS, resulting in increased secretion of proinflammatory cytokines and upregulation of the adhesion molecules necessary for immune cell recruitment. Mishra et al. [205] introduced a simplified PDL model. In this system, PDLSC spheroids were placed into microwells within a multichamber microfluidic chip. The authors evaluated the effect of fluid flow on spheroid morphology, cell viability, and osteogenic differentiation. The resulting platform provided a physiologically relevant system with controlled fluid flow and showed potential utility for biomaterial testing and studies of host–microbe interactions.

Microfluidic organ-on-chip technologies have emerged as powerful and versatile tools for advancing craniofacial biofabrication. By enabling precise control over cellular microenvironments, mechanical stimuli, and biochemical gradients, these systems facilitate the *in vitro* reconstruction of complex tissue interfaces and organ-specific functions. The development of models such as oral mucosa-on-chip, tooth-on-chip, and PDL-on-chip has enhanced the current understanding of tissue physiology and provided valuable platforms for testing dental materials, therapeutic interventions, and regenerative strategies. As these microfluidic systems continue to evolve with improved design complexity and the integration of biosensors, they hold significant promise for personalized medicine and disease modeling. Ultimately,

the integration of microfluidics with biofabrication represents a critical step toward more accurate, efficient, and ethically responsible approaches to craniofacial tissue regeneration and treatment.

4 Future challenges and advancements

Craniofacial and oral cell-based biofabrication holds immense promise for improving dentistry and maxillofacial surgery, with the potential for personalized, functional, and aesthetically optimized tissue regeneration and modeling. However, several challenges must be addressed before these innovative approaches can be effectively translated from laboratory research to clinical practice. The craniofacial region comprises diverse tissue types—including bone, cartilage, muscle, and skin—each with unique structural and functional properties. Replicating this intricate architecture requires advanced bioprinting techniques capable of precise, multimaterial, multicellular fabrication. The development of multilayered constructs that imitate not only individual tissues but also their complexes represents the future of biofabrication and tissue engineering. Nevertheless, research aimed at achieving this goal is already underway. For example, this review discusses the *in situ* bioprinting of constructs for bone and skin regeneration in the maxillofacial region, which consist of multiple layers with different hydrogel compositions and include various cell types [177]. From a structural standpoint, the periodontium exhibits a similarly sophisticated “sandwich” architecture, in which the PDL, cementum, and alveolar bone form a multilayered complex surrounding the tooth. To recreate this architecture *in vitro*, bioprinting with bioinks of different compositions is used [112], or fabricating individual tissue components separately and subsequently assembling them [122]. Additionally, combining two or more distinct bioprinting technologies for the fabrication of complex tissue-like constructs enables the selection of the most suitable method for each specific tissue type. This combined approach makes it possible to account for the specific characteristics of each structural element within the tissue complex [122, 177].

Vascularization—the formation of functional blood vessel networks within engineered tissues—is one of the most significant challenges in bioprinting and regenerative medicine. Achieving effective vascularization is essential for the supply of nutrients and oxygen and for the removal of waste products, all of which are vital for the survival, growth, and functionality of thick or structurally complex tissue constructs. Without proper vascular networks, bioprinted tissues risk necrosis and eventual failure after implantation. In native tissues, blood vessels form an intricate network that ensures cellular viability and tissue homeostasis [206]. The craniomaxillofacial region and soft oral tissues, such as

dental pulp and PDL, are highly vascularized to support their complex structures and functions. Successful vascularization of engineered constructs enhances tissue integration with the host vasculature, accelerates healing, and improves long-term functional outcomes. Accordingly, several common strategies have been developed to promote vascularization in bioprinted craniofacial and oral tissues. One established strategy for promoting vascularization involves embedding endothelial cells, such as HUVECs, directly within bioinks, which can lead to the formation of capillary-like structures inside bioprinted constructs or organ-on-chip systems [175, 176, 195, 200, 204, 207]. Furthermore, incorporating growth factors, including VEGF and platelet-derived growth factor (PDGF), into bioinks stimulates blood vessel formation by promoting endothelial cell proliferation and migration [179, 208, 209]. Another widely used approach is the application of microfluidic devices and perfusion bioreactors, which enable the generation of artificial vascular networks that supply nutrients and oxygen to engineered tissue equivalents [112, 210]. Although these approaches have been employed for a long time and have exhibited measurable effectiveness, they are sometimes insufficient for achieving full vascularization of bioprinted tissues. Therefore, current research efforts are increasingly focused on developing more advanced strategies for vascular network formation. Advanced bioprinting techniques, such as VBP, allow for the direct fabrication of predefined microchannels within tissue constructs that structurally mimic native blood vessels. These channels can subsequently be lined with endothelial cells to establish functional vasculature within the engineered tissue [162]. Another promising approach involves using multicellular spheroids and organoids containing endothelial cells as vascularization units [206]. Organoids derived from endothelial cells and DPSCs [74–76] or PDLSCs [211] have emerged as valuable tools for the regeneration and vascularization of dental pulp and PDL tissues. Incorporating vascular organoids into bioinks for the bioprinting of dental and craniofacial tissues is therefore expected to facilitate more complete and effective vascularization of tissue-engineered constructs.

Combining two cell-based biofabrication strategies—organoids and 3D bioprinting—is a relatively recent and emerging approach. It seeks to generate functional tissues by using 3D miniorgans as building blocks. Within this framework, employing organoids or microtissues, rather than cell suspensions, as the cellular components of bioinks enables more controlled and physiologically relevant morphogenesis of printed tissue equivalents. This advantage is due to the ability of organoids to self-organize and form an ordered tissue architecture. Additionally, incorporating organoids into bioinks addresses key challenges in 3D bioprinting by enabling relatively high cellular density and providing a physiological microenvironment that enhances cell viability and metabolic activity [13, 212, 213]. To date, bioprinting

approaches that utilize organoids or microtissues have been successfully applied to generate tissue equivalents of the liver [159], cartilage [214], and skin dermis [215]. Recently, Duan et al. [216] generated prevascularized organoids composed of MSCs, HUVECs, and osteogenic microparticles, and subsequently employed these constructs for 3D bioprinting of a cranial bone equivalent. In vivo animal experiments revealed effective vascularization and osteogenesis following the transplantation of the organoid-laden hydrogel construct into a bone defect. In this system, organoids serve simultaneously as units of vascularization and as building blocks for bone formation. Overall, the use of organoids in bioprinting represents a promising strategy that is likely to continue evolving, with expanding applications anticipated in craniomaxillofacial biofabrication.

Another critical challenge in craniofacial biofabrication is developing approaches to generate patient-specific tissue constructs with complex geometries. Personalization of tissue constructs is particularly important in reconstructing facial bones and skulls, which vary in shape and size depending on factors such as sex, age, and individual anatomical characteristics. The prebioprinting stage involves designing a CAD model of the target tissue and selecting suitable biomaterials. Imaging techniques, such as computed tomography (CT), cone beam CT, and magnetic resonance imaging, are used to capture patient-specific anatomy. These images, usually in digital imaging and communications in medicine format, are then converted into a standard tessellation language format for input into a 3D printer or bioprinter [217]. This approach is readily implementable in cell-free biofabrication, where 3D printing with sufficiently rigid biomaterials, such as bioceramics or titanium, enables stable construct shapes and accurate reproduction of the patient-specific 3D model. Researchers are developing personalized 3D-printed implants for craniofacial bone defects [218], including the zygomatic [219, 220], mandibular [221, 222], and skull [223, 224] bones, as well as the TMJ [225]. However, when bioprinting with bioinks containing live cells within a hydrogel, additional limitations arise. Factors such as bioink viscosity, printing resolution, crosslinking processes, and potential reductions in cell viability during prolonged or complex manipulations constrain the complexity of the constructs that can be achieved. Consequently, current bioprinting efforts are mostly limited to simple, small-scale structures, often possessing basic cubic or cylindrical geometries.

The post-processing maturation of biofabricated tissues is a critical step in determining the functionality, structural integrity, and long-term viability of tissue constructs. After bioprinting, engineered tissues often require an in vitro maturation period to facilitate cell differentiation, ECM production and deposition, and the development of appropriate tissue architecture before transplantation or experimental use. The use of bioreactors for the maturation of printed

tissues provides controlled environments with regulated oxygen levels, nutrient supply, and waste removal. Additionally, mechanical stimulation of the tissue equivalent during maturation is essential for imparting functional features inherent to native tissues. For example, in the maturation of PDL constructs, cyclic stretching and compression cycles are necessary to guide the proper orientation of cells and collagen fibers, support ECM remodeling, and promote osteogenic differentiation [226–228]. Bioreactors can also provide specialized conditions, such as ALI exposure, which is necessary for the maturation and stratification of keratinocytes in gingival tissue equivalents [227]. Nevertheless, achieving complete maturation remains challenging due to the complexity of native craniofacial tissues. Continued development of standardized maturation protocols and the design of bioreactors for effective in vitro tissue maturation are essential, and successful implementation will represent a significant step toward the realization of personalized regenerative medicine.

Finally, regulatory gaps continue to hinder the clinical translation of biofabricated products, particularly bioprinted tissue constructs. In the European Union (EU), cell-based therapies are regulated as advanced therapy medicinal products, whereas in the USA, the Food and Drug Administration (FDA) classifies them as cellular and gene therapies. When cell therapies are combined with biomaterials or scaffolds, they are regarded as combination products. Bioinks, under both EU and FDA frameworks, fall into this category because they integrate biologics—such as somatic cells, engineered cells, or growth factors—with a medical device, such as hydrogels and their delivery systems [229]. However, there is still no unified regulatory pathway for bioprinted constructs, primarily due to challenges in standardizing hydrogel formulations, cell sources, and bioprinting protocols. This circumstance continues to pose a significant hurdle for the regulatory approval of biofabricated tissues [229].

5 Conclusions

In summary, the high prevalence and complexity of craniofacial and oral defects require advanced solutions beyond current clinical methods. Cell-based biofabrication of craniofacial tissues holds significant promise for generating personalized, functional tissues. Advanced strategies in this field—including organoids, sophisticated 3D bioprinting, and microfluidic organ-on-chip systems—represent a promising frontier for producing highly organized biomimetic tissues that can effectively restore both the architecture and function of this intricate region. Translating this potential into clinical practice hinges on addressing several key challenges, including accurately replicating complex multi-tissue

architectures, achieving effective vascularization for cell survival, developing reliable post-processing methods to ensure tissue maturation and functionality, and navigating the regulatory gaps that currently hinder the approval and clinical adoption of bioprinted constructs.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This study does not contain any studies with human or animal subjects performed by any of the authors.

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