

Facial Muscle Fiber Composition: Novel Morphological Muscle Cartography for Clinical and Neuromechanical Implications

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Abstract

Facial muscles differ from limb muscles in their functional specialization and are essential for facial expression, social interaction, and oral and ocular competence. Their contractile properties are determined in part by myosin heavy chain (MyHC) isoform composition. However, comprehensive MyHC-based fiber typing has not previously been applied across human facial muscles.

We characterized the fiber-type composition of 21 facial muscles, together with the masseter and sternocleidomastoid, from eight cadavers. Samples were processed for immunofluorescence staining using MyHC isoform-specific antibodies and quantified with a machine-learning algorithm.

Facial muscles were dominated by pure fibers ($88 \pm 4\%$), but also contained a substantial proportion of hybrid fibers ($12 \pm 4\%$). Type IIA fibers were the most abundant overall ($66 \pm 16\%$). By contrast, the sternocleidomastoid contained comparable proportions of type I and IIA fibers, whereas the masseter was predominantly composed of type I fibers ($73 \pm 18\%$). Hierarchical clustering identified three groups: orbicularis oculi and platysma with increased IIX content; nasal muscles and zygomaticus major with high IIA, low I, and absent IIX content; and the remaining facial muscles with higher proportions of fatigue-resistant type I fibers.

These findings provide a map of human facial muscle fiber composition and establish a basis for biomechanical modelling, neuromuscular research, surgical reconstruction, and rehabilitation.

Introduction

Facial muscles have been investigated far less extensively than skeletal muscles, despite their vital roles in ingestion, vision and respiration.¹ In addition, they enable facial expressions and thus underpin human interaction and communication.² The fine and complex movements required for these functions are possible only because of the unique morphology and histology of facial muscles.³ Although both, facial- and skeletal muscles are striated, they differ substantially at both the macro- and microscopic levels^{4,5}. Consequently, detailed analyses of facial muscle fiber composition are needed to better understand their distinctive properties.

On a macroscopic level, facial muscles are characterized by a relative absence of tendons and by their close proximity to, and often direct insertion into, the skin⁶. Additionally, some facial muscles, such as the orbicularis oris or oculi muscle, follow a circular anatomic course that is otherwise only found in smooth muscles⁶. However, the fiber composition of facial muscles has not yet been investigated based on all pure and hybrid types, and not by determining the expression of different myosin heavy chain (MHC) isoforms.

Such fiber type cartography provides information about the fundamental physiological properties of muscles, including the velocity of contraction, the maximal tension, and the susceptibility to fatigue⁷. These characteristics of a muscle are predetermined in embryological development by the expression of

different MHC types, but may change due to usage, age, disease or trauma⁸. The muscles performance is largely dictated by these different types of muscle fibers^{7,9}. There are two types of human muscle fibers: Slow (Type I) and Fast (Type II), later being further divided into IIA and IIX. Furthermore, hybrid fibers can occur, namely I/IIA, IIA/IIX and I/IIA/IIX.^{10–12}

Using MHC analyses, a detailed fiber cartography of the mimic muscles can be established to enhance our understanding of the physiology and pathophysiology and thus improve future treatments.

^{13,14}, ^{15,16} This is relevant for example in, facial nerve injury or diseases like Varicella Zoster virus.¹⁷ Such conditions often result in loss of facial expression, facial droop, sagging of the oral commissure and drooling.^{17–21}

A more precise understanding of facial muscle fiber composition may therefore lead to a deeper understanding of facial neuromechanics, neuromuscular pathologies and optimization in surgical treatment of facial paralysis. The present study aims to provide a detailed fiber type cartography of the facial muscles to gain new insight into the physiological properties and sets a baseline for further comparison.

Materials and Methods

Patient collective: This study has been approved by the institutional review board of the Medical University of Vienna (Approval Protocol: 1372/2024) and was performed in accordance with relevant guidelines and regulations. The Medical University of Vienna, Division of Anatomy have written consent of all donors for the use of their bodies in teaching and research purposes. 20 muscles from hemi-faces of 8 fresh cadavers each were included. Patients age ranged from 66 to 97 years, with average of 80 years. All muscles were harvested within 24 hours after death. Following events in donor history were considered as exclusion criteria: central and peripheral facial nerve paralysis, neurological diseases, stroke and skull base trauma. Cause of death of donors were cardiac events or tumor outside the craniofacial region.

Dissection Procedure

All facial muscles were visualized by removing the skin and adjacent tissue and a cross-sectional biopsy was harvested at the point with the widest diameter.

The muscle samples were embedded into Tissue-Tek® O.C.T.™ Compound (Sakura Finetek Europe B.V., Alphen aan den Rijn, Netherlands) using liquid nitrogen-cooled isopentane. The samples were stored at -80°C until cut at -20°C into 10 µm thick cross-section with a freezing microtome (CM3050 S, Leica Microsystems, Germany).

Immunohistochemistry: For visualization of the muscle fiber types the protocol from Bloemberg and Quadri²⁰¹² et al.¹¹ was used in combination with machine learning assisted analysis. To identify

all muscle types, two cross sections of each muscle were selected and stained with Setup 1 and 2 respectively according to the Table 1. Firstly, the cross sections were washed in a phosphate-buffered-saline + Triton (PBST) bath for 5 minutes. Then Alexa Fluor 594 (1:250) wheat germ agglutinin (WGA) (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) mixed with phosphate-buffered saline (PBS) containing 10% goat serum was applied for 10 min. Followed by a 5-minute bath in PBST. 10% goat serum mixed with PBST was applied for 60 minutes. Then the primary antibodies (Developmental Studies Hybridoma Bank, Iowa City, Iowa, USA), concentrated for Human Set-up 1 and 2 as described in Table 1, were applied for 60 minutes. Combining the two set-ups allows calculation of all fiber types, pure and hybrid. After washing the slides with PBST, the second antibody-mix (Invitrogen, Thermo Fisher Scientific, Waltham, MA, USA) was applied for 60 minutes. For 10 slides, 1 mL of PBST was mixed with 2 drops of NucBlue DAPI and left for 15 minutes to incubate. The slides were fixated with DAKO fluorescent mounting medium and stored at 4°C without light exposure.

Table 1

Immunofluorescent Antibody Cocktails. *Muscle Fiber Typing was performed in two set-ups for full analysis of all fiber types. This table shows the antibody cocktails and concentrations used, as well as their reactivity for MHC isoforms as previously established ¹¹.*

	Primary Antibody Concentration	MHC Reactivity	Secondary Antibody Concentration	
Setup 1	BA-F8 (1:50)	I	AF690 (1:250)	red
	SC-71 (1:600)	Ila + IIX	AF 520 (1:250)	green
	6H1 (1:50)	IIX	AF570 (1:250)	yellow
Setup 2	BA-F8 (1:50)	I	AF690 (1:250)	red
	BF-35 (1:100)	I + Ila	AF520 (1:250)	green
	6H1 (1:50)	IIX	AF570 (1:250)	yellow

Imaging and Image Analysis

The slides were visualized with the multispectral imaging and slide scanning device Vectra Polaris (Akoya Biosciences, Marlborough, MA, USA). Images were analyzed by quantifying muscle fiber types using a deep learning phenotyper Halo AI (V 3.3 Indica Labs, Albuquerque, NM, USA).

Statistics

In GraphPadPrism version 10 (GraphPad Software, San Diego, CA, USA) differences between facial, masseter and sternocleidomastoid muscles were visualized using box plots and assessed using the Mann-Whitney test. Furthermore, facial muscle fiber type composition data (% Type I, Type IIA, Type IIX, and hybrid fibers I/IIA, IIA/X, I/IIA/IIX) were analyzed using hierarchical cluster analysis. Prior to clustering, all variables were standardized (z-scores, mean = 0, standard deviation = 1) to ensure equal weighting across measures and to prevent dominance of variables with larger numerical ranges.

Hierarchical clustering was performed in XLStat using Ward’s minimum variance method and Euclidean distance as the dissimilarity metric. The resulting dendrograms were inspected to identify natural groupings of muscles, and the number of clusters was further evaluated by silhouette analysis to assess cohesion and separation of the identified clusters. The outcome defined three main groups and various sub-groups, as illustrated in the Fig. 2.

Results

Comparison of Facial Muscles to Skeletal Muscles:

Table 2

Average and standard deviation of all facial muscles, masseter and sternocleidomastoid muscle. Facial muscles predominantly consist of pure fibers (only one fiber type; average: $88 \pm 4\%$), hybrid fibers (more than one fiber type; average: $12 \pm 4\%$). Lowest abundance was Type IIX (mean: $2 \pm 6\%$), followed by Type I (mean: $20 \pm 14\%$). Type IIA can clearly be identified as the most abundant muscle fiber type in all facial muscles (mean: $66\% \pm 16$). The sternocleidomastoid muscle showed equal amounts of I and IIA (mean, I: $48 \pm 22\%$, IIA: $41 \pm 20\%$) and oppositely, the masseter muscle presented Type I (mean: $73 \pm 18\%$) as the most abundant fiber type. No difference can be seen in hybrid fiber composition between the three groups.

Muscle	Number of specimens	Fiber Type % ($\pm$ Std Dev %)					
		I	IIA	IIX	I/IIA	IIA/IIX	I/IIA/IIX
Facial Muscles	118	20 ± 14	66 ± 16	2 ± 6	8 ± 5	3 ± 6	1 ± 3
M. masseter	5	73 ± 18	15 ± 11	3 ± 8	8 ± 7	0 ± 1	1 ± 2
M. sternocleidomastoid	6	48 ± 22	41 ± 20	0 ± 0	8 ± 5	2 ± 3	1 ± 2

Facial muscle fiber composition:

The resulting fiber type composition of facial muscles reflects their specialized functional demands. The first group of muscles shows both, a high IIX and I/IIX proportion (IIX: 6–14%, IIA/X: 7–16%) in combination with low type I (8–15%) and moderate type IIA (55–71%) fibers in comparison to the other groups. As IIX fibers are fast glycolytic fibers responsible for high rapid force and thus reflex, this represents the functional demand of the orbital region. Furthermore, our data confirms the previous findings, as the palpebral part shows an even higher percentage of IIX fibers, as it is responsible for the fastest reflex, the blinking reflex^{4,22,23}.

The second group contains muscles of the nasal region and the zygomatic major muscles. It shows a high type IIA (71–91%), low Type I (3–22%) and no Type IIX (0–1%) fiber composition. Furthermore, hybrid fibers are the lowest of all group (0–6%). The fast oxidative IIA fibers are known as intermediate,

they are fast but also more fatigue resistant. This correlates to their functional demand, prolonged nasal dilatation, frowning and smiling.

The third group shows the greatest endurance capacity with proportionally high Type I fibers known as slowly contracting and fatigue resistant fibers. A balanced profile between Type I, IIA and I/IIA (I: 7–34%, IIA: 55–76%, I/IIA 7–12%) with little to none IIX fibers (0–1%). This profile allows sustained contractions for cheek, lip and eyebrow movements. Therefore, representing postural stability, controlled movement and endurance, all essential for facial expression and articulation.

Table 3

Fiber type composition. Percentage of Fiber Type (I,IIA,IIIX,I/IIA,IIA/IIIX,I/IIA/IIIX) occurrence in facial muscles, including the standard deviation and number of analyzed specimens. No muscle showed the fiber type I/IIIX and is therefore not included in the table. Since calculation of fiber types involved two staining procedures (Setup 1&2) on two cross-sections, total percentages did not always sum up to 100%. Therefore, values were transformed to range between 0 and 100% for interpretability. Standard Deviations seen in this data set are comparable with other muscle fiber results. A higher standard deviation may be due to a natural variability of fiber types caused by alterations in training, metabolic changes or through changes in microenvironment around the muscle.^{24,25}

Muscle	Number of specimens	Fiber Type % (± Std Dev %)					
		I	IIA	IIX	I/IIA	IIA/IIX	I/IIA/IIX
Group 1							
M. orbicularis oculi pars palpebralis	6	8 ± 4	55 ± 18	14 ± 15	4 ± 5	16 ± 11	2 ± 1
M. orbicularis oculi pars orbitalis	6	10 ± 7	71 ± 13	6 ± 9	3 ± 1	7 ± 10	2 ± 4
M. platysma	5	15 ± 3	58 ± 15	8 ± 13	11 ± 7	7 ± 7	1 ± 1
Average		11	61	9	6	10	2
Group 2							
M. nasalis	6	3 ± 3	91 ± 7	0 ± 0	4 ± 3	1 ± 1	1 ± 1
M. levator labii superioris alaeque nasi	7	5 ± 3	86 ± 7	0 ± 1	5 ± 3	3 ± 4	1 ± 1
M. zygomaticus major	7	18 ± 9	72 ± 6	0 ± 0	6 ± 4	0 ± 1	3 ± 8
M. levator anguli oris	4	22 ± 8	72 ± 9	0 ± 0	6 ± 2	1 ± 1	0 ± 1
M. procerus	6	14 ± 9	79 ± 10	0 ± 0	5 ± 3	2 ± 1	0 ± 1
M. depressor supercilii	5	16 ± 15	71 ± 23	0 ± 0	5 ± 2	4 ± 4	3 ± 4
Average		13	79	0	5	2	1
Group 3							
M. depressor labii inferioris	8	31 ± 16	57 ± 20	0 ± 0	8 ± 4	1 ± 3	2 ± 3
M. corrugator supercilii	9	32 ± 17	59 ± 16	0 ± 0	7 ± 3	1 ± 2	0 ± 1

Muscle	Number of specimens	Fiber Type % (± Std Dev %)					
		I	IIA	IIX	I/IIA	IIA/IIX	I/IIA/IIX
Group 1							
M. buccinator	5	34 ± 10	56 ± 6	0 ± 0	8 ± 3	1 ± 1	1 ± 2
M. depressor anguli oris	9	34 ± 12	56 ± 12	0 ± 0	8 ± 4	2 ± 2	1 ± 2
M. frontalis	5	28 ± 8	55 ± 12	0 ± 1	10 ± 3	5 ± 9	1 ± 1
M. risorius	1	27	61	0	9	3	0
M. orbicularis oris superior	6	7 ± 7	76 ± 10	0 ± 0	11 ± 7	4 ± 6	2 ± 2
M. depressor septi nasi	2	12 ± 12	71 ± 17	0 ± 0	12 ± 4	1 ± 1	1 ± 1
M. zygomaticus minor	3	19 ± 11	62 ± 8	1 ± 1	11 ± 9	5 ± 6	3 ± 4
M. levator labii superioris	7	25 ± 13	62 ± 11	0 ± 0	11 ± 4	2 ± 2	0 ± 1
M. orbicularis oris inferior	5	22 ± 6	63 ± 9	0 ± 0	12 ± 6	1 ± 2	2 ± 2
M. mentalis	6	21 ± 5	68 ± 8	0 ± 0	11 ± 5	1 ± 1	0 ± 0
Average		24	62	0	10	2	1

Discussion

Fiber types define the fundamental physiological properties of skeletal muscle. These neurophysiological relevant characteristics include contraction velocity, maximal tension, and susceptibility to fatigue.^{9,26,27} Across the spectrum: I < IIA < IIX there is a sequential increase of the diameter of the motor axon, size of motor unit, muscle fiber diameter, fusion frequency, contraction speed and tetanic force, accompanied by reduced fatigue resistance.^{9,24,26,27} The unique functional role of facial muscles becomes evident when compared to the sternocleidomastoid and masseter muscle. Mimetic muscles display a distinct fiber composition with a high abundance of IIA fibers. This profile renders them fast, relatively fatigue-resistant and adaptable - properties required for frequent, rapid, and repetitive activities such as speech, blinking, smiling and frowning. Within the group of mimetic muscles, certain muscles, particularly the orbicular oculi muscles and platysma exhibit a higher proportion of IIX fibers. Consequently, these muscles generate higher force and contract more rapidly but are less suited for sustained activity due to reduced fatigue resistance.

In neuromechanics fiber types play an important role. Motor unit recruitment follows the trend of first activating slow contracting muscle fibers (Type I) through slow conducting axons and then activating fast conducting axons, recruiting fast fibers (Type IIA, IIX)²⁸. A detailed understanding of this specialized muscle group is therefore essential for electromyographical investigations, which are used to characterize, grade, and predict outcomes of neuromuscular disorders²⁹. Moreover, a key role in neuromechanics is understanding electrical outputs from muscles and estimating muscle activation in virtual models.^{2,30} For this, the physiological properties of muscles are relevant.³⁰ Such models exist for extremities, however, could also be useful for the analyses of facial muscles. Knowledge of fiber composition and activation properties may therefore improve the fidelity of simulation models.³⁰

Muscle fiber typing can be achieved through different approaches. One way is to analyze the ATPase activity either by using different pH levels or by assessing its activity in comparison to an oxidative enzyme such as immunoperoxidase.¹¹ However, this has the disadvantage that multiple cross-sections of the muscle are required and pH pre-incubation lead to irregular inactivation of the ATPase in the myosin for the same fiber type.¹¹ The currently most accurate method, used in this study, is to analyze the expression of MHC isoforms, indicating the composition of muscle fibers within the muscle.^{10,24,26,31} Because MHC isoforms are the primary determinants of fiber type, this approach allows for deeper physiological insight.³² MHC can be analyzed with an electrophoretic separation or by immunohistochemical staining of different MHC isoforms.^{9,11} Bloemberg et al.¹¹ compared the limitations of the different approaches and established a method using immunohistochemical staining with antibodies against the different MHC isoforms to identify all major fiber types, pure and hybrid, with a reduced time expenditure for processing and analyzing.^{11,12} This allows direct characterization of muscle fibers through determination of the MHC isoforms, which can display co-expression of isoforms (MHC I, IIA and IIX) in muscle fibers.¹²

Based on MHC expression, fibers are classified into Type I, IIA and IIX. Some authors additionally describe the IIC muscle fiber type, which expresses MHC I and IIA and is therefore the hybrid fiber type I/IIA.³³ Furthermore, fiber nomenclature changed throughout the years of research. Previously, it was thought that IIb fibers were present in human muscle since the IIX fibers were only found later. Previous work showed that fibers classified using ATPase as IIb actually contained IIX MHC when analyzed using monoclonal antibodies.^{34,35} Knowing this, it was found that the presumable IIb fibers expressed IIX MHC transcripts and the conclusion was drawn that IIb MHC does not exist in human skeletal muscles.^{1,11,36} Therefore, in formerly published work using ATPase method, authors are referencing to IIb fibers.

The specific physiological role of hybrid fibers remains incompletely defined; however, our data is comparable to the findings in literature. Hybrid fibers may occur naturally in a muscle fiber co-expressing multiple MHC isoforms, however can also be more frequent during the process of fiber transitions.^{10,24} Transition can be induced due to alterations in training, metabolic changes or through changes in microenvironment around the muscle.^{24,25} A higher frequency of IIA/X MHC has been found in less

active individuals and a higher amount of I/IIA in well trained individuals.²⁶ Nonetheless, I/IIA fibers have also been described as immature muscle fibers and that they make up less than 5% in skeletal muscle.³³ In our data the hybrid fibers (I/IIA,IIA/X, I/IIA/IIIX) were always below 16%. Since the prevalence of hybrid fibers increases with age, it's relevant to point out that the average age of the donors included in this study is 80 years. (Purves-Smith et al. 2014, Medler 2019) Total amount of hybrid fibers, I/IIA and IIA/IIIX, in rodent extraocular muscles occur naturally as a majority, showing yet again the significant difference of craniofacial muscles.^{37,38} Our data showed that mimic muscles show a relevant amount of total hybrid fibers ($12 \pm 4\%$), indicating the significance of including hybrid fibers in studies when looking at muscle fiber compositions.

Until now, histological characterization of facial muscles has only been performed using ATPase analysis by Freilinger et al.^{4,7,39}. Their studies classify the mimetic muscles only based on the percentage of fiber type I present and found three groups: <20%, 20–40%, > 40%. When comparing the results for Type I fibers, we found lower type I percentages in our study. However, Freilinger only performed ATPase staining after preincubation at a pH of 4.3. This is relevant, because at this pH level not only Type I fibers but also hybrid I/IIA fibers stain intermediate positive.³³ When comparing with the sum of type I and Type I/IIA fibers in our dataset, the fiber distribution aligns for the groups < 20% and 20–40%. Only in the group > 40% we have found slightly lower Type I fiber composition. (M. corrugator supercilli: 39% I + I/IIA, M. depressor labii inferior: 39% I + I/IIA, M. frontalis: 38% I + I/IIA, M. buccinator: 42% I + I/IIA), however still presenting high percentage I-I/IIA% in our data set. Freilinger et al. found different zones for the orbicularis oculi in fiber diameter and a 2% higher type I quantity in the orbital part compared to the palpebral zone. Similarly in our study we found a 2% difference between the two zones in fiber type I and 8% difference in type IIX. Highlighting the importance of analyzing all fiber types when clustering muscles.

Stal et al.^{40,41} described four human mimic muscles using staining for ATPase. In the M. buccinator the authors found 53.4% (± 17.2) Type I, 46.4% (± 17.7) Type IIA and 0.4% (± 0.45) Type I/IIA, with no staining for type IIB.⁴⁰ Comparison to our data shows a similar percentage of Type IIA (56%) fibers. Type I (34%) is lower and I/IIA (8%) is higher in our findings. The same comparison can be drawn for the orbicularis oris muscle.⁴⁰ He also analyzed the zygomatic major and minor muscle, showing a similar Type I and IIX occurrence as in this study.⁴¹ Nevertheless, we found 40–50% more IIA fibers and 50 % less I/IA in both muscles.

Understanding the morphological individuality of mimic muscles may improve reconstructive strategies and facilitate selection of appropriate or more suitable donor muscles. For this reason, a fiber type comparison of potential donor muscles is often performed, especially when suggesting a new donor muscle for facial restoration.^{42,43} Previously, the sternohyoid flap was evaluated and the fiber composition compared to the gracilis and zygomaticus major muscle.⁴³ A key finding of this study is the functional similarity between the orbicular oculi muscles and the platysma. Together the present the

group of fast twitching facial muscles, with high expression of IIX fibers. This highlights the platysma flap for functional restoration of the eye sphincter.^{42,44,45}

More broadly, the fiber type map generated in this study may serve as a reference for comparing donor muscles with native mimetic muscles. Facial reanimation outcomes could thus be evaluated, in relation to the fiber types present in reinnervated muscle, as muscle fibers differentiate dependent on the neural activity of the reinnervated muscles.^{42,46,47} This property of neuromuscular reinnervation shows that muscles can change in their function based on the reinnervating nerve. Therefore, fiber changes indicate functional outcome of reinnervation.^{46,47} This has recently been investigated in reinnervated rat facial muscles, however never in humans.⁴⁶ Therefore, the cartography could aid in the assessment of the reinnervation result, by indicating the muscle fiber changes and similarity to the original mimic muscles. Further research into the influence of original muscle fiber composition on the reinnervation is needed to find the best fit based on functional need and to use these results as outcome measure for accurate reinnervation and restoration of original mimic function.

In conclusion, facial muscles represent a specialized group of muscle fibers characterized by fast, adaptable and fatigue-resistant fibers. Within this cohort the orbicularis oculi muscles and the platysma form a distinct subgroup with a higher proportion of fast type IIX fibers. Comprehensive fiber typing of facial muscles provides the groundwork for refining clinical practices in the diagnosis, treatment, and rehabilitation of facial muscle-related disorders, contributing to improved patient outcomes and a deeper understanding of facial biomechanics.

Declarations

Data Availability:

All relevant data are within the paper.

Author contributions:

Concept: OCA, JK, VT, KB; Definition of intellectual content: VT, JK, OCA; Literature search: JK, Statistical analysis: JK, VT; Manuscript preparation: JK; Manuscript editing: all authors, Manuscript review: all authors. OCA takes responsibility for the integrity of the work as a whole, from inception to publication, and is designated as the guarantor. All authors approved the final version of the manuscript.

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Conflict of interests:

All authors declare that there are no competing interests in the conduct of the research or the preparation of the manuscript. The authors further state that no potential conflicts of interest exist regarding the equipment or reagents used in this study.

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Figures

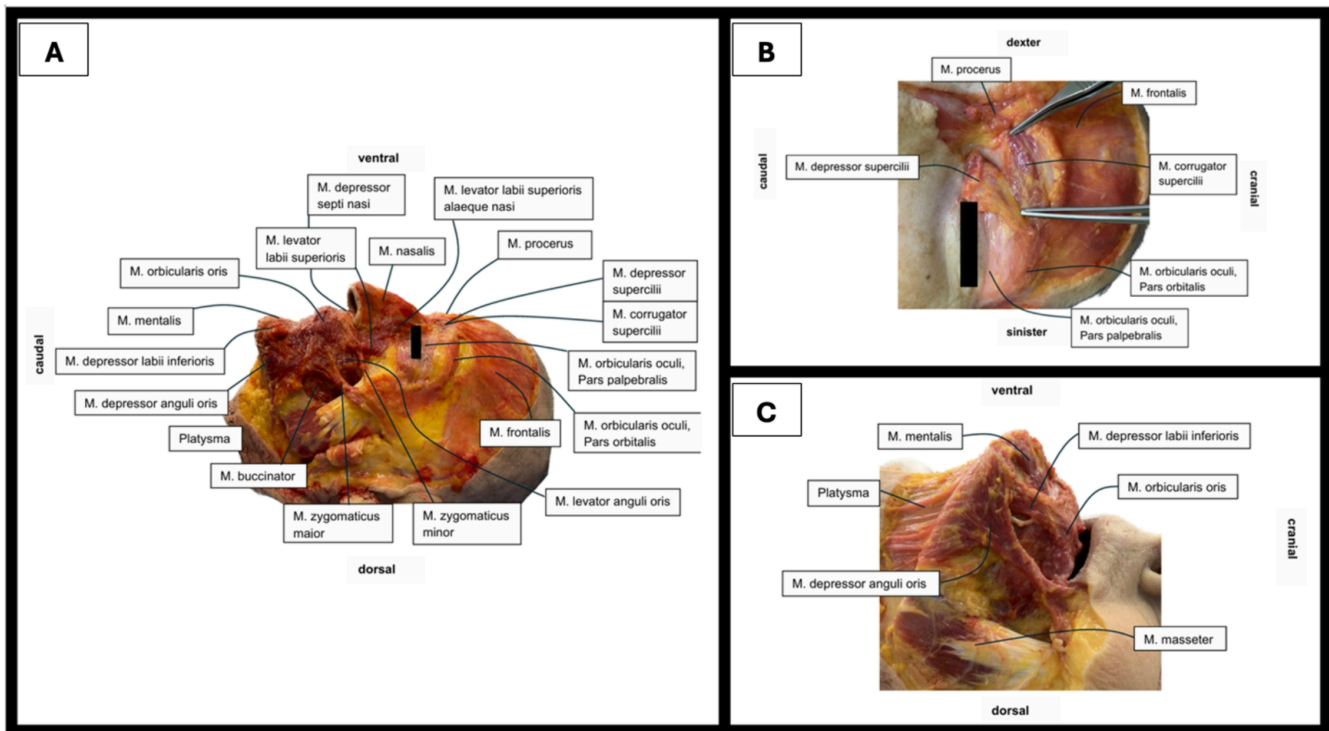


Figure 1

Dissection of facial muscles in fresh cadavers. (A) Overview of the face with the facial muscles. (B) Overview of the forehead and orbicular region. (C) Overview of the lower face and mental region.

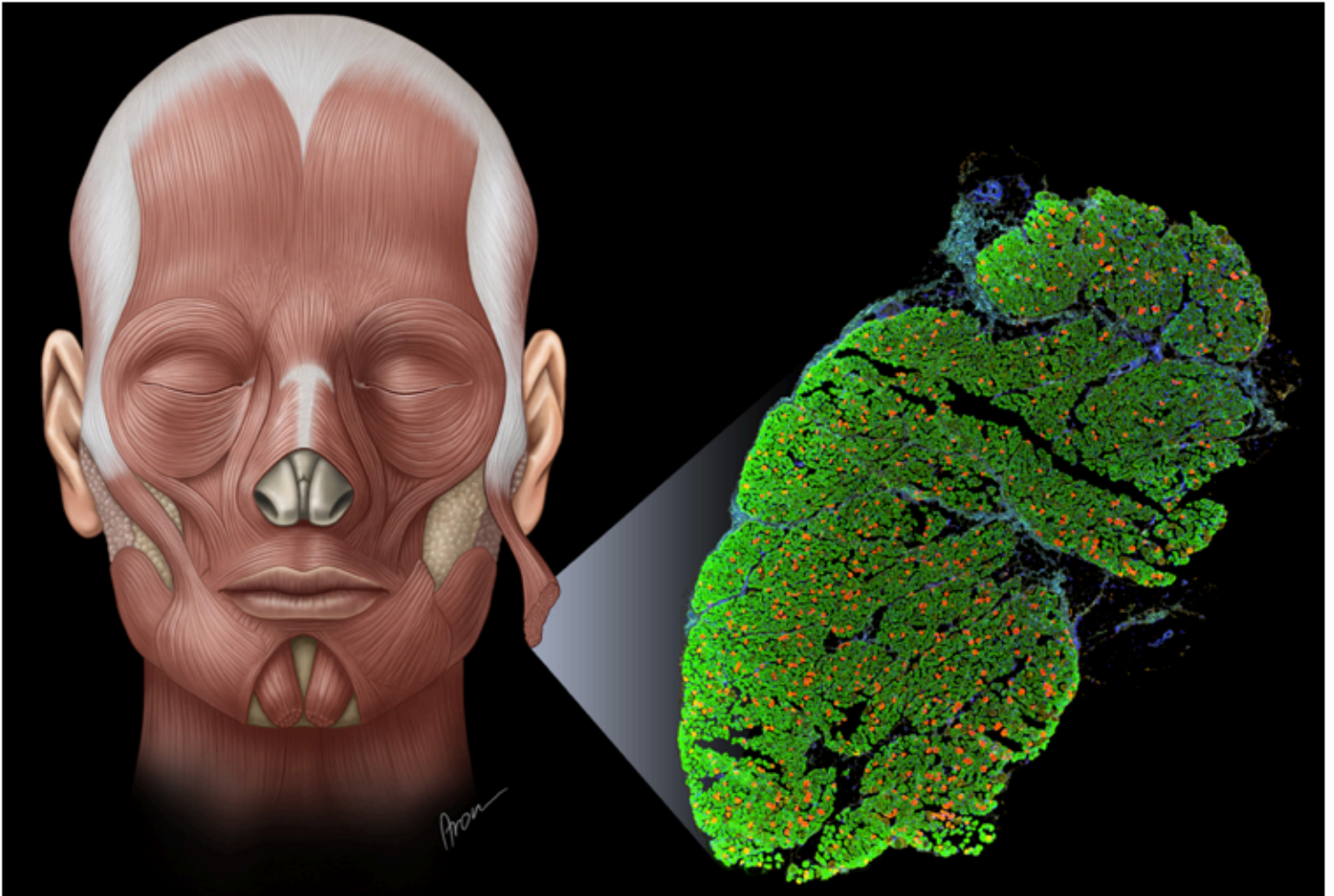


Figure 2

This figure presents the mimetic muscles and demonstrates the zygomatic muscle after staining the muscle fibers based on their MHC type.

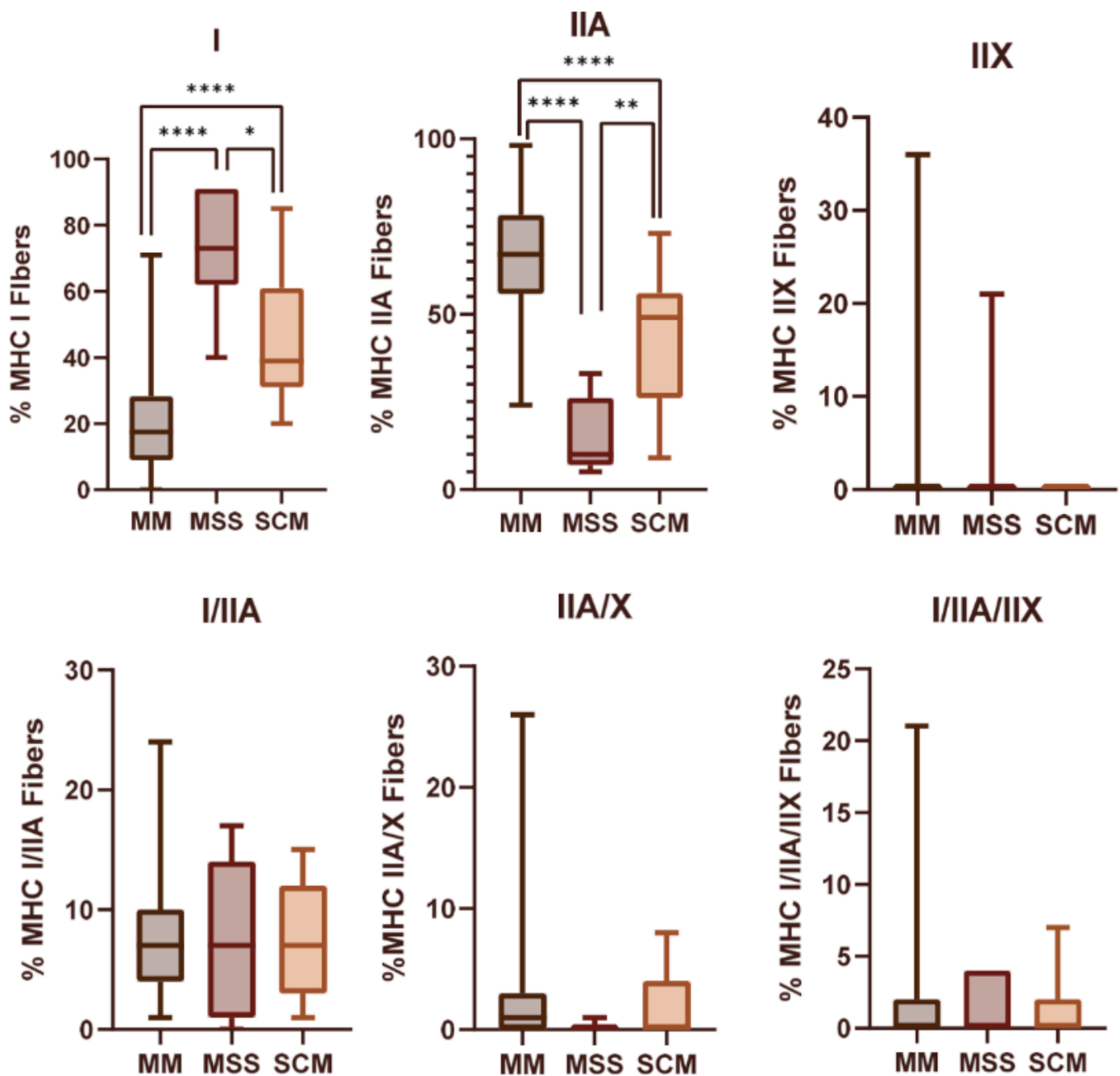


Figure 3

Comparison of fiber type occurrence between facial muscles (MM), the masseter muscle (MSS) and sternomastoid muscle (SCM). The uniqueness of facial muscles is seen in the difference in fiber type I and IIA composition when compared to the masseter and sternocleidomastoid muscle respectively and confirmed with the Mann Whitney test showing statistical significance ($p < 0.0001$ ****). The masseter and sternocleidomastoid muscle present a lower significant difference ($p < 0.0114$ *, $p < 0.0035$ **). All other fiber types showed no relevant difference between the three groups.

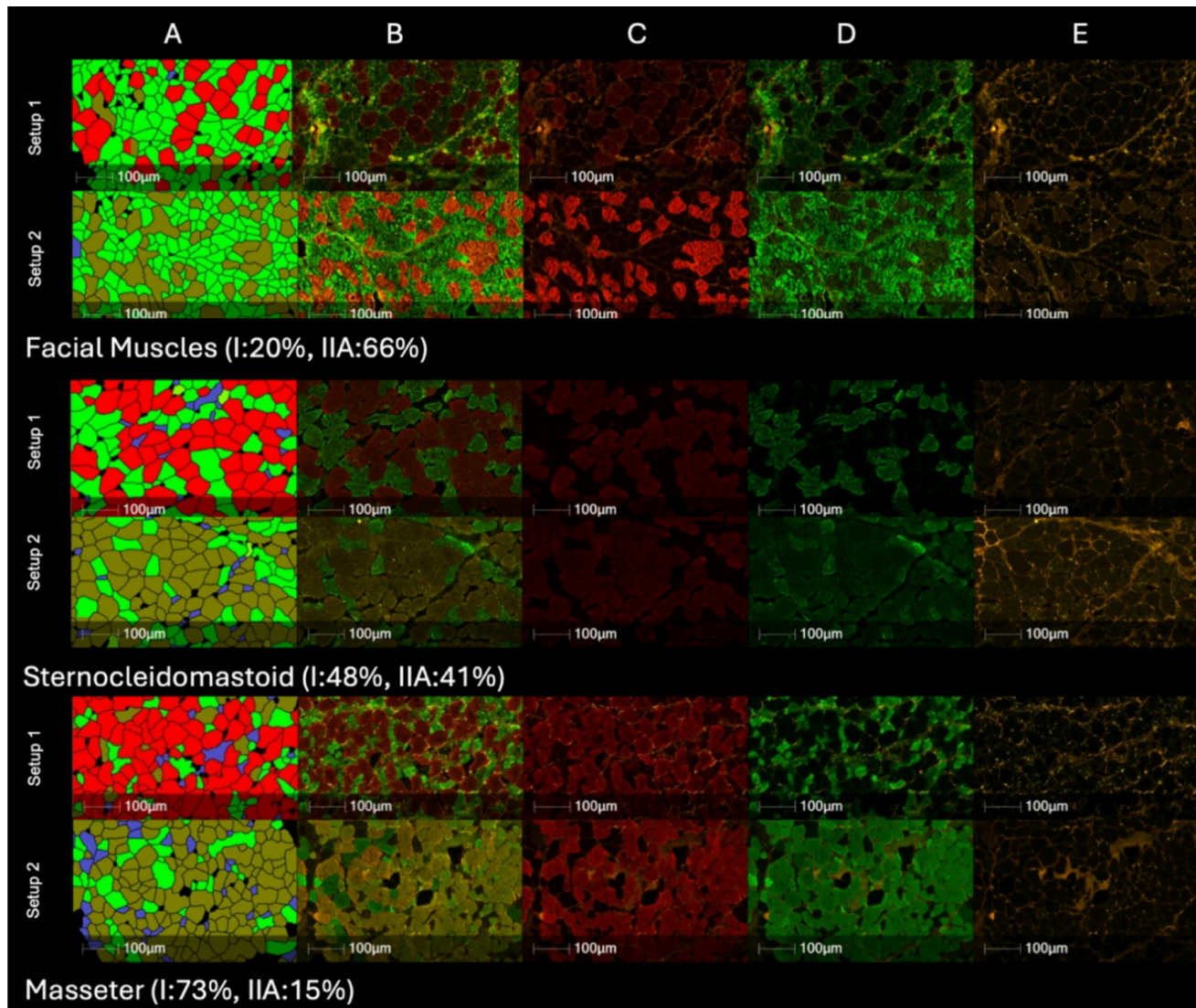


Figure 4

Confocal Microscopic Images show the different fiber distributions between facial muscles and the sternocleidomastoid and masseter muscle. Corresponding images of Staining Set-up 1 (A) and Set-up 2 (B) as described in table 1 from Bloemberg et al. The cell membrane is shown in orange. (A) Halo AI analysis shows the fiber types detected. Double positive muscle cells are presented as color merge of the two types. Negative cells and intercellular spaces are shown as blue. In both staining for BA-F8 (C,red) is positive for MHC I and 6H1 (E,yellow) is positive for MHC IIX. In Set-up 1 SC-71 (D,green) stained positive for IIA and IIX, while in Set-up 2 BF-35 (D,green) stained positive for I+IIA. (B) Fiber types and hybrids can be seen when all channels are activated and compared between the two set-ups.

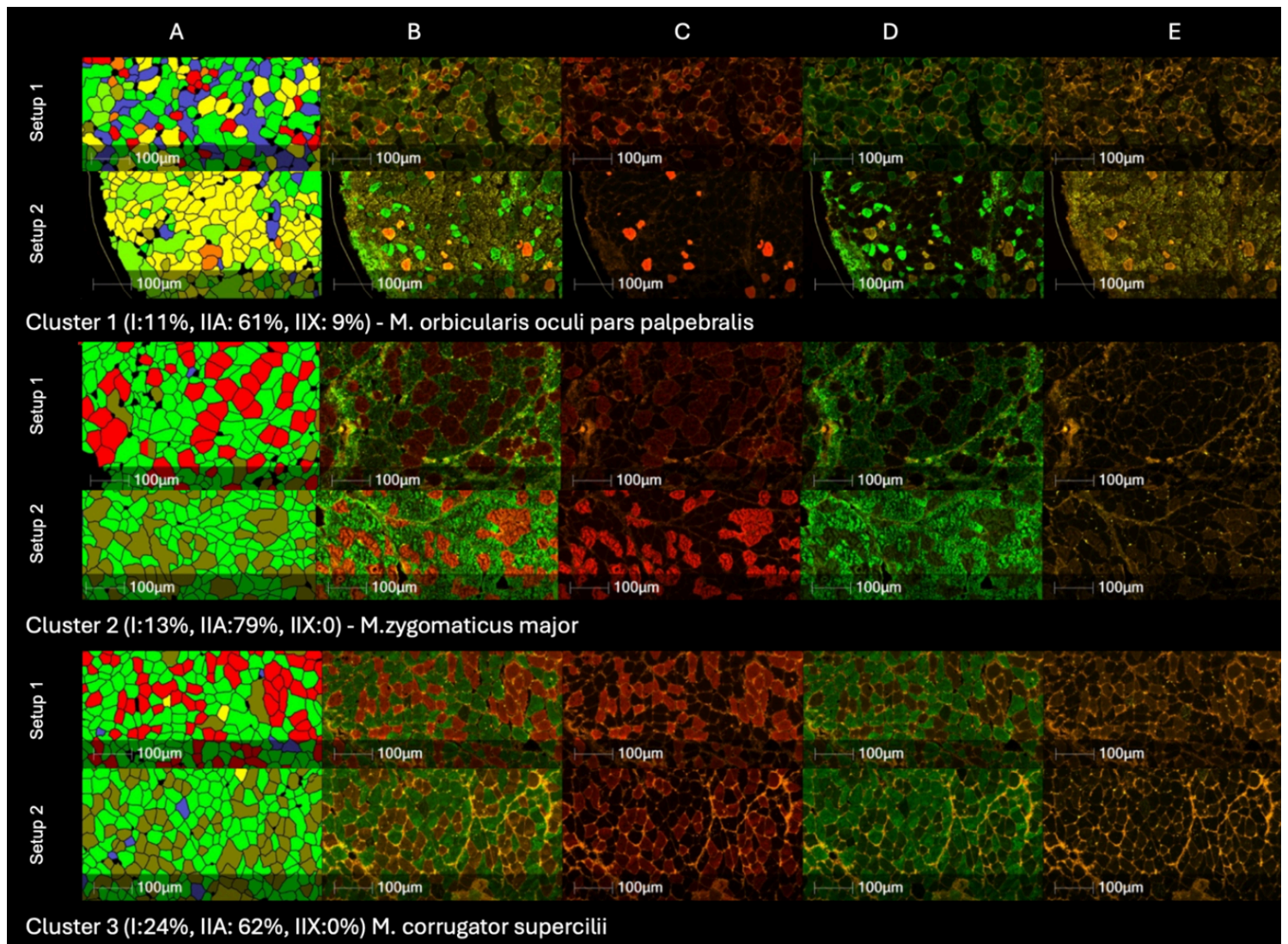


Figure 5

Confocal Microscopic Images show the different fiber distributions as described in figure 3 using exemplary muscles from the three clusters found for facial muscles. Cluster 1 are the only muscles presenting MHCIIX fibers. The antibody 6H1 presented a lower staining intensity than the others used. Therefore, as previously discussed by other authors, IIX fibers and their hybrids may be underrepresented in this study. Nevertheless, the significant difference from cluster one shows that in relation this cluster has the most IIX fibers from all other muscles analyzed.

Dendrogram with choice of clusters

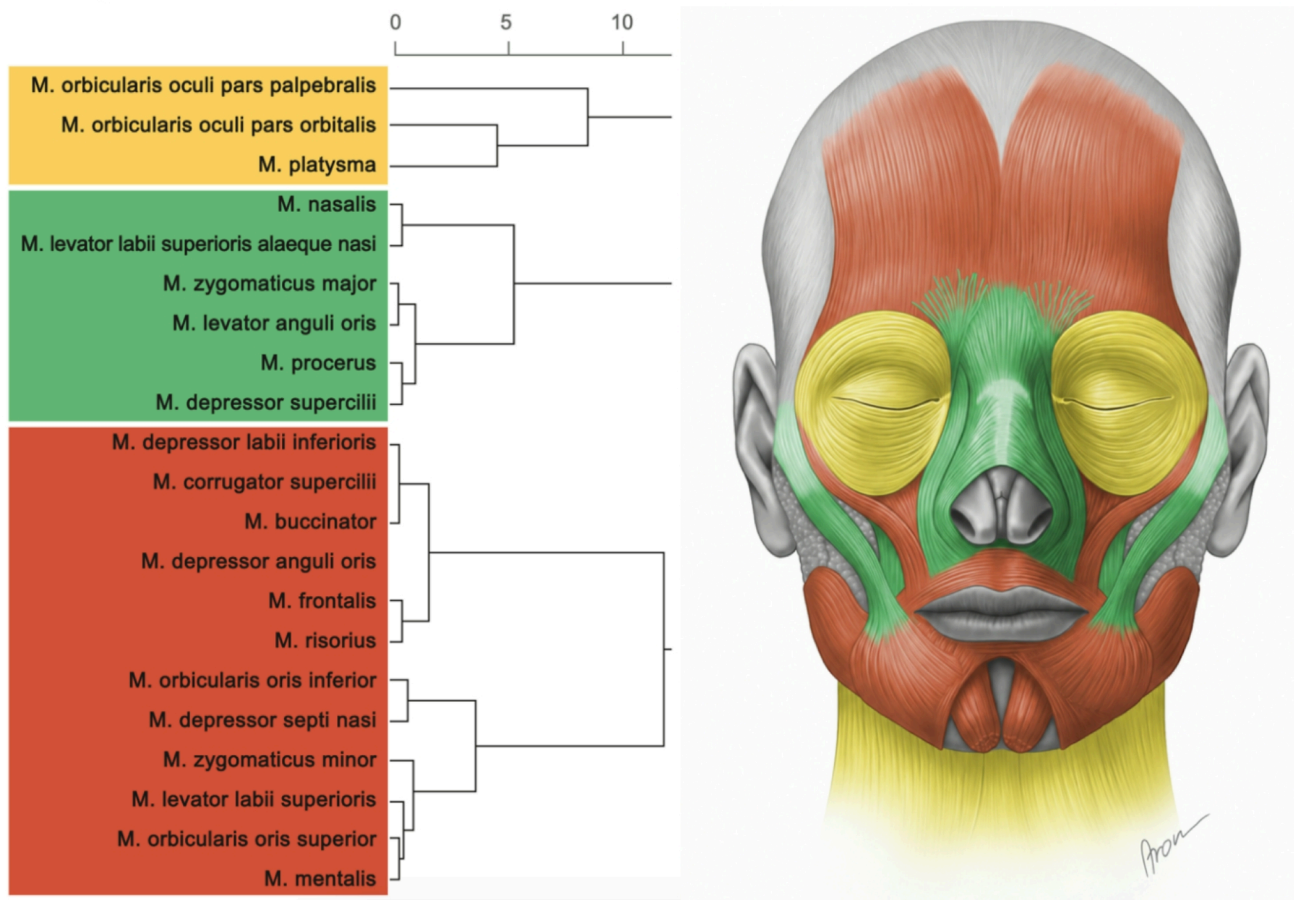


Figure 6

This dendrogram shows hierarchical clustering of facial muscles with similar muscle fiber type distributions. The dataset includes observations from 20 muscles (listed in Table 3), with average percentages of muscle fiber types (I, IIA, IIX, I/IIA, IIA/IIX, I/IIA/IIX) calculated across 8 patients. Not all patients had available data for every muscle. The colored boxes (yellow, green, red) indicate the three clusters, as indicated by the applied hierarchical clustering.