



# DELIVERABLE

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**Report on integration of cryo-blades and cryo-  
imaging into the Titan-Holo microscope at FZJ  
(release 2)  
D5.2**

**Draft: Version 1**

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**Authors:**

Vincenzo Grillo (CNR)  
Amir Tavabi (FZJ)  
Rafal Dunin-Borkowski (FZJ)  
Peter Tiemeijer (FEI)  
Moumita Ghosh (FEI)  
Raimond Ravelli (MU)

**Contributors:**

Abril Gijsbers (MU)  
Kevin Knoops (MU)

**Reviewers:**

Enzo Rotunno (CNR)  
Stefano Frabboni (UMR)

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## Revision History

Revision	Date	Author	Organisation	Description
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0.2	15/1/2020	Vincenzo Grillo	CNR	Rewriting and organization of the material
0.3	6/ 2/ 2020	Peter Tiemeijer, Moumita Ghosh	FEI	Addition of further information
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1.0	24/3/20	Vincenzo Grillo	CNR	Final version

### Statement of originality:

This deliverable contains original unpublished work except where clearly indicated otherwise. Acknowledgement of previously published material and of the work of others has been made through appropriate citation, quotation or both.

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## TABLE OF CONTENT

Table of Content	3
EXECUTIVE SUMMARY	4
1. Cryo-Blades for Cryo-EM	5
1.1 Cryo-EM	5
1.2 Cryo-Box (Cryo-Blades)	5
1.2 Compatibility with the MEMS aperture holder element	6
2 Cryo-Blades for TITAN HOLO	8
2.1 Octagon with novel cryo-blade	8
2.2 Installation steps and problems	13
3. Experimental test of imaging of proteins	14
4 Conclusion	16
ANNEX 1: Temperature control	17
ANNEX 2: REFEREE	18
ANNEX 3: ABBREVIATIONS	18
SHORT NAME OF PARTICIPANTS	18
LIST OF ABBREVIATIONS	19

## EXECUTIVE SUMMARY

The purpose of this deliverable is to explain the actual installation of cryo-blades inside the Titan HOLO electron microscope and to illustrate difficulties and delays that characterised the installation.

This report is strongly based on the report 5.1 and shows the comparison between the initial design and the actual realisation of the parts.

The long term objective has been to introduce retractable cryo blades in TITAN HOLO microscope protecting a frozen biological specimen (cooled to liquid nitrogen temperature) to be safely studied with no contamination effect from the residual organic contaminants inside the electron microscope column and allowing also to implement Sorter phase element for the actual experiments since the microscope must be operated in non-standard conditions.

The principal challenge of this implementation is the possibility to adapt a machine normally suited for materials science and in particular holography to perform cryomicroscopy without losing the functionality in materials science. This makes the machine an “unicum” in the electron microscopy landscape. Moreover, new elements for QSORT such as the special MEMS aperture have been taken in to consideration as a constraint for the realisation of the project.

This deliverable complies with the Q-SORT description of work outlined in “*Work Package 5, task 5.1 Integration of cryo-elements in the TEM at FZJ*”.

The deliverable has been produced thanks to confidential information about the microscope column, previous designs of commercially existing cryo-blades, engineering drawings of internal ports TEM octagon provided by the support of the partner FEI.

It is intended to benefit directly the work of the following interrelated tasks:

- *Task 5.2 Spiral phase plate test.*
- *Task 5.3 Experimental application of the sorter to protein orientation problem.*
- *Task 5.4 Minimally destructive wave recognition of proteins*

Due to the delay in realization for 5,2 we used a different microscope as per contingency plan.

In passing the possibility to still use the microscope for materials science is necessary for the development of WP4.

This current document is comprised of three main Chapters, an Executive Summary and Conclusions. The first chapter introduces the essence of cryo-blades for cryo-EM experiments on biological samples, its features and design and the compatibility issue.

The second chapter describes the actual realization in the TITAN HOLO in the Juelich with a comparison between the original design and the actual realisation

The third chapter demonstrates the first experimental imaging of protein with the TITAN

# 1. CRYO-BLADES FOR CRYO-EM

## 1.1 Cryo-EM

The imaging of frozen hydrated samples by cryo-TEM is nowadays used to characterise a wide variety of structures. This technique improves the results obtained by standard imaging in some cases where the classical methods are difficult to use. Moreover, there is a whole class of structures that cannot be visualised in dry specimens because they only exist under the action of forces which are acting in liquid water. Examples of structures that have been characterised with this technique include viruses, ribosomes, actin filaments, several kinds of **proteins** and other macromolecules as lipid and lipid vesicles.

Characterization of frozen hydrated samples by cryo-TEM requires several specialised steps from sample preparation to visualization.

Cryo-immobilization of the protein samples was done both using plunge freezing (Vitrobot Mark IV) and jet freezing (VibroJet, UM). We used 0.5 - 4mg/ml protein concentration, and applied it on quantifoil R1.3/1.2 300 mesh grids. Liquid ethane was used as cryogen, both for plunging and jetting.

Transfer of sample to cryo-holder: the holder must be previously prepared to ensure isolation of its dewar and cooling transmission from liquid nitrogen to the sample in its tip. Several hours of holder evacuation in the turbo-pumping station and zeolite cycle with the cold stage controller are required. Sample transfer is performed under liquid nitrogen in the cryo workstation

The holder is introduced in the microscope and visualised under the cryo-protection of cryo-blades and using Low Dose mode.

## 1.2 Cryo-Box (Cryo-Blades)

The idea of cryo microscopy is to getter the organic compounds inside the microscope through a cold driven diffusion. There are different implementations depending on the geometry of the cold elements. These can be in the form of 2 metallic blades on top and below the specimen (cryoblades) or in the form of a nearly complete box surrounding the specimen in all directions (cryobox).

The main problem with cryoblades and boxes is the typical size of the object that should be within the polar expansion of the objective lens.

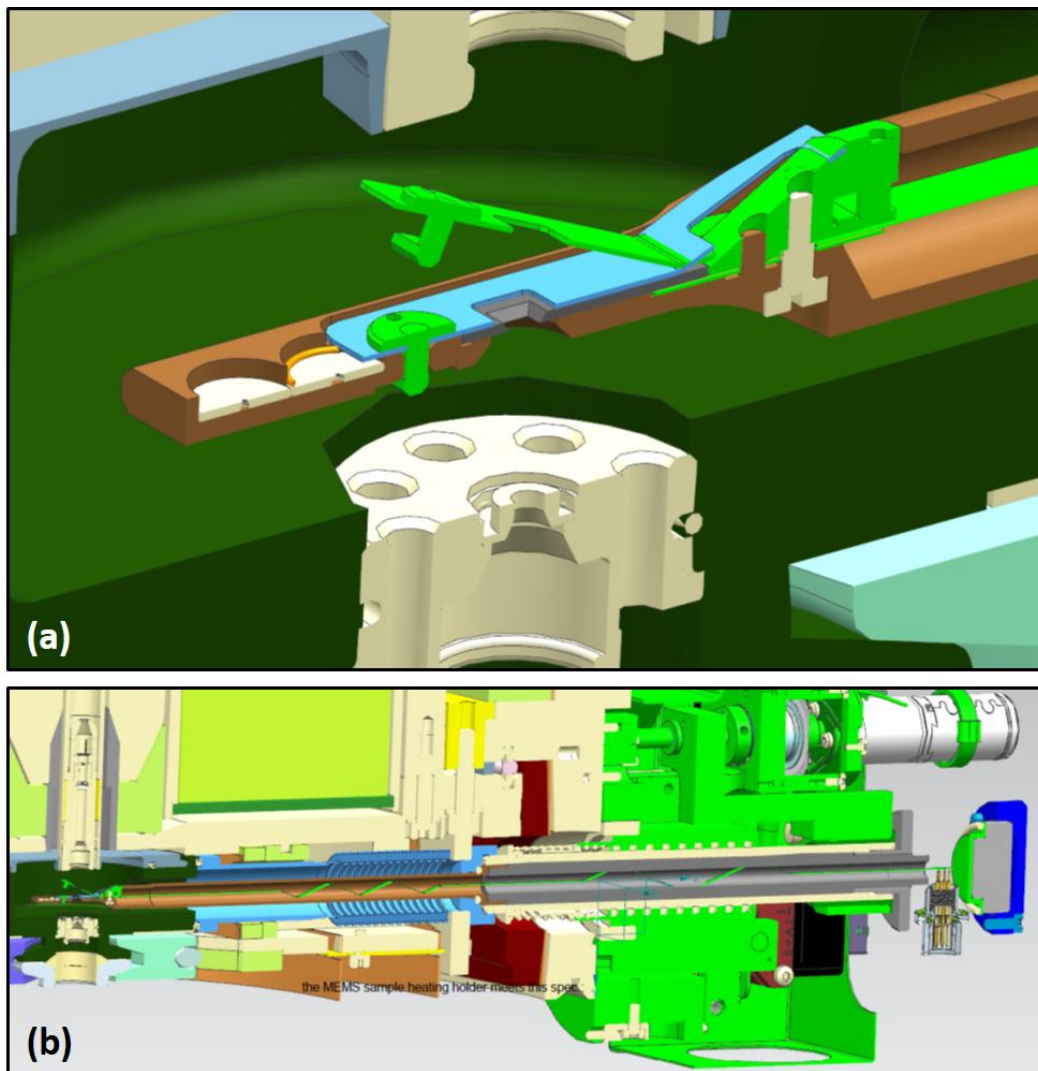
The cryoblades are retractable or, more often, fixed and substitute the normal cold trap that is used in conventional microscopy as cryo getter.

The objective lens region of the microscope where the specimen is hosted has 8 flanges that permit the access to the specimen region.

The conundrum of this deliverable is that we need to find the space for hosting retractable cryoblades compatible with the rest of the column. Moreover we have a smaller dimension in the Z direction with respect to standard

## 1.2 Compatibility with the MEMS aperture holder element

One of the most important restrictions that makes the standard cryo-blades very difficult to instal on the TITAN HOLO microscope resulted from the installation of the First Sorter phase element in the objective aperture position, port “3”. A new MEMS aperture holder was designed and fabricated to implement this element in appropriate position, see Fig 1. This aperture is much thicker than standard objective aperture due to electrical contact and MEMS chip of the phase element which may interfere with the standard cryo-box or cryo-blade.



**Fig 1.** MEMS aperture holder for Sorter phase element (a) MEMS mounting mechanism, (b) overall configuration of the MEMS aperture inside the microscope column.

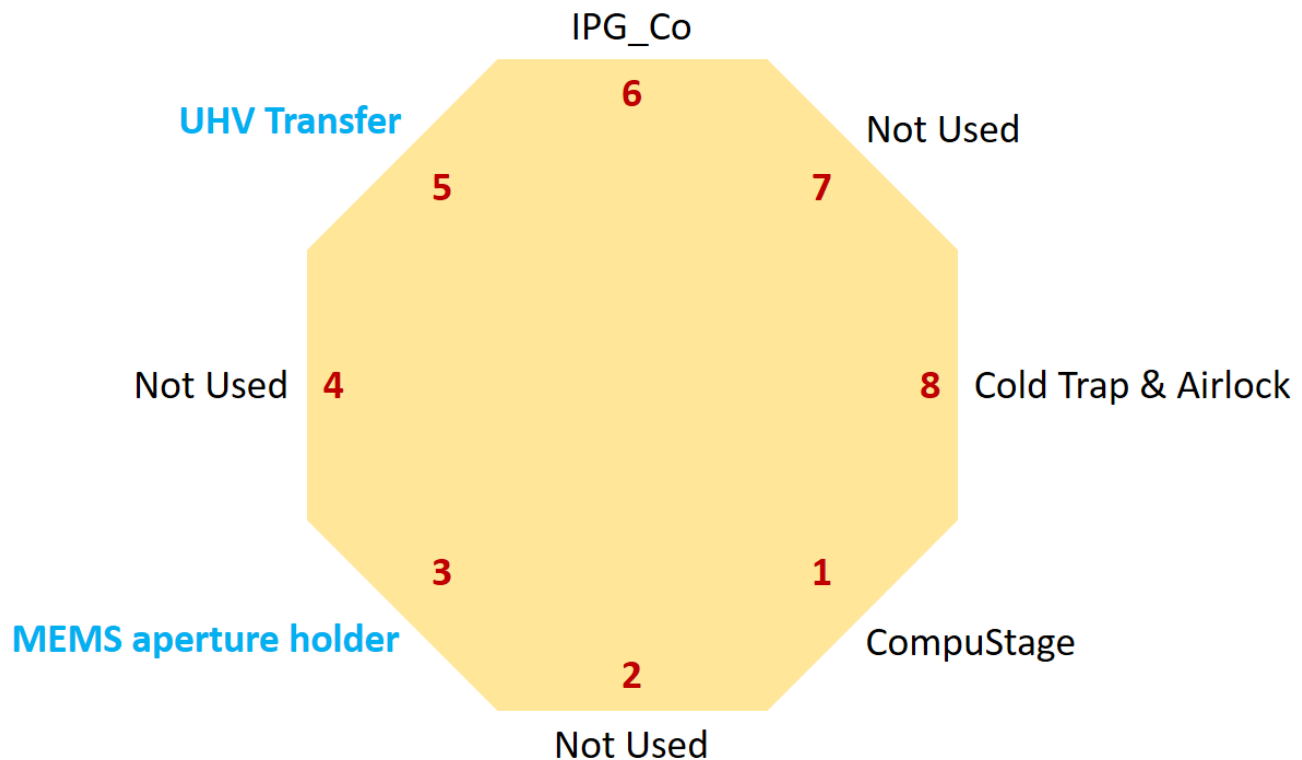


**Fig 2** The actual MEMS aperture holder for Sorter phase element

The design and real object are shown in Fig 1 and 2. It was important to have the device ready to check together with the new cryoblades.

Before installation of the cryoblades the entry of the port of the octagone ( the section of the microscope containing the objective lens) are configured as in the scheme in fig 3.





**Fig 3.** Scheme of TTAN HOLO's Octagon configuration for new cryo-blades.

## 2 CRYO-BLADES FOR TITAN HOLO

According to the mentioned limitations in section 1, a novel cryo-blade system has been produced according to the specification in the previous release of the deliverable ( 5.1).

We have checked

- Mechanical interoperability of cryoblades and sorter MEMS holder
- Complete retractability of the cryoblades for normal operability
- Good anti-contamination action even when the cryoblades are retracted.
- X-ray safety after installation.
- Sufficient tilt possibility when cryoblades is on.

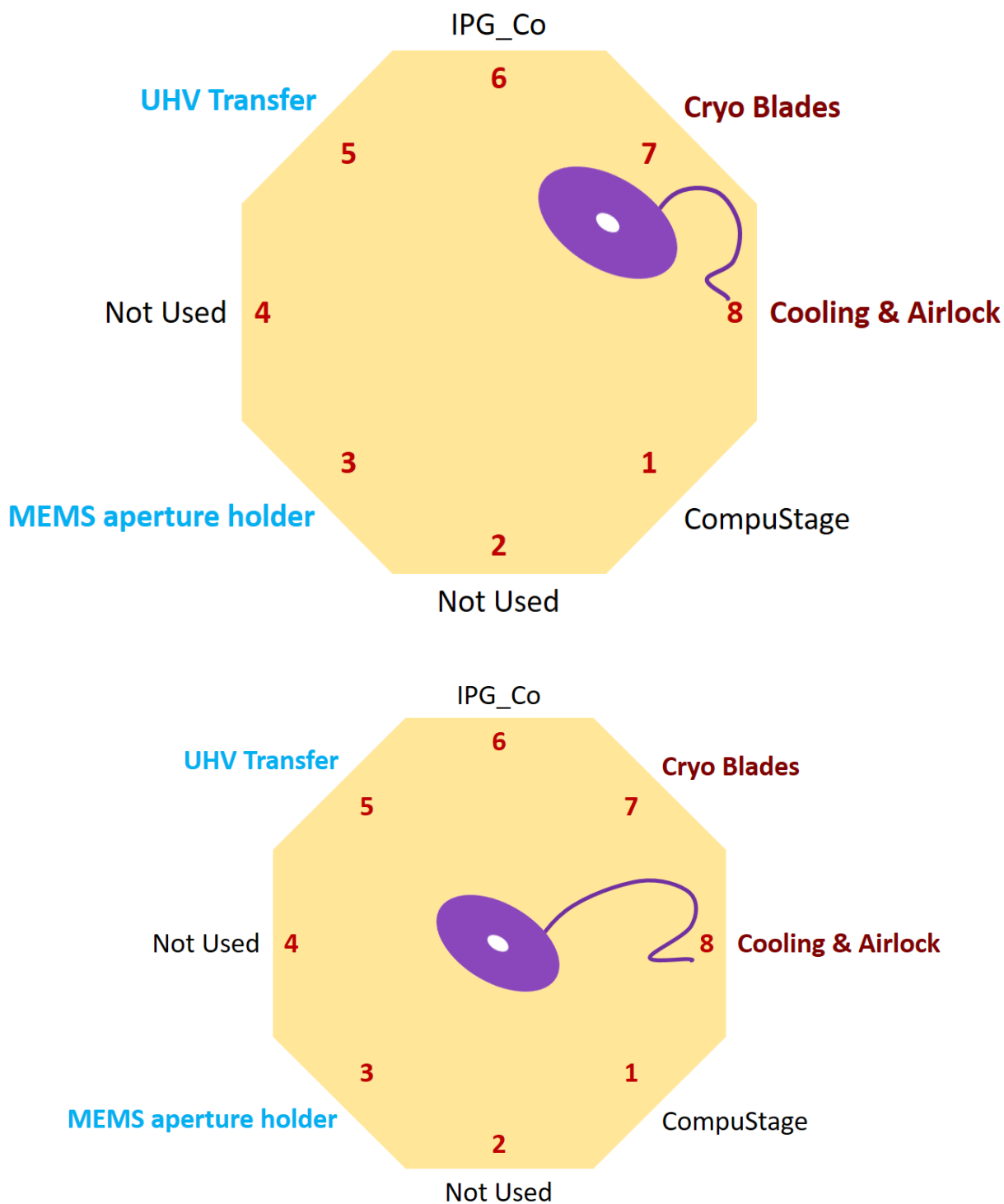
In addition to the Cryoblades introduction we took the opportunity to improve the projection system of the titan HOLO microscope by substituting the SAD2 mechanisms in order to allow a safe functionality of MEMS holder and its mechanical positioning.

## 2.1 Octagon with novel cryo-blade

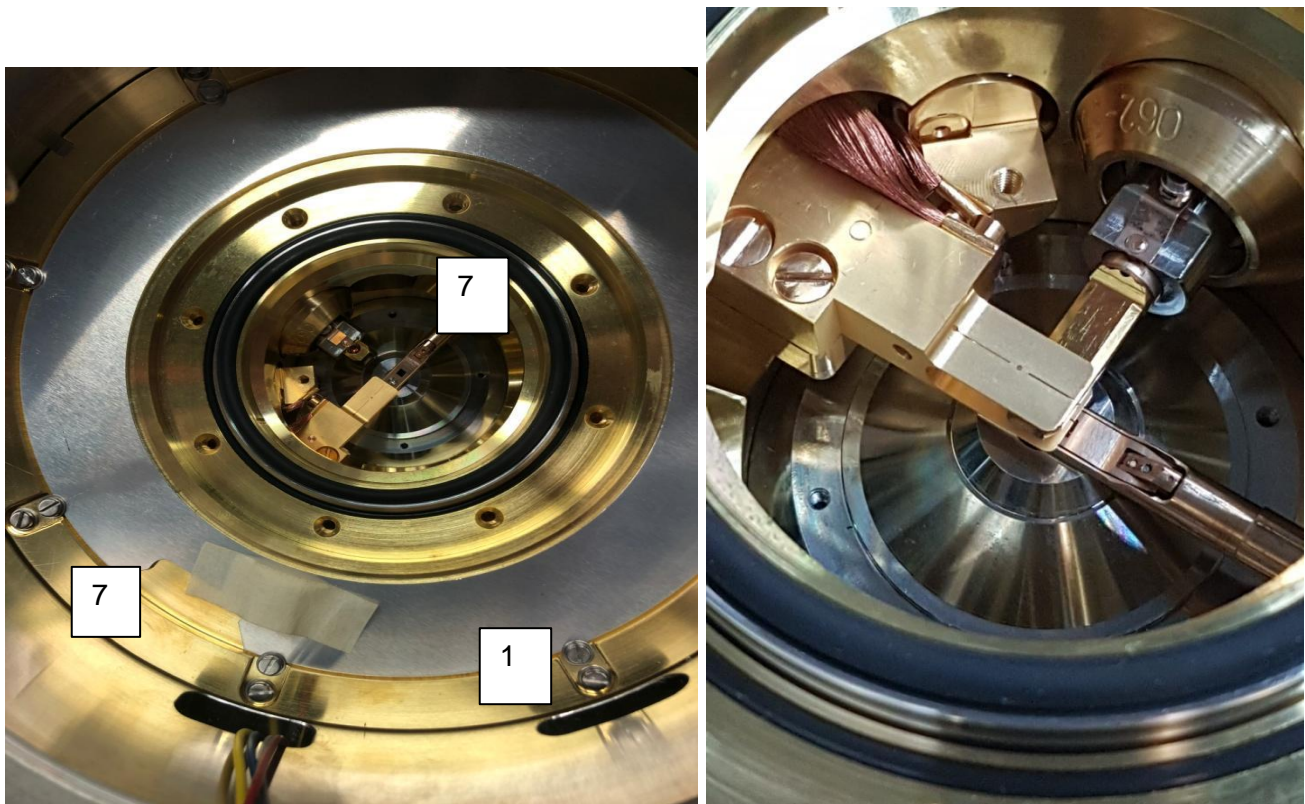
As explained in the design time it has been decided to mount the cryo-blades at 90° to the specimen holder. The configuration of the blades in retracted and non retracted mode are shown in fig 5. Notice that the blades are connected by a socket to the cool finger connected to the dewar of liquid nitrogen

Fig 5 shows the actual configuration with the element numbered according to fig 4. The column in this moment is split allowing for a direct view of the area.

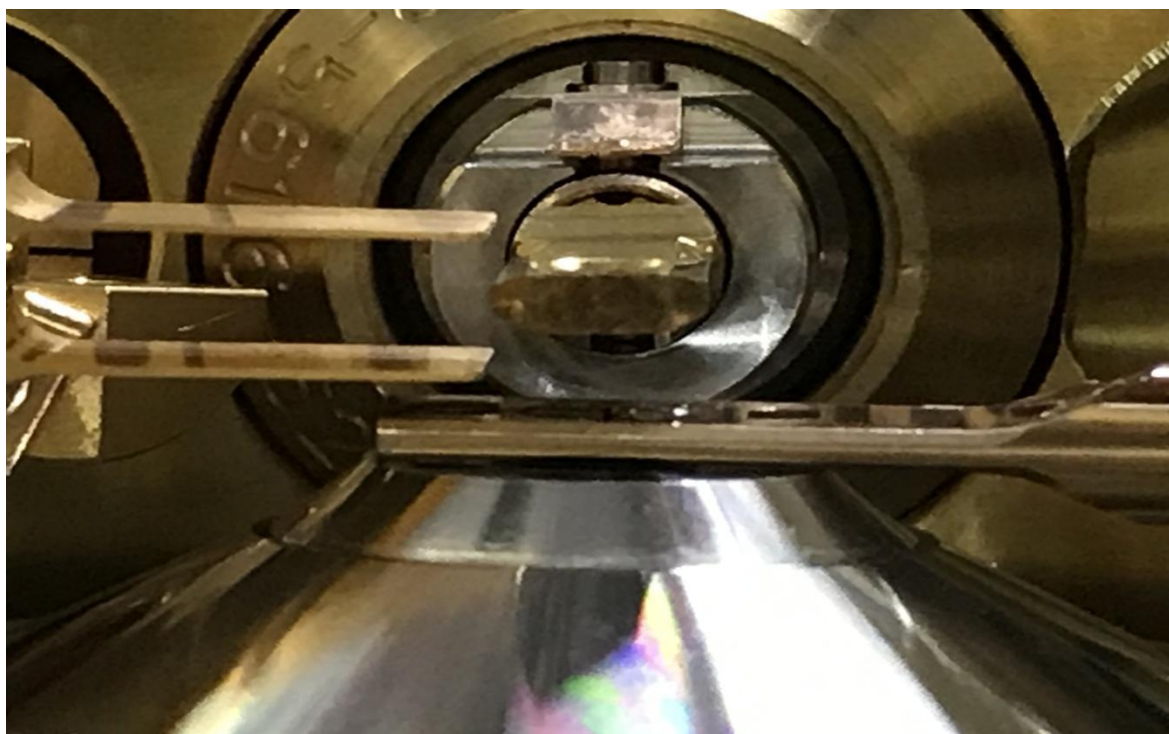
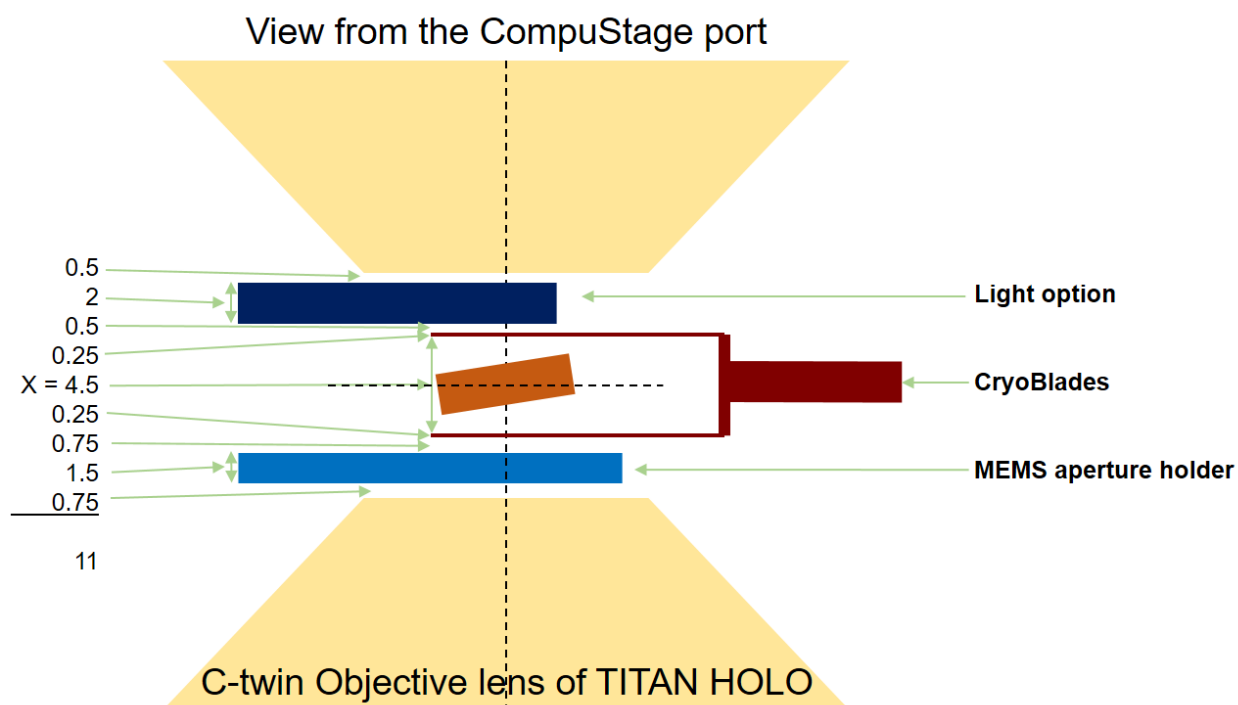
Fig 6 shows a comparison between the designed and actual lateral view and the specimen area. It can be appreciated that the MEMS holder, the cryoblades and the specimen don't touch each other allowing for normal operation.



**Fig 4.** Scheme of TITAN HOLO's Octagon configuration with new cryo-blades not inserted ( up) and inserted (down).



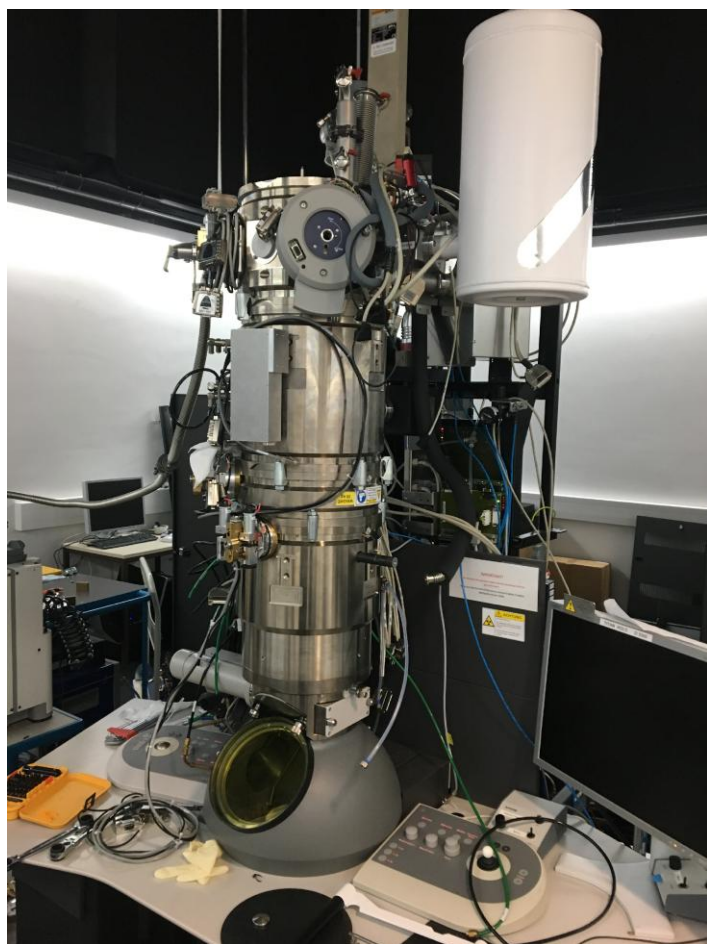
**Fig 5** Actual configuration with non-inserted and inserted cryoblades.



**Fig 6.** Scheme and real image of TITAN HOLO's Octagon configuration with new cryo-blades, side view.

While inserted cryoblades do normally ensure a strong cryo protection from contaminant, in the non-inserted mode the cryoblades system is potentially a less efficient getter for contaminants with respect to the traditional cold finger. We experimented that this is not a problem having a very good vacuum and a “normally acceptable” level of contamination.

Another part of the modification has been to take into account that the cryoblades as configured are in contact with large parts of the column the consumption of liquid nitrogen is larger, a new large volume LN2 dewar is therefore foreseen to be mounted on microscope column allowing long stand time operation, e.g. > 8 hrs. The new dewar is visible in fig 7



**Fig 7.** Microscope during reinstallation with the new Dewar directly visible.



## **2.2 Installation steps and problems**

The splitting of the column and its reassembly took a relatively long time.

The main operations have been

- Stopping the microscope vacuum
- Splitting of the column piece by piece.
- Change of the projection system
- Installation of the new elements ( mainly cryoblades) in the octagone
- Installation of the new dewar
- Reassembly of the microscope
- Realignment of all the microscope

To these operations it must be added that after the reassembly there have been failures of the electron gun and of a lens circuit and these have been replaced.



**Fig 8.** Images of various moment of the reassembly of the microscope

### 3. EXPERIMENTAL TEST OF IMAGING OF PROTEINS

As a final test we realised a full experiment of imaging of protein in cryomicroscopy.

Whereas the methodology is not state of the art, as we obviously could not fit an autoloading, we could still obtain images of proteins. Fig 9a shows the Gatan 626 side entry cryo-holder used to mount cryosamples. 9B is the image of the shadow of the cryoblades aperture once inserted.

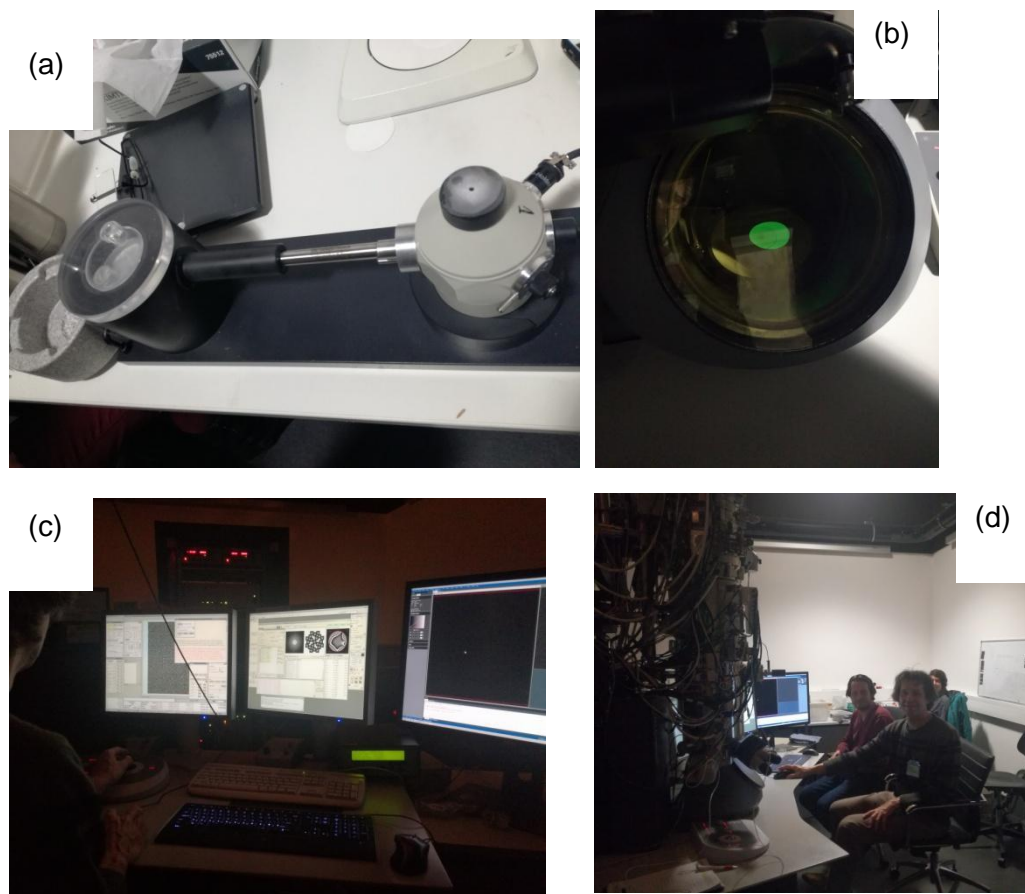


We had to take particular care for the insertion of the holder in the microscope that needs to be done in a relatively short time. At the same time the sequence of insertion operation requires a large tilt of the specimen holder that is not easily compatible with the Liquid nitrogen container. This means that a very quick insertion is necessary considering the fact that the sample is kept at low temperature with refrigeration.

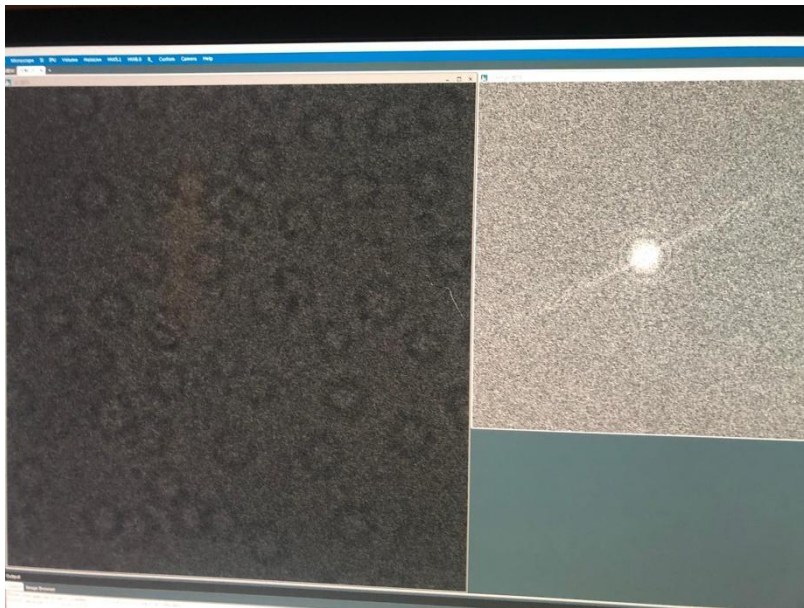
After a few attempts we optimised the procedure and reduced the insertion time to 1 min and kept the temperature below  $-177\text{ }^{\circ}\text{C}$  (fig c).

In addition it has been necessary to retract the cryoblades during the holder insertion since the goniometer was proposedly tilted to reduce the nitrogen loss and the tilted holder would not fit the blades with such a tilt.

Therefore the full operation required very accurate programming and cooperative work.



**Fig 9.** Images of the final testing of the cryoblades. (a) mounting of the sample on holder. (b) Test of electron beams through the cryoblades hole (c) monitoring of the temperature (d) image during experiments



**Fig 10.** First image of the protein EspB as taken in the acquisition system of the TITAN HOLO

The final results is a successful image of the EspB proteins as in fig 10. The image shows a typical contrast of protein with slight out of focus conditions. This demonstrates the full functioning of the system at least for basic cryomicroscopy

## 4 CONCLUSION

In this deliverable we demonstrated the efforts to produce a dedicated design for a cryo-blades system to be installed in TITAN HOLO microscope enabling to conduct cryo-microscopy experiment on biological specimen within Q-SORT project and still retain the compatibility with the day by day materials science use of the microscope.

The cryoblades introduced in the microscopes are highly nonstandard and required a complete redesign by FEI engineer and the specific fabrication out of the normal processing chain.

The splitting of the column is, by itself was a delicate operation that required a complete stop of the microscope operation and a realignment procedure. The whole procedure took more than 1 month and ended up with as successful installation.

Anticontamination effects , mechanical interoperability of the parts, retraction of the blades and finally normal operation of the microscope have been demonstrated and here documented.

## ANNEX 1: TEMPERATURE CONTROL

Tests have been conducted in Thermofisher with a simplified chamber shown below.

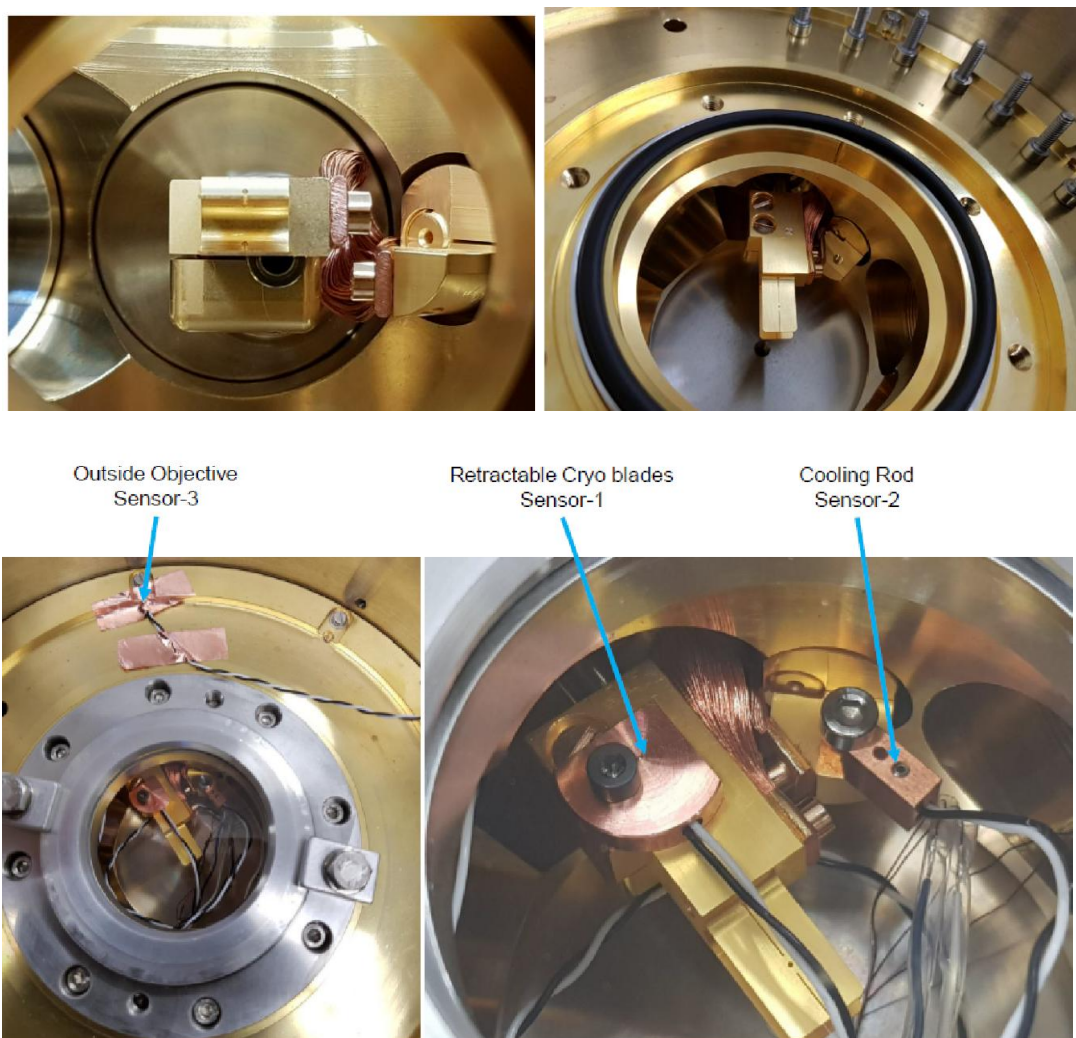


Fig S1 special measurement chamber for the cryoblades and temperature probe positions.

The measurements show that after 100 minutes after cooling the following temperatures have been reached

- Retractable cryoblades 110.2K
- Tip cooling Rod in Objective 102.7
- Outside inner block objective 297.1

Therefore the cryoblades reach a reasonable operational temperature

## ANNEX 2: REFEREE

### Referee 1

The deliverable describes one of the main objectives of the Q-sort project, i.e. the design, the realization and the installation of cryo-blades inside the Titan HOLO microscope.  
This device should allow for frozen biological specimens (cooled to liquid nitrogen temperature) to be safely studied in the microscope protecting them from contamination effect.

The deliverable is structured in three chapter.

In the first chapter the authors briefly introduce the very concept of cryo-blades for cryo-EM experiments on biological sample.

In the second, they describe the actual realization of the cryo-blades for the TITAN HOLO in Juelich: all the challenges encountered while adapting a machine meant for materials science to perform cryomicroscopy without losing its performances, are described very clearly and the main differences between the original project and the final realization are highlighted.

Finally, in the third chapter, they demonstrate the first experimental imaging of protein obtained with the HOLO.

The cry-EM experiment reported in fig.11 is a clear evidence of the correct functioning of the installed cryoblades and represent the main and noticeable result of the deliverable.

Still, I would like to suggest the authors to discuss the following topic:

1. In the introduction it is mentioned that one of the main requirements of the cryo-blades design is the compatibility with the OAM-sorter. Have the authors tested this compatibility?

*Yes The microscope has been largely used for the experiments of Holography both for this project and outside it. In particular in January we could align the OAM sorter. However due to the changes in the column the alignment, that is highly custom, required a large contribution of Thermofisher.*

Have the authors noticed any interference between the two devices?

*As said no interference but some extra work was required to re align the OAM sorter*

2. It appears to me that the cryblades drastically limit the tilt range of the sample holder. What it is the new tilt range? Will this affect the normal operation of the microscope?

*The new tilt angle is 40° that does not affect the normal microscope operation. Ift would only affect tomography*

3. According to the authors' experience, how well do the cryoblades installed on the Titan HOLO in Juelich compare with a dedicated cryomicroscope?

*The Cryoblades have comparable performances as normal cryoblades. Of course the rest of the microscope is not optimized for cryomicroscopy. The most important limitations arise from the sample loader. As explained the insertion with a manual sample holder is very outdated with respect to*



*autoloader technology. Moreover, we will need to use specialized software for standard operation that allows us to adjust automatically focus for every point.*

In addition:

- Figure 1 appears to be missing or misnumbered.

*Thanks we corrected the mistake*

## **Referee 2**

This deliverable describes the design and installation of cryoblades in a TITAN Holo microscope. It reports the technical details, the problems encountered and the solutions adopted. The final result is an image of proteins acquired with a reasonable contrast.

I have only few questions. Some comments are put in the text.

Q1: The detector used for image acquisition was single electron?

*Yes the detector was a Gatan K2 that is considered a good product for single electron imaging. We used "counting" mode that is expecially made for low dose measurements.*

Q2: The cryoblades, OAM sorter and Low-Mag STEM mode are all compatible?

*Yes we already made some preliminary OAM SORTER, scanning experiment in cryo mode. At that time the sample insertion was not working well but still we could verify the effective use of all these mode together.*

Q3: Is a new LN specimen holder under study?

*The side entry standard is an outdated technology and since this is only a demonstrator we can still reasonably work in these condition. If the QSORT technologies were to affirm on a commercial level an integration with real cryo microscopy with autoloader should be studied.*

Moreover I corrected typos all over the text

*Yes thanks, we applied the changes*

## ANNEX 3: ABBREVIATIONS

### SHORT NAME OF PARTICIPANTS

Partner	Country	Short Name
National Research Council (Project scientific coordinator)	Italy	CNR
Forschungszentrum Jülich, Ernst Ruska-Centre for Microscopy and Spectroscopy with Electrons	Germany	FZJ
FEI - Thermo Fisher Scientific	Netherlands	FEI
The Max Planck Institute for the Science of Light	Germany	MPI
University of Glasgow, Department of Physics and Astronomy	United Kingdom	UG
QED Film & Stage Productions Ltd. - UK	United Kingdom	QED
University of Modena and Reggio Emilia, Department of Physics, Informatics, and Mathematics - IT	Italy	UMR
Maastricht University, MultiModal Molecular Imaging Inst.	Netherlands	MU

### LIST OF ABBREVIATIONS

Consortium Agreement	CA
Description of Action	DoA
Description of Work	DoW
European Commission	EC
Grant Agreement	GA
Kick-off Meeting	KoM
Project Management Board	PMB
Work Package	WP
Work Package Leader	WPL