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ACRONYM: FLUFET
TITLE: FLow detection of virUses by graphene Field Effect Transistor microarrays

Instrument: HORIZON-EIC-2023-PATHFINDEROPEN-01

DELIVERABLE 4.1

VIRUS STOCK

Project Starting Date: 1 March 2024
Project End Date: 31 August 2027
Duration: 42 months

PROJECT CO-ORDINATOR: UDC
Coordinator's Organization Name: Universidade da Coruña

REPORTING PERIOD: **n.1** from 1 to 12

Reference Work packages:
WP4 Test and validation of the final device

Project funded by the European Commission within HORIZON-EIC-2023-PATHFINDEROPEN-01		
Dissemination Level		
PU	Public	X

Project Officer: LUCIA FARINA

Research funded by HORIZON EUROPE

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G.A. n° 101130125

European Innovation Council
Funded by the European Union

Document information

GA Number	101130125		Type of Action	EIC Pathfinder
Project name	FLow detection of virUses by graphene Field Effect Transistor microarrays			
Project name:	FLUFET			
Start Date	March 1st, 2024		Duration	42 month
End Date	August 31st, 2027			
Deliverable	D4.1 Viral Stock			
Work Package	WP4			
Project Month of Delivery	Contractual	M3	Actual	M3
Nature	Report	Dissemination Level		PU
Lead Beneficiary	ICGEB			
Responsible Author	Tea Carletti			
Contributions from	Alessandro Marcello			

Background on FLUFET

Infectious zoonotic diseases that jump from animals to humans are on the rise, and the risk of a new pandemic is higher now than ever before. Future health models need to consider the close connection between human and animal health, and new technologies capable of continuously monitor places where the risk of pathogens transmission is higher (shared by animals and humans) are urgently needed to prevent the human, socio-political and economic cost from pandemics.

Continuous monitoring and harmonized data collection of animal farms are required by the European Parliament. However, current methods are not suitable for an in-situ, continuous and automatic detection, so today only a limited number of specific pathogens are monitored.

FLUFET will be the first automatized sensor able of continuously detecting a broad spectrum of viral targets, and with the unprecedent capability of detecting unknown viruses. This sensor will be based on graphene Field Effect Transistors (gFETs). FLUFET will detect infectious zoonotic threats before they spread to humans and create potential outbreaks, opening the door for a pandemic's prevention continuum. It will bring the possibility to incorporate the long-distance external factors heavily affecting human health at worldwide level.

FLUFET brings interesting opportunities for Health and pandemics experts and managers, Policymakers and regulatory/ standardization bodies, Animal farmers and their associations, Precision livestock farming solution providers, Investors and researchers in the multiple disciplines involved in the consortium.

FLUFET requires an interdisciplinary consortium including partners from computational biophysics, graphene technology, nanotechnology, sensing, microfluidics, virology, surface engineering and sensor design and electronics

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Consortium Members

Nº	Role	Short Name	Legal Name	Country	PIC
1	COO	UDC	Universidade da Coruña	ES	999629718
2	BEN	BCMaterials	FUNDACION BCMATERIALS - BASQUE CENTRE FOR MATERIALS, APPLICATIONS AND NANOSTRUCTURES	ES	928273511
3	BEN (IO)	INL	Laboratorio Iberico Internacional de Nanotecnología	PT	988145985
4	BEN	BIOMA	ASOCIACION CENTRO DE INVESTIGACION COOPERATIVA EN BIOMATERIALES- CIC biomaGUNE	ES	998347572
5	BEN (IO)	ICGEB	INTERNATIONAL CENTRE FOR GENETIC ENGINEERING AND BIOTECHNOLOGY	IT	999470444
6	BEN	GSEMI	GRAPHENEA SEMICONDUCTOR SL	ES	910983940
7	BEN	VTT	TEKNOLOGIAN TUTKIMUSKESKUS VTT OY	FI	932760440

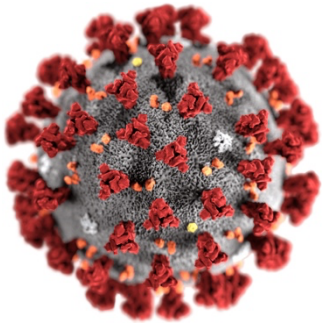
History of Changes

Version	Issue Date	Stage	Description	Comments	Contributor
1.0	13.05.2024	Draft	First Draft of D4.1		Tea Carletti
1.1	29.05.2024	Draft 2	Internal Review		Alessandro Marcello
1.2	30.05.2024	Final Verision	Report on D4.1		Tea Carletti Alessandro Marcello

D4.1 - VIRUS STOCK: Report on the procedures, methods, and results pertaining to the production and characterization of stocks of CoV-2, A/H1N1, and VSV

1. SARS-COV-2

Severe acute respiratory syndrome coronavirus 2 (**SARS-CoV-2**) is the causative agent of COVID-19 respiratory disease, responsible for the COVID-19 Pandemic. SARS-CoV-2 is a Betacoronavirus belonging to the family of Coronaviridae. It is an enveloped, positive-single stranded RNA virus with a spherical shape and diameter of about 100nm.



SARS-CoV-2 was originally isolated by the Molecular Virology Laboratory in ICGEB from a swab of an infected patient, as described in Licastro et al. 2020 (DOI: 10.1128/JVI.00543-20).

1.1 PROTOCOL FOR VIRUS PRODUCTON

- Day 1: seed $3x10^6$ Vero E6 cells into 3 cell culturing 175 cm^2 flasks with filtered cap;
- Day 2: infected cells with SARS-CoV-2 at a Multiplicity of Infection (MOI) of 0.01 in minimum medium (DMEM/2% heat inactivated Fetal Bovine Serum (FBS))
- Day 5: after evaluation of cytopathic effect the surnatant of the infected cells is collected and centrifuged at 4000 rpm, 4°C, for 15 min and subsequently filtered with a membrane filter 0.22 μm pore size. 0.5 ml aliquotes of the solution are prepared and stored at -80°C.

In order to determine the viral concentration the following protocol for Plaque Assay was performed:

1.2 PROTOCOL FOR VIRUS TITRATION

- Day 1: seed $6x10^4$ Vero E6 cells into each well of a 48-well plate;
- Day 2: one aliquot of SARS-CoV-2 was thawed and serially diluted (1:10) into DMEM. 100 μl of each solution was used to infect duplicate wells for 1 hr at 37°C. After 1 hour the virus was removed and the cells were washed with a solution of Phosphate Buffered Saline (PBS). Cell were then covered with a solution of DMEM/2% heat inactivated FBS/1.5% Carboxy Metil Cellulose (CMC)
- Day 5: the solution of DMEM/2%FBS/1.5%CMC was removed and cells were washed with PBS. 100 μl of 4% Paraformaldehyde (PFA) was added into each well to fix the cells for 15 minutes. After PFA fixation the monolayer of cells was stained for 20 minutes with a solution of 1% Crystal Violet/80% Methanol/20% PBS. The solution of crystal violet was then removed, and the plate washed with water.

Number of plaques was counted and multiplied for the dilution factor in order to determine the concentration of infectious viral particle in the solution.
SARS-CoV-2 STOCK CONCENTRATION = 1.4 x 10^6 PFU/ML

The SARS-CoV-2 virus was also sequenced with the Oxford Nanopore MK1c platform using the Artic workflow and version 4.1 of primers pool. The analyses confirmed that the virus produced was compatible with the original isolation and did not mutate during passaging in cells. The full sequence is available in the Appendix section of this report.

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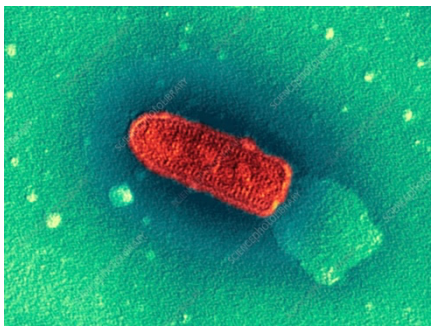
2. VSV

After a discussion with project partners during the kick-off meeting we decided to substitute Puumala Virus (PUUV) in the project with **Vesicular Stomatitis Virus (VSV)**. The reasons of this change are several. VSV belongs to the family Rhabdoviridae and is closely related to Rabies virus and Lyssavirus. VSV cause a contagious disease of livestocks. It mainly affects horses and cattles but can occasionally infect swine, sheep and goats making it an important pathogen of concerns to farmers. Moreover, VSV is a zoonotic virus and can lead to a flu-like illness in infected humans.



Finally, interesting for the aim of the project, is that VSV has different shape and size compared to the other 2 virus chosen, which are spherical and with a size of about 100 nm. In fact, VSV has a bullet shape and a size of about 70 nm diameter x 200 nm length. This characteristic will help "challenging" the system to understand if FLUFET device can detect viruses that vary significantly in terms of shape and size. Also, VSV is used as a model tool in virology for its characteristics of fast replication and safety, indeed it is classified as a Class 2.

For producing the stock of virus to be used during the FLUFET project an aliquot of VSV, Indiana Strain (ATCC VR-158), was used to infect Vero E6 cells as per the following protocol.

2.1 PROTOCOL FOR VIRUS PRODUCTON

- Day 1: seed $3x10^6$ Vero E6 cells into 3 cell culturing 175 cm^2 flasks with filtered cap;
- Day 2: infected cells with VSV at a MOI of 0.01 in minimum medium (DMEM/2% FBS);
- Day 3: after evaluation of cytopathic effect the surnatant of the infected cells is collected and centrifuged at 4000 rpm, 4°C, 15 min and subsequently filtered with a membrane filter 0.22 μm pore size. 0.5 ml aliquots of the solution are prepared and stored at -80°C.

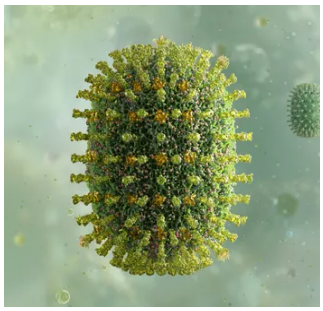
In order to determine the viral concentration, the following protocol for Plaque Assay was performed:

2.2 PROTOCOL FOR VIRUS TITRATION

- Day 1: seed $1.2x10^5$ Vero E6 cells into each well of a 24-well plate;
- Day 2: one aliquot of VSV was thawed and serially diluted (1:10) into DMEM. 200 μl of each solution was used to infect duplicate wells. After 1 hour at 37°C the virus was removed and the cells were washed with PBS. Cells were covered with a solution of DMEM/2% heat inactivated FBS/3% CMC;
- Day 3: the solution of DMEM/2%FBS/3%CMC was removed and cells were washed with PBS; 200 μl of 4% PFA was added into each well to fix the cells for 15 minutes at room temperature. After PFA fixation the monolayer of cells was stained for 20 minutes at room temperature with a solution of 1% Crystal Violet/80% Methanol/20% PBS. The solution of crystal violet was then removed and the plate washed with water;

Number of plaques was counted and multiplied for the dilution factor in order to determine the concentration of infectious viral particle in the solution.
VSV STOCK CONCENTRATION = 7 x 10^7 PFU/ML

3. A/H1N1



Human Influenza Avirus subtype H1N1 (**A/H1N1** or **H1N1**) is a subtype of influenza A virus. Different strains of A/H1N1 have been responsible for several pandemics as the 1918 Spanish Flu pandemic, the 1977 Russian Flu pandemic and the 2009 Swine Flu pandemic.
H1N1 is an orthomyxovirus with and elliptical shape and a size of about 80-120 nm diameter.

During the first months of project ICGEB worked on obtaining the permits to work with H1N1 virus and established a protocol and safety procedure for the proper handling of this pathogen in the BSL3 laboratories of the institute.

We initiated the procedure to obtain from the European Virus Archive Global (EVAg) the Human Influenza A(H1N1)pdm09 virus, A/Bretagne/06091/2023 reference strain.
The virus we will receive has been isolated in 2023 from a nasopharyngeal swab of an infected patient and has a concentration of $5.5x10^8$ PFU/ml. Full genome sequence is available in the appendix section of this report.

Here below we describe the protocol that will be performed to amplify and titrate the virus:

3.1 PROTOCOL FOR VIRUS PRODUCTON

- Day 1: seed MDCK cells into 175 cm^2 flasks with filtered cap;
- Day 2: infected cells with A/H1N1 at a MOI of 0.01 in minimum medium (MEM/0.3% FBS);
- Day 4: after 48 hours incubation at 37°C collect the surnatant, centrifuge it at 4000 rpm, 4°C, 15 min and subsequently filter it with a membrane filter 0.22 μm pore size. 0.5 ml aliquotes of the solution are prepared and stored at -80°C.

In order to determine the viral concentration the following protocol for Plaque Assay will be performed:

3.2 PROTOCOL FOR VIRUS TITRATION

- Day 1: seed $3x10^5$ MDCK cells into each well of a 12-well plate;
- Day 2: prepare serial dilution (1:10) of virus. 200 μl of each solution will be sed to infect duplicate wells. After 1 hour at 37°C the virus was removed and the cells were washed with PBS. Cells were covered with a solution of DMEM/Avicel 1:1;
- Day 5: the solution of DMEM/Avicel will be removed and cells washed with PBS before fixation with PFA for 15 min. The cells are then stained with crystal violet for 20 minutes and washed with water.

Number of plaques will be counted and multiplied for the dilution factor in order to determine the concentration of infectious viral particle in the stock.

ANNEXES

SARS-COV-2 SEQUENCE

NNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNTTGTAGATCT
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