



MONALISA: MOlecular **NA**nototechnology for **Life Science A**pplications

NANOTEC-CNR (LECCE)

G. SCOLES, & D. COGLITORE

**FROM MY PROBLEM TO THE DIAGNOSTICS
OF RARE NEURO-DEGENERATIVE DISEASES**

SCIENTIFIC EVENING OF OCTOBER 16, 2019

**Collaborators: Daniela CESSELLI (Pathology DEPT. UNIUD)
Cristina Diprimio (SNS) L. Viggiano & A. Gerbino(UniBari)**

Solutions (even cute 😊) in search of a problem to solve or PROBLEMS (even bad) in search of a solution that we want and can provide?

Metastatic cancer and Liquid biopsies

This has been the work of the last years. Now it is time to **Understand degenerative diseases**

(maybe it is too late for me, but I am trying anyhow!)

BUT FIRST LET ME NOTE THAT: IT IS VERY IMPORTANT TO GET THE CORRECT DIAGNOSIS FOR ANY DISEASE! my problem started in 2006 (micrografia) and for reasons that will become clearer later I lost several years

THERE I WAS in JANUARY '18 JUST BEFORE GOING TO PHILADELPHIA

I WAS STILL CONVINCED THAT I HAD A RARE FORM OF PD (Parkinsonism) WITH THE FOLLOWING SYMPTOMS: (**PARKINSONISM: A WORD** the use of which SHOULD BE FORBIDDEN)

- 1) COMPLETE LACK OF RESPONSE TO LEVODOPA THERAPY
- 2) NO TREMORS WHATSOEVER.
- 3) ALMOST CONSTANT FREEZING OF THE GAIT
- 4 TOTAL Micrographia (FIST SYMPTOM APPEARING)
- 5) RAPIDLY PROGRESSIVE BLEPHAROSPASM APPEARED AFTER A FEW YEARS SINCE DIAGNOSIS

BUT already 5 or 6 years before I HAD FOUND A 2002 paper that maintained that exactly these symptoms corresponded to a different illness that is: PURE AKINESIA WITH FREEZING OF THE GAIT (now a form of PROGRESSIVE SUPRANUCLEAR PALSY or PSP) Unfortunately this paper did not mention that the presence of TAU protein was an essential part of the diagnosis

BUT I HAD ALREADY DISCUSSED WITH MY NEUROLOGIST IN 2008

A 2002 PAPER THAT DISCUSSED THE POSSIBLE EXISTANCE
OF A PURE AKINESIA WITH GAIT FREEZING SYNDROME

HOWEVER NOTHING CAME OF IT MAINLY BECAUSE

1) THE DOCTOR HAD NEVER SEEN A PATIENT LIKE ME and

2) THE 2002 PAPER NEVER SPOKE OF THE TAUOPATHIC
NATURE OF THE SYNDROME THAT WOULD HAVE
ESTABLISHED ITS LINK WITH THE PROGROESSIVE
SUPRANUCLEAR PALSY (PSP)

3) THIS LINK WAS ESTABLISHED LATER WITH A VERY CAREFUL
CLINICAL POST MORTEM STUDY THAT USED THE LONDON
QUEEN SQUARE BRAIN BANK

After having wasted some time, I started looking out of

the BOX. But before we look into that we need to do a few preliminary things SUCH AS ASKING OURSELVES THE FOLLOWING QUESTION:

IS IT POSSIBLE TO ARRIVE AT A GOOD DIAGNOSIS BY CLINICAL OBSERVATION AND HOW DOES IT GET IT DONE?

POST VS PRE-MORTEM DIAGNOSTICS!

So how a good diagnose gets done at present? ,,

The first condition is that the doctors that evaluate you **MUST** have seen a few patients like you (a dozen or so).

The second is that the doctor that examine you must be up to date with the literature.

The **center for movement disorders** at U PENN where dr. DAVID COUGHLIN USED TO WORK TILL A FEW MONTHS AGO with a fresh PhD in PSP (12 cases)(NOW @ U. of C. San DIEGO) is one of the 2 places in the USA (THE OTHER BEING THE Mayo CLINIC IN Arizona (40 cases) where difficult cases can be found.

Pure akinesia with gait freezing a third phenotype of PSP

Abstract

The clinical syndrome of pure akinesia has most often been associated with progressive supranuclear palsy (PSP) and is characterized by difficulty initiating gait and "freezing" during walking, writing and speaking. Similar syndromes have been described under the rubrics of primary progressive freezing gait and primary gait ignition failure. We investigated the specificity of the clinical syndrome of pure akinesia with gait freezing (PAGF) for PSP-tau pathology. INVESTIGATING the BRAINS at the Queen's Square Brain Bank or QSBB **POST-MORTEM!!**

THE STUDY USING THE QSBB

Among 749 patients archived at the QSBB, only 7 fulfilled proposed diagnostic criteria of: gradual onset of freezing of gait or speech; absent limb rigidity and tremor; no sustained response to levodopa; and no dementia or ophthalmoplegia in the first 5 years of disease. **In these cases detailed pathological examination was performed.** PSP was the pathological diagnosis in six patients, and alpha synuclein(PD) in the seventh. Since the prevalence of PD is approx. 1% and PSP-Richardson. 1% of that (i.e. 1 part in 10,000) 1% of that gives a 1 case per 1/2M population.

In these six with PSP there were no additional features of coexistent vascular or PD (alphasyn) and the median PSP-tau score was 3, reflecting relative mild tau load.

The clinical syndrome of PAGF appears to have a high specificity for PSP-tau pathology. This relatively uncommon presentation of PSP-TAU pathology has less severe TAU accumulation than in the more common, "classic" PSP clinical phenotype: Richardson's disease which kills you in 8 years +/- 6 months a very narrow distribution!

And this adds another diagnostic criterium because for instance if I had RICHARSON syndrome I would be already dead (SEE THE NEUROLOGIST IN UDINE)

(c) 2007 Movement Disorder Society

Uses of TAU & state of the art

Comparative studies using **autopsy-confirmed samples** suggest that CSF total-tau (t-tau) and A β (1-42) levels can accurately distinguish FTLD from AD, with a high t-tau to A β (1-42) ratio diagnostic of AD.

.ALZHEIMER!!!!

The limit of detection of the assay, which requires 30 μ L of plasma, is 0.02 pg/mL (or 0.02 ng / L) (or \sim 0.5 fM concentration) [2], which is more than 1,000-fold more sensitive than conventional immunoassays. [Indeed elisa tests are said to have \sim 1 pM conc. detectability]

BEST NOT TO LET THE PROTEIN OUT OF THE CELLS

Because this level of sensitivity is unheard about, we need to look carefully into it!

Enter Daniela Cesselli and her study of Niemann Pick disease!

DURING HER TENURE IN MY ERC GROUP WHILE I WAS GOING IN AND OUT OF HOSPITALS IGNORING THE GAIT FREEZING (THREE SHOULDER OPERATIONS AND ONE SHOULDER PROTESIS INSTALLED) SHE DECIDED TO USE STEM CELLS TO ARRIVE AT A MOLECULAR MODEL OF A GENETIC DISEASE: NIEMANN PICK!

A human neuronal model of Niemann Pick C disease developed from stem cells isolated from patient's skin

Natascha Bergamin^{1†}, Andrea Dardis^{2†}, Antonio Beltrami¹, Daniela Cesselli¹, Silvia Rigo¹, Stefania Zampieri², Rossana Domenis¹, Bruno Bembi² and Carlo Alberto Beltrami^{1*}

**AUTOSOMAL RECESSIVE INHERITANCE
NEUROVISCERAL *LYSOSOMAL STORAGE DISORDER*, CAUSED
BY THE ABNORMAL FUNCTION OF **NPC1** OR **NPC2**
PROTEIN.**

THE DEFICIENCY OF EITHER OF THEM LEADS TO THE ACCUMULATION OF THE ENDOCYTOSED UNESTERIFIED CHOLESTEROL, **GANGLIOSIDES** AND **OTHER LIPIDS** WITHIN THE **LYSOSOME/LATE ENDOSOME (LE)** COMPARTMENT.

NPC1 GENE IS MUTATED IN 95% OF THE FAMILIES, *NPC2* IS INVOLVED IN RARE FAMILIES.

Niemann Pick-C disease: open questions

DESPITE THE PROGRESS IN CHARACTERIZING THE BIOCHEMICAL AND GENETIC DEFECTS IN NPC DISEASE, THE MECHANISMS UNDERLYING THE PATHOPHYSIOLOGY OF THIS DISORDER ARE NOT CLEAR AND THE CURRENTLY AVAILABLE THERAPEUTIC INTERVENTIONS ARE LIMITED:

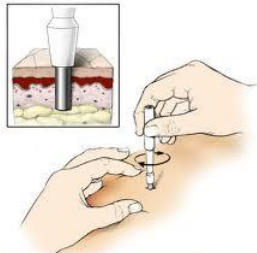
NP-C PATIENT MANAGEMENT IS OFTEN SYMPTOMATIC

TRANSLATION OF DATA OBTAINED FROM ANIMAL MODELS TO HUMAN PATIENTS IS NOT SIMPLE

LIMITED AVAILABILITY OF HUMAN NEURONAL MODELS OF THE DISEASE:

- FIBROBLAST ACCUMULATE CHOLESTEROL ONLY
- NEURONS ACCUMULATE CHOLESTEROL AND GANGLIOSIDES GM2 AND GM3

FROZEN
FIBROBLAST
CULTURE:
n=3 NP-C patients
n=3 Healthy Controls



FRESH SKIN BIOPSY

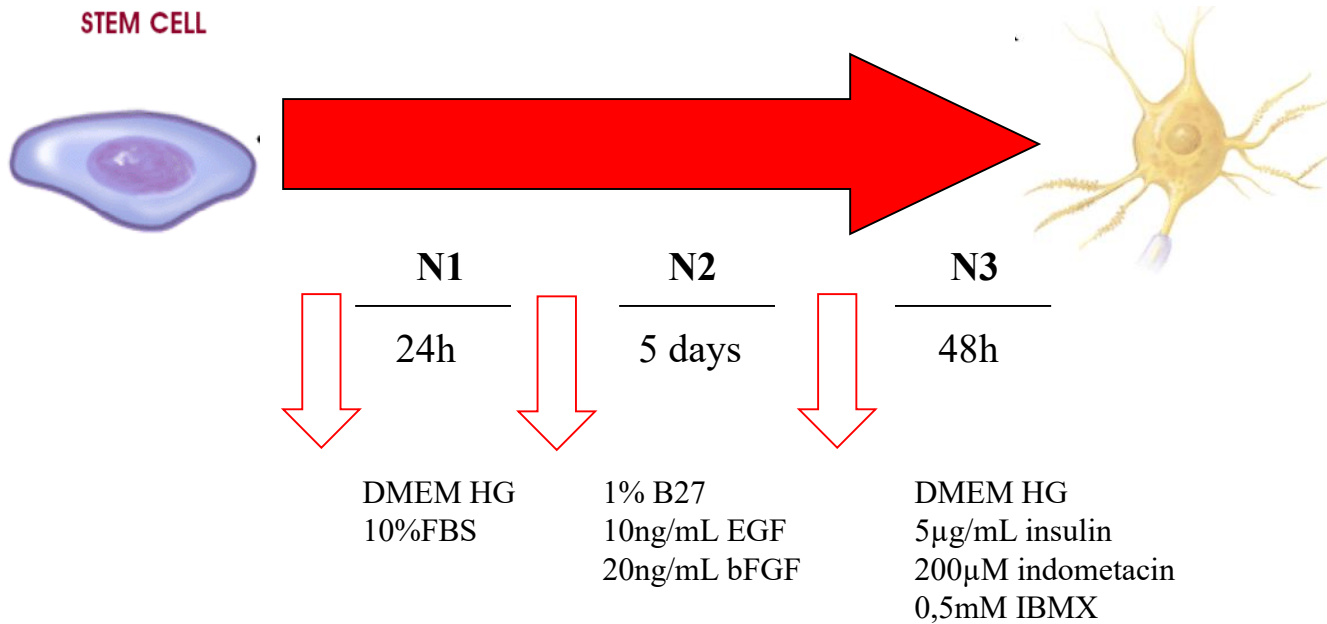


CULTURE IN A
MEDIUM SELECTIVE
FOR THE GROWTH
OF **HUMAN
MULTIPOTENT
ADULT STEM CELLS**
(hMASC)



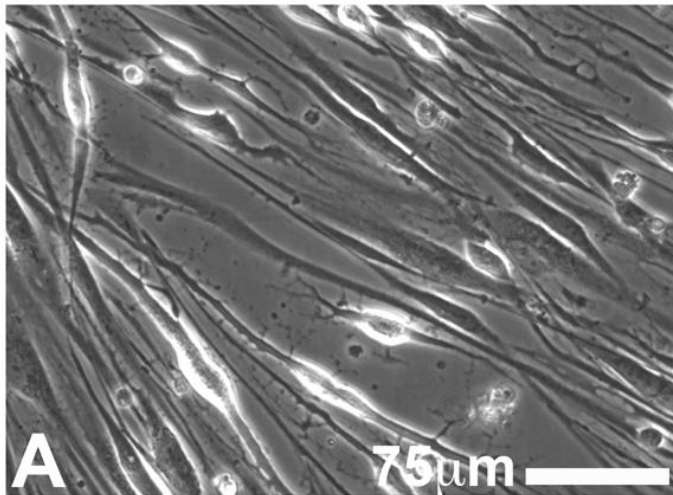
IN VITRO INDUCTION OF
NEURAL DIFFERENTIATION

NEURAL DIFFERENTIATION PROTOCOL

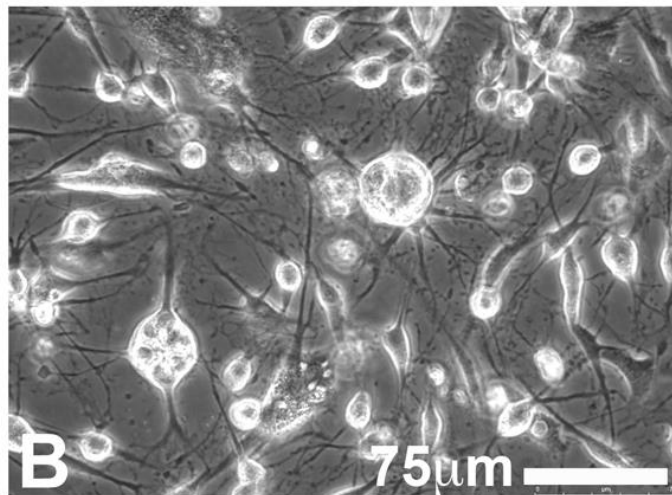


Neural differentiation

CTRL



NP-C

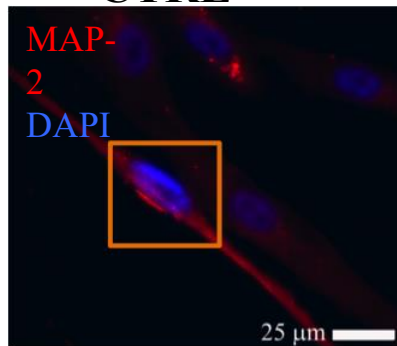


AFM characterization of differentiated cells

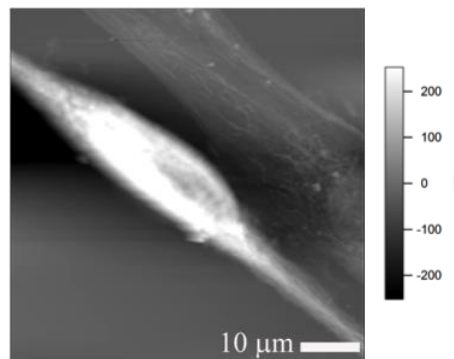
Dr. Anita
PALMA



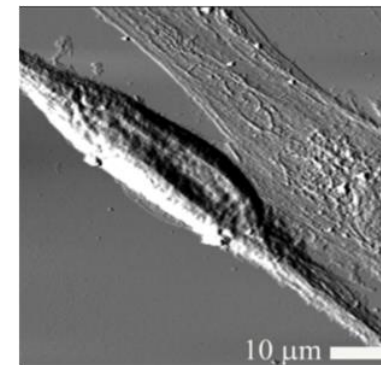
CTRL



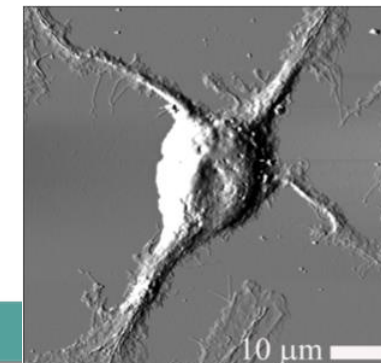
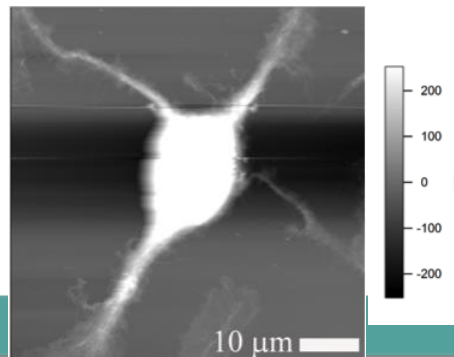
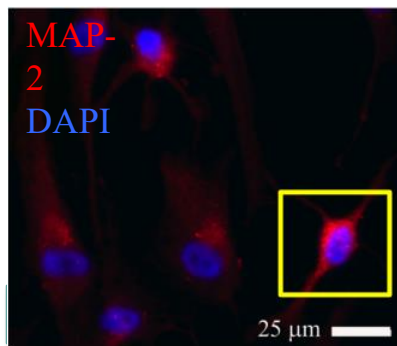
HEIGHT IMAGE



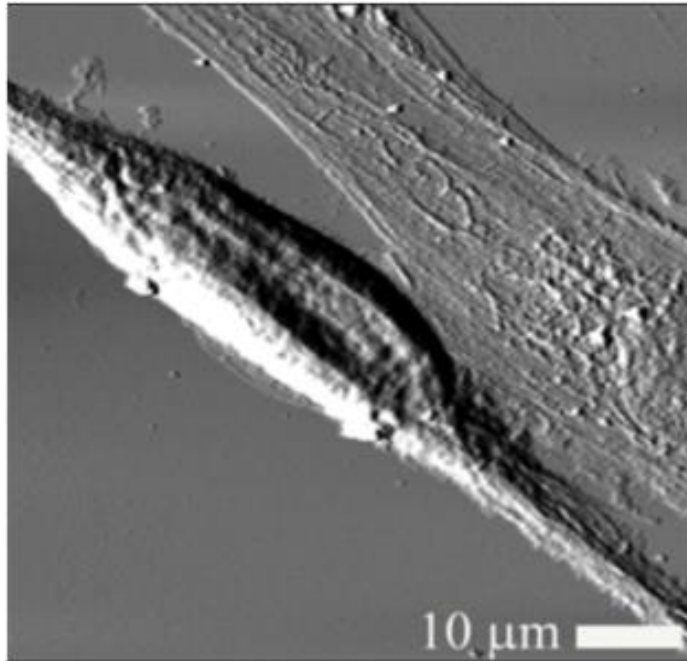
AMPLITUDE IMAGE



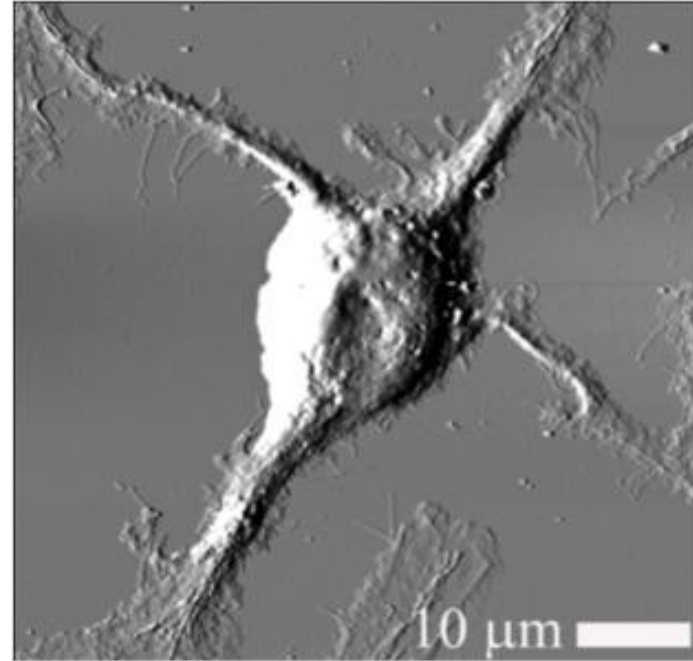
NP-C



CTRL



NP-C




Meganeurite


Ectopic dendritogenesis

Conclusions


It is possible to develop from NP-C patients a neural in vitro model resembling some crucial NP-C in vivo alterations:

1. Abnormal lipid accumulation (cholesterol and GM2 ganglioside)
 2. Morphological changes (meganeurite, ectopic dendritogenesis)
- 

This “PERSONALIZED” in vitro model can be used to:

1. understand the molecular basis of NPC neurodegeneration;
 2. evaluate the impact of different mutations on the pathological phenotype;
 3. perform drug screening on cells obtained from NPC patients presenting different genotypes;
 4. generate human neuronal models of other neurodegenerative diseases
- 

Main advantages :

1. it was developed directly from patient's cells and therefore it would be useful to analyze the effect of specific NPC1 mutations within the context of the patient genetic background;
 2. it was obtained through the differentiation of cells obtained from accessible sources;
 3. it did not involve the forced expression of transgenes in target cells, thus avoiding confounding results due to the reprogramming process (iPS).
- 

PROJECT:SCALES



Uses of TAU & state of the art

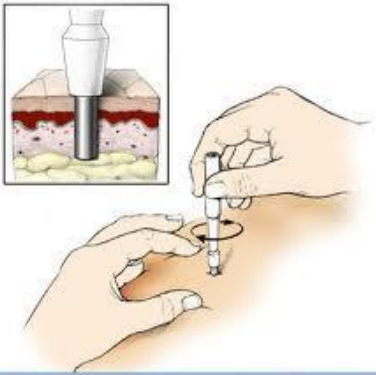
Comparative studies using **autopsy-confirmed sample** suggest that CSF total-tau (t-tau) and A β (1-42) levels can accurately distinguish FTLD from AD, with a high t-tau to A β (1-42) ratio diagnostic of AD. ALZHEIMER NOW THAT IT IS INVOLVED.....

The limit of detection of the assay, which requires 30 μ L of plasma, is 0.02 pg/mL (or 0.02 ng / L) (or \sim 0.5 fM concentration) [2], which is more than 1,000-fold more sensitive than conventional immunoassays. [Indeed Elisa tests are said to have \sim 1 pM conc. detectability]

Because this level of sensitivity is unheard about, we need to look very carefully into it!

BY INTUITION I DECIDED TO LOOK
INTO USING THE SAME SYSTEM
FOR SPORADIC DISEASES AND SHE

WENT ALONG WITH ME AND TRIED! ONLY
DIFFERENCE WAS THAT WE STARTED FROM
ADIPOSE TISSUE INSTEAD THAN FROM SKIN!



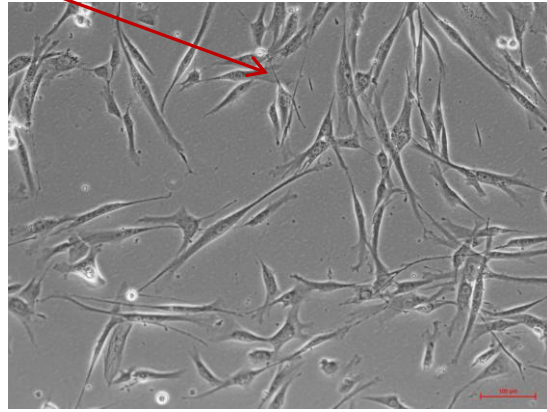
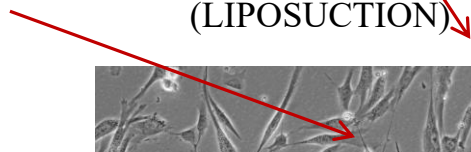
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IN VITRO INDUCTION
OF
**NEURAL
DIFFERENTIATION**

FRESH SKIN BIOPSY

ADIPOSE TISSUE
(LIPOSUCTION)

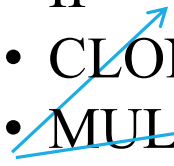


SUB-CULTURE IN A MEDIUM SELECTIVE
FOR THE GROWTH OF **human
MULTIPOTENT ADULT STEM CELLS**
(hMASC)

CHARACTERIZATION:

- FACS
- IF
- CLONOGENICITY
- MULTIPOTENCY

**STORAGE OF
FROZEN VIALS**



THE VERY ITALIAN STORY OF ANNA BERTOLINI

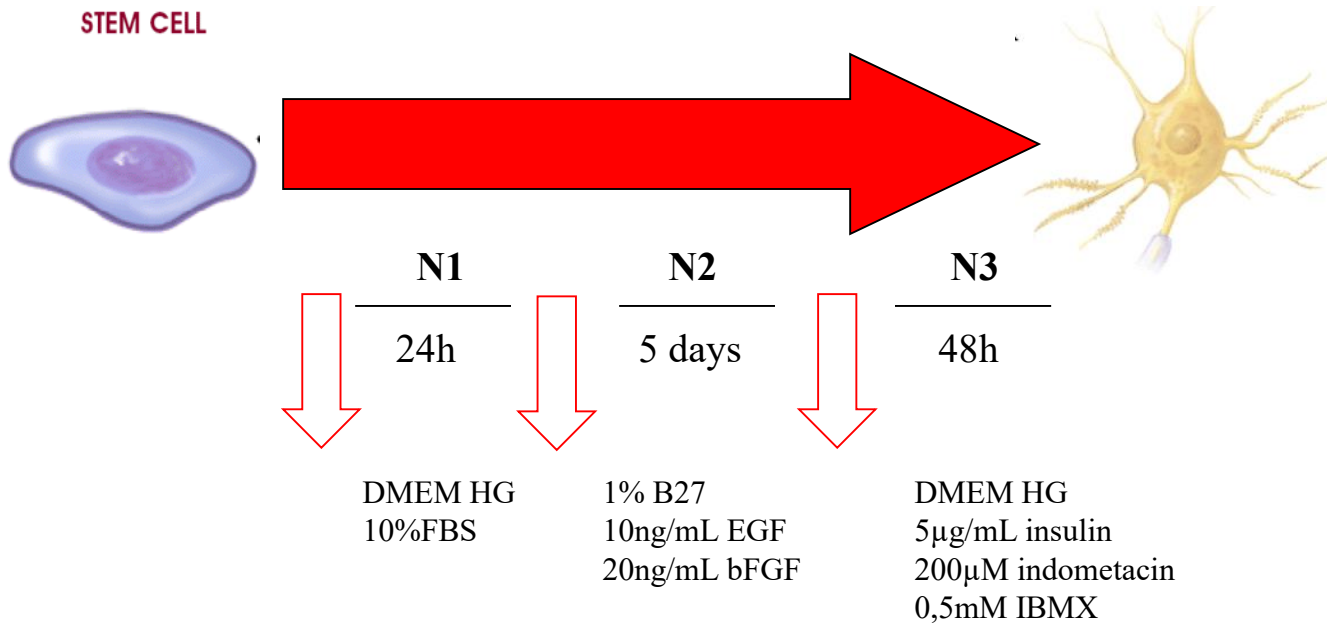
A TECHNICIAN HAPPY TO BE THAT.....

Seconda prova di differenziamento

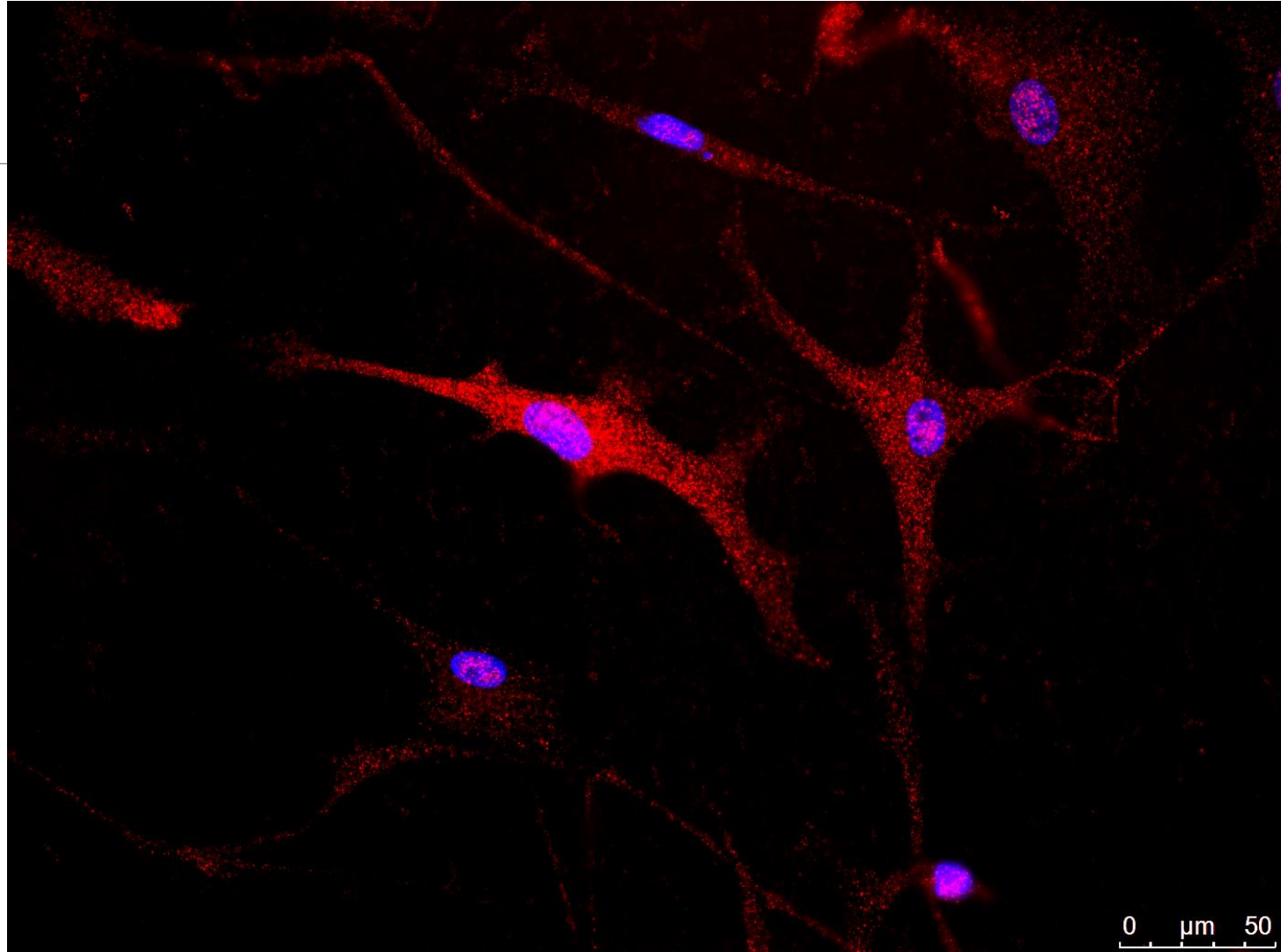
DONATORI SANI: H-SLIM 121 (75 ANNI); H-SLIM 130 (50 ANNI)

PAZIENTE: GS (84 ANNI)

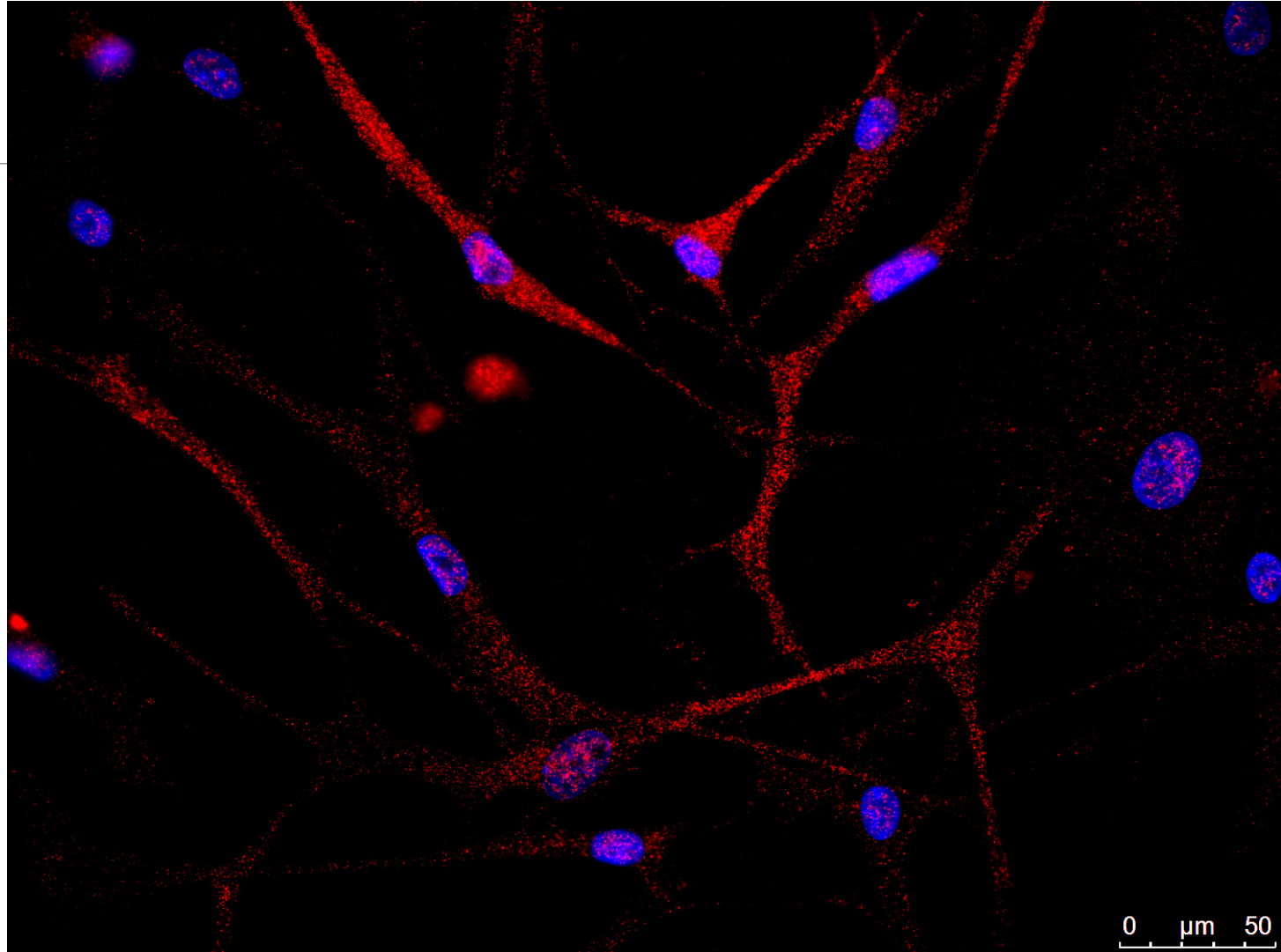
NEURAL DIFFERENTIATION PROTOCOL



TAU:
H-SLIM
121

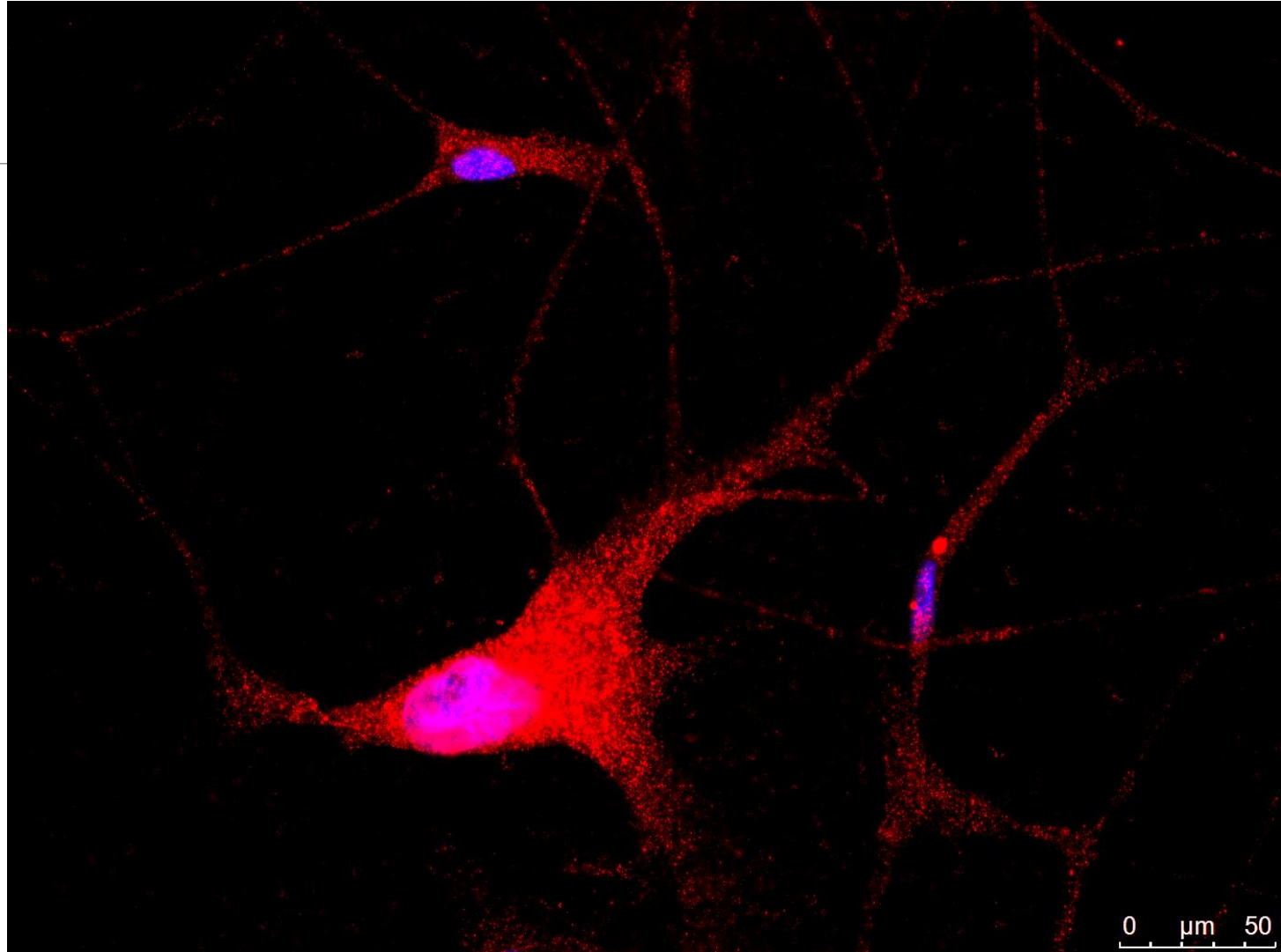


TAU:
H-SLIM
130



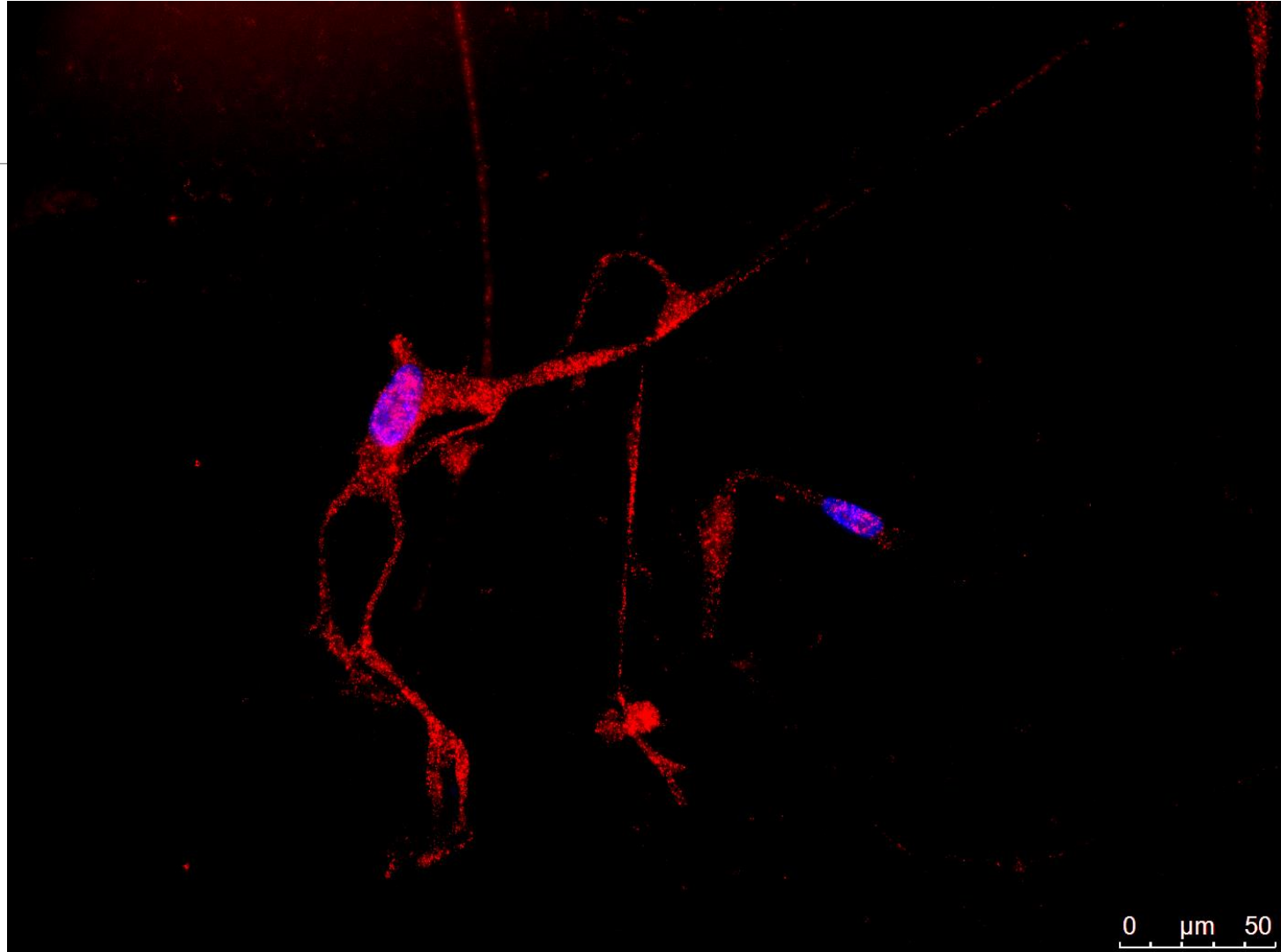
TAU: GS

Nota 1: presenza di
cellule molto più grandi
(ingrandimento
invariato)



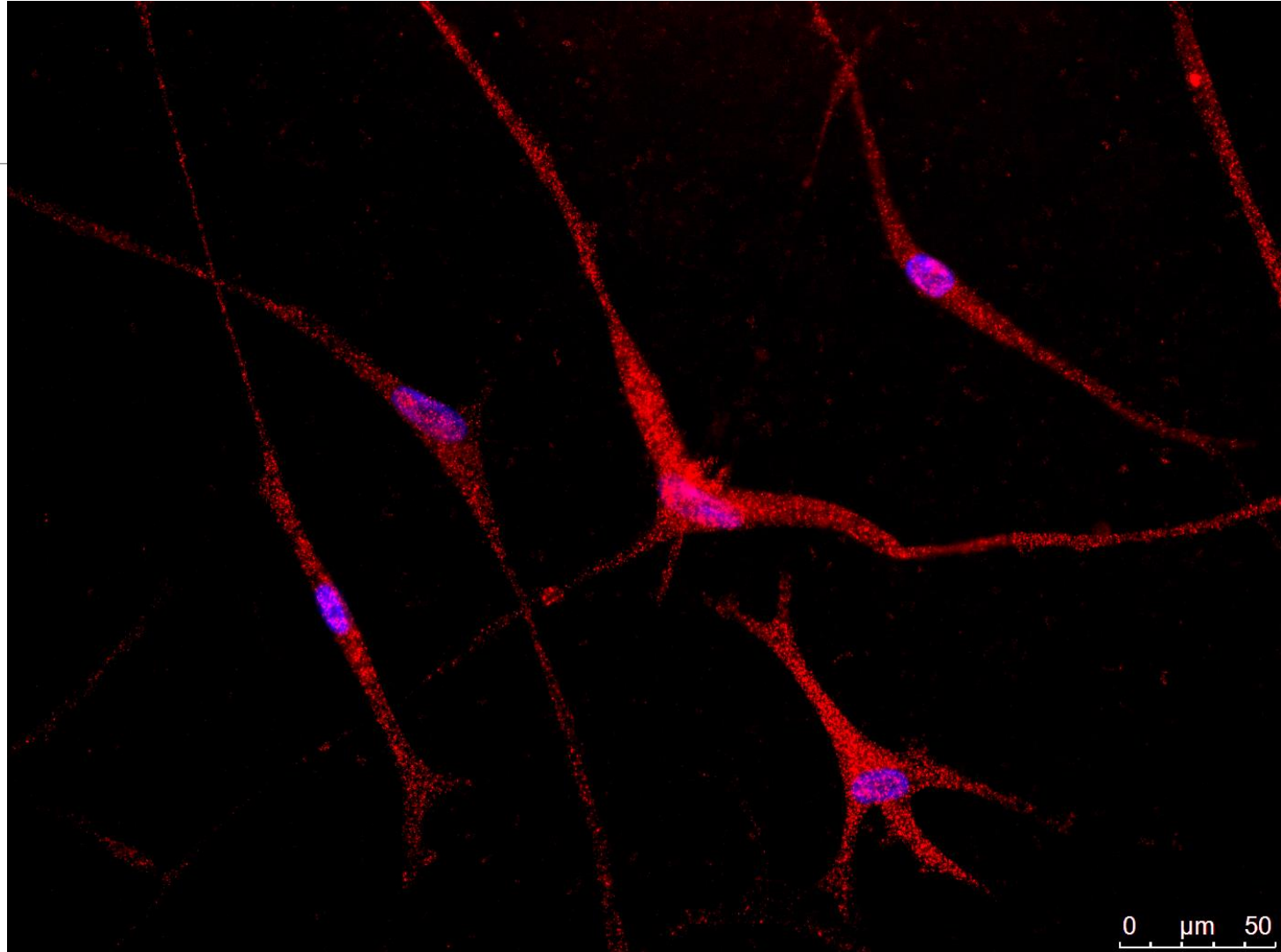
TAU: GS

Nota 2: presenza di cellule con prolungamenti così lunghi da staccarsi e risultare deformati (credo durante le varie procedure di colorazione). Forse la densità in questi vetrini era molto più bassa e ha spinto alla formazione di prolungamenti.



TAU: GS

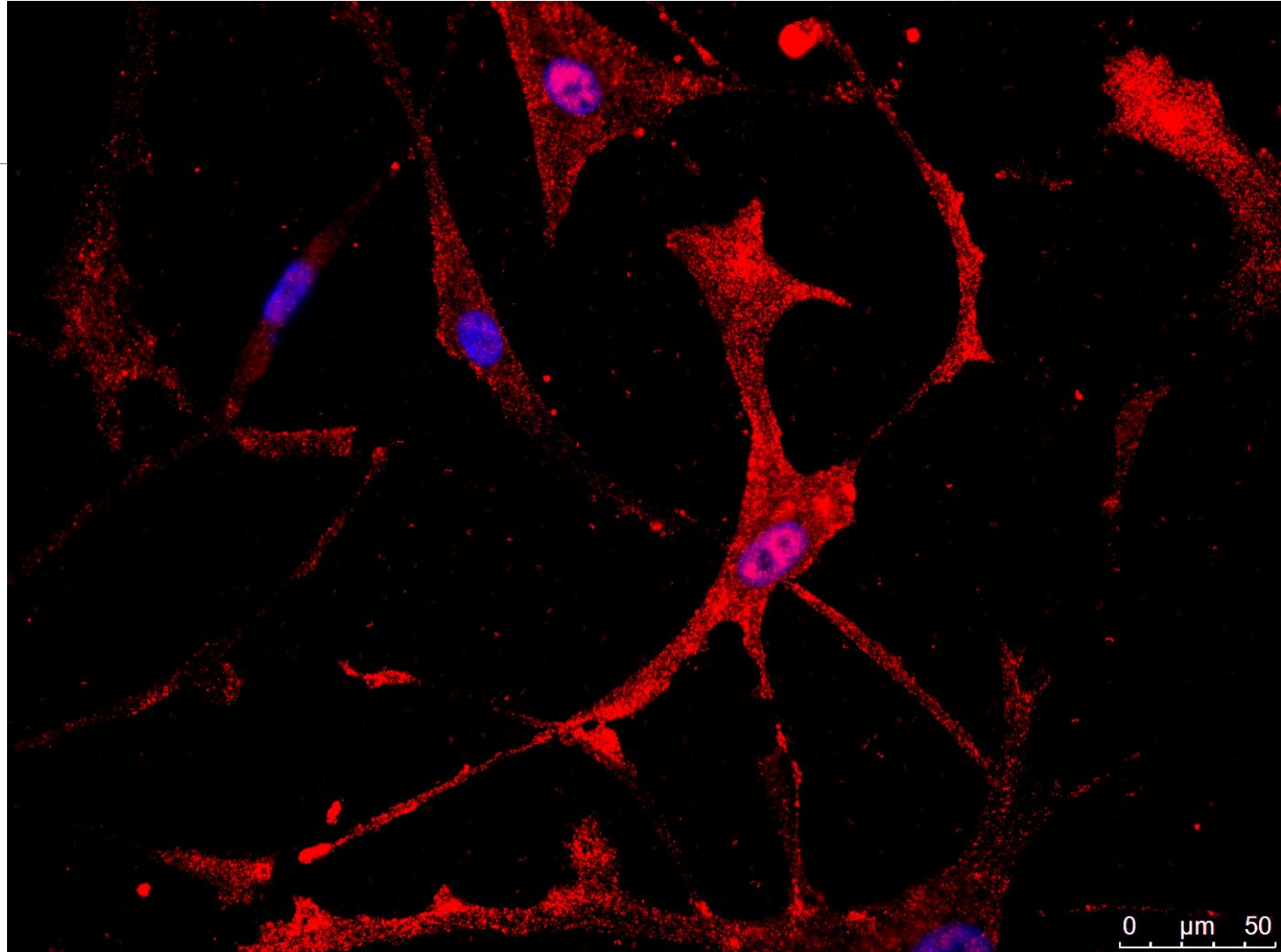
Nota 2: altro esempio di
cellule con lunghi
prolungamenti



MN1000: H-SLIM 121

L'anticorpo ha un pattern diverso rispetto a TAU:

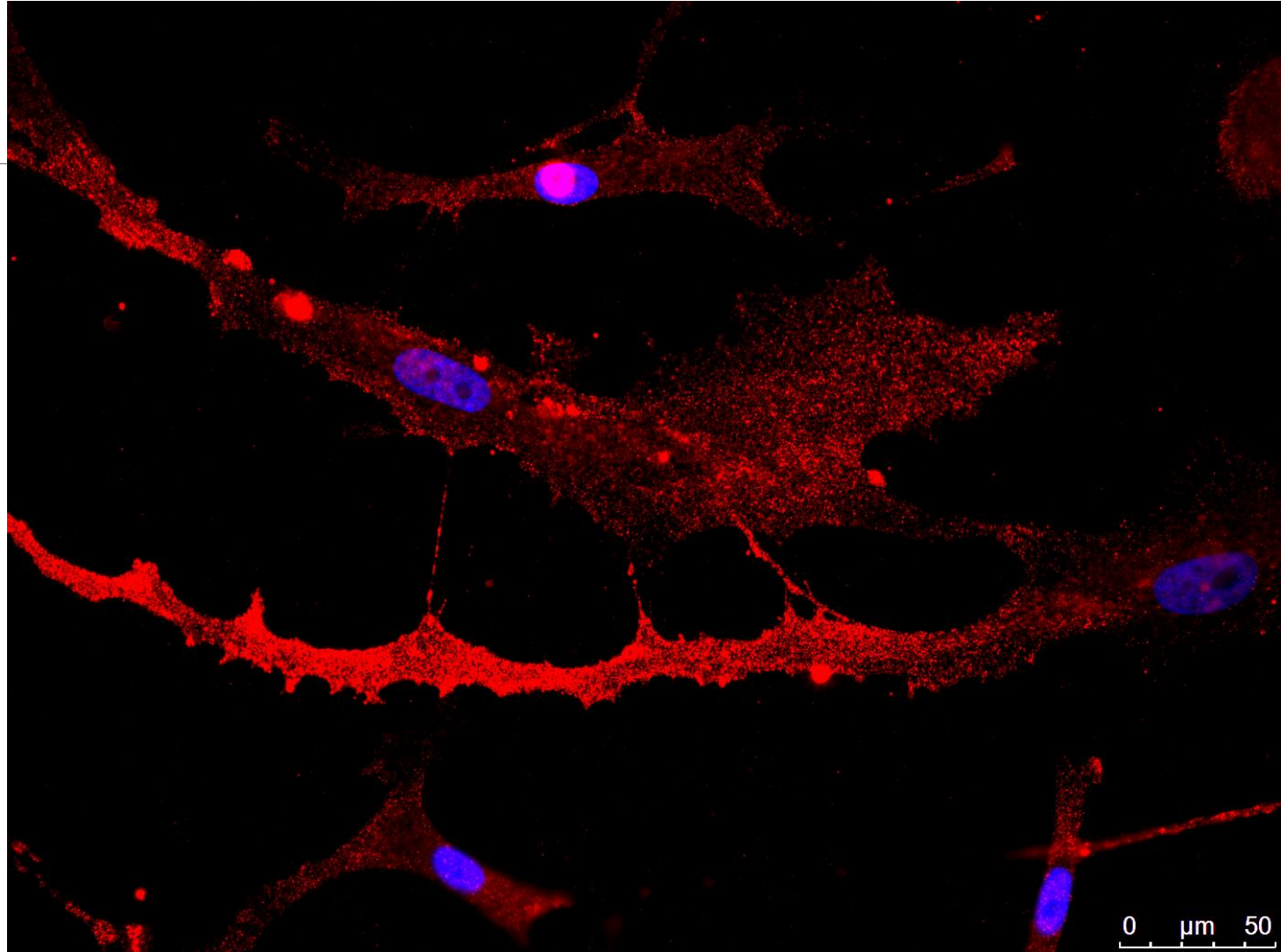
1. Marca le membrane, soprattutto nei prolungamenti e nelle estremità;
2. Si marcano formazioni vescicolari
3. Nel caso 121, ci sono cellule molto positive altre di meno.



MN1000: H-SLIM 121

L'anticorpo ha un pattern diverso rispetto a TAU:

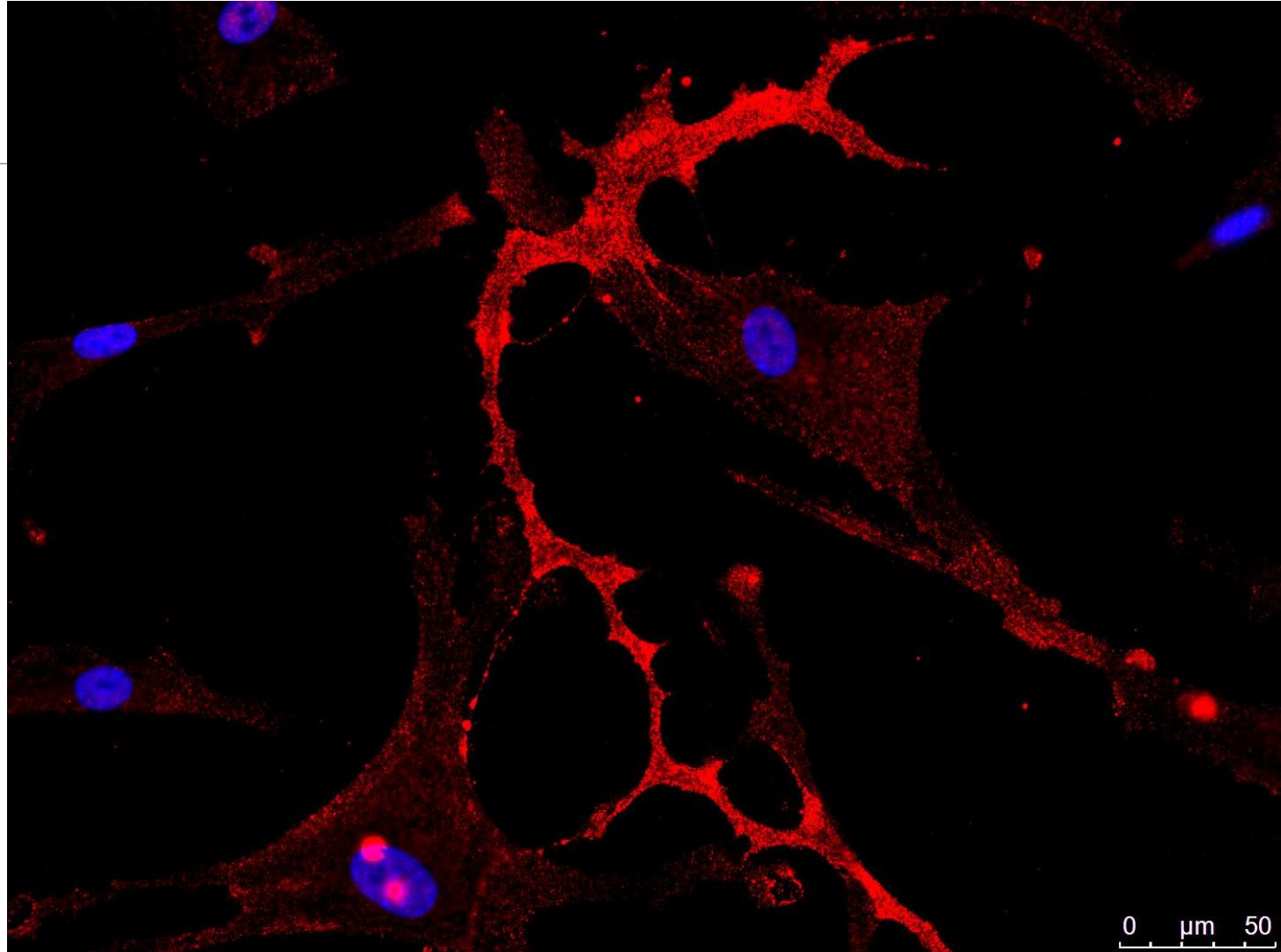
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MN1000: H-SLIM 121

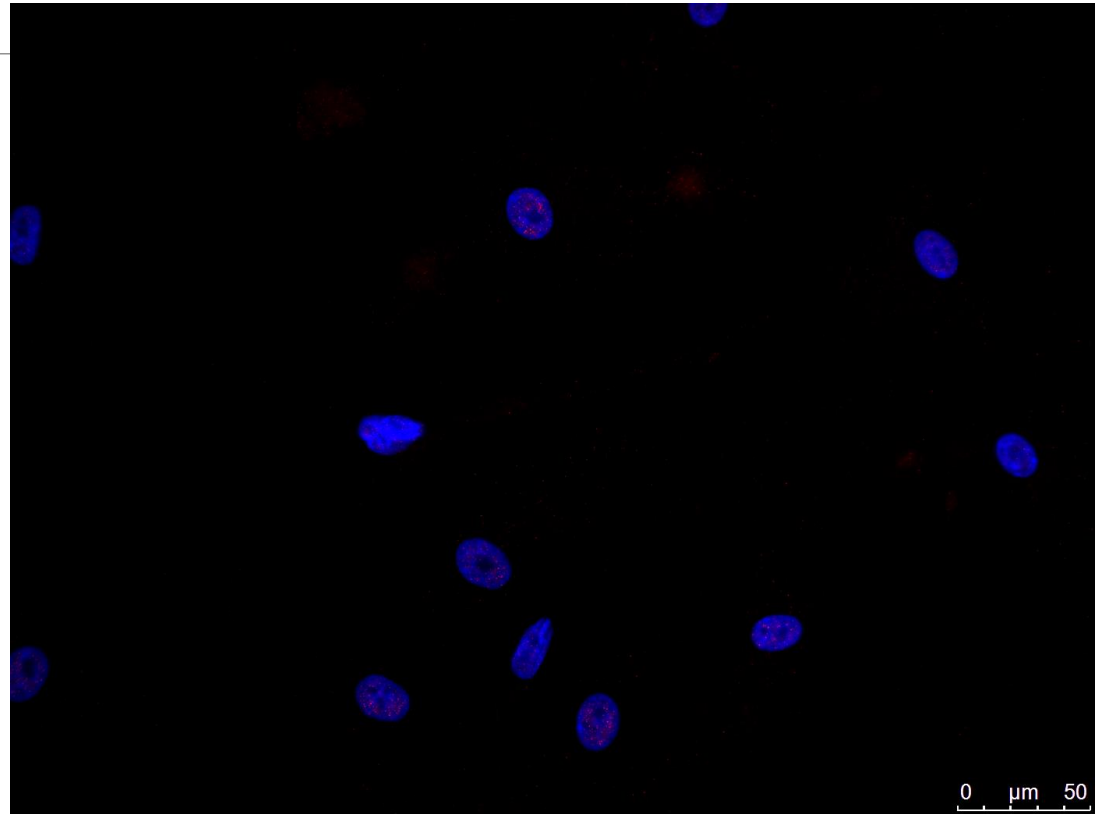
L'anticorpo ha un pattern diverso rispetto a TAU:

1. Marca le membrane, soprattutto nei prolungamenti e nelle estremità;
2. Si marcano formazioni vescicolari
3. Nel caso 121, ci sono cellule molto positive altre di meno.



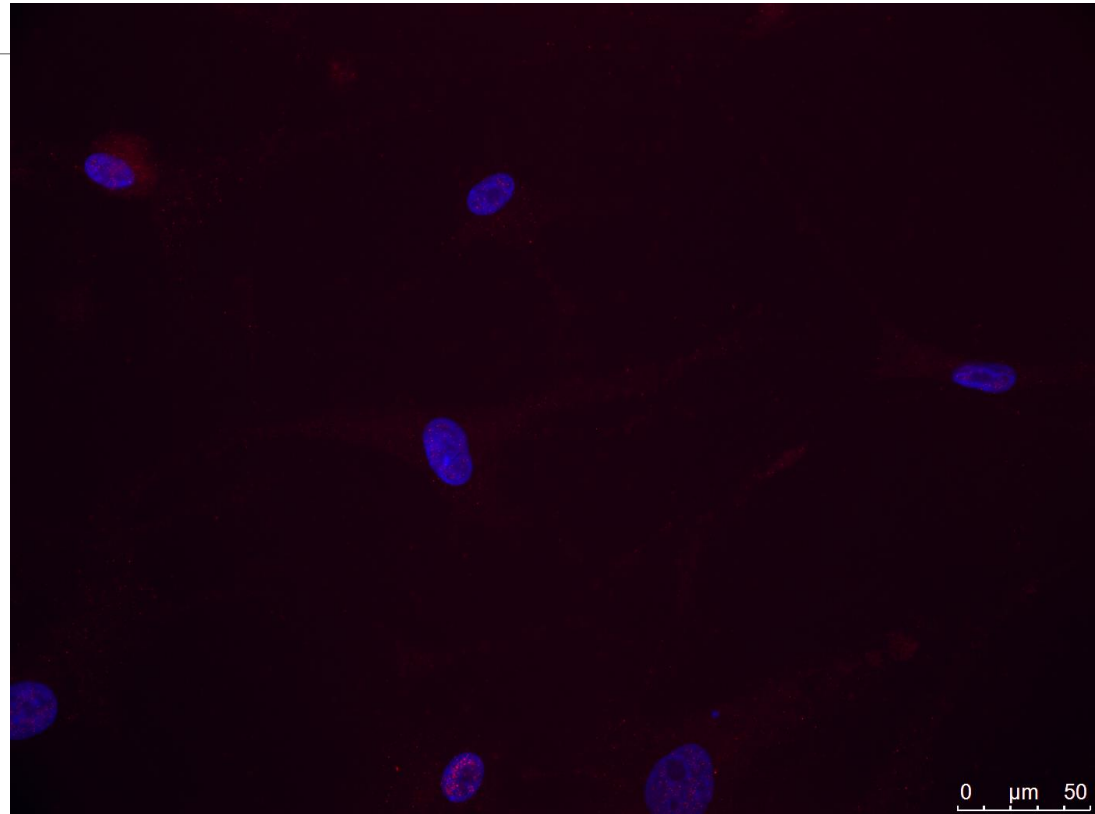
AT8: H- SLIM 121

Nota: si nota solo
una debole
positività
nucleare
puntinata



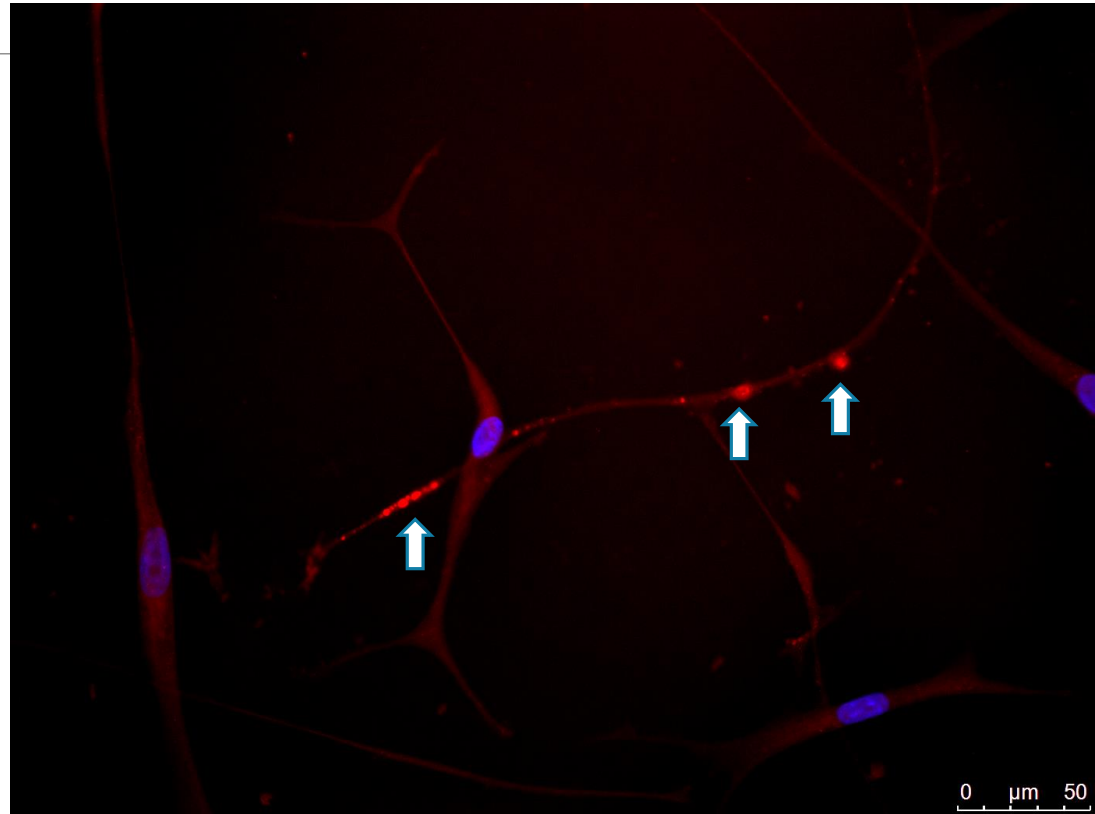
AT8: H- SLIM 130

Nota: si nota solo
una debole
positività
nucleare
puntinata

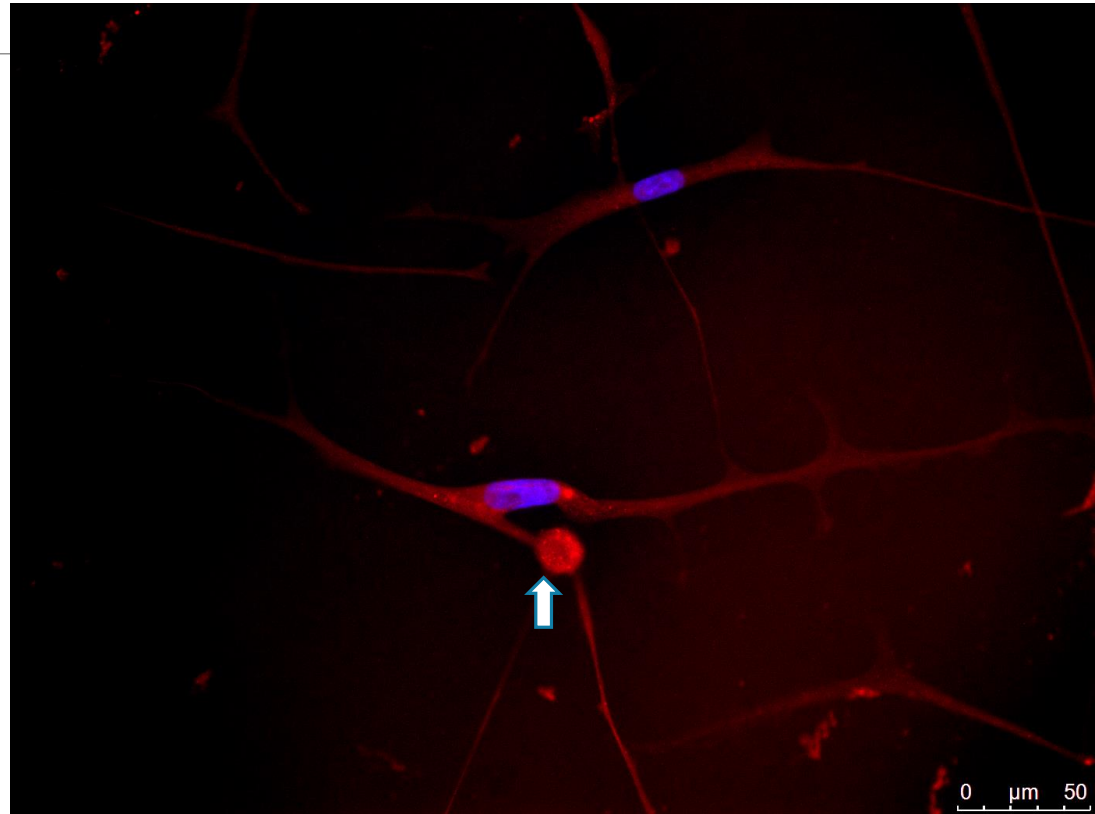


AT8: GS

Nota: il pattern è diverso. C'è una debole positività del citoplasma e la presenza di alcuni «accumuli»: vedi MN1000/GS2



AT8:
GS



ALL OF MY MARVELLOUS TEAM FOR HELP ACKNOWLEDGEMENTS

«MENTORS»: A. De Marco (N.Go) A. Laio (Sissa) S. Gustincich (Sissa) L. Casalis (Elettra) M. Lazzarino (IOM-CNR) S. Fortuna & M. Soler (N. Gorica & Sissa)

AND MY MOST PATIENT WIFE GIOK-LAN









In my opinion now is the time to start looking for a cure!

NOT BEFORE WE HAVE “COPLETED” THE CURRENT REVIEW OF THE DIAGNOSTICS METHODS.

FOR INSTANCE LOOKING INTO THE METHOD OF EVALUATING THE DNA DAMAGE IN LEUCOCITES OBTAINED FROM THE PERIFERAL BLOOD PLASMA

(see paper by VEA & Porcellini) VEA=

V. ENRICO AVVEDIMENTO (Mr. Epigenetics)

CESSELLI'S WORK ON NIEMANN PICK DISEASE

Genetic diseases are rel easy

WP1: Sviluppo di un modello in vitro di taupatia a partire dalle cellule staminali multipotenti dell'adulto (MASC) isolate da biopsie cutanee. Leader: UniUD.

Risultati preliminari ottenuti confrontando 3 linee MASC ottenute da donatori sani e da un paziente con PSP, hanno evidenziato che aggregati di tau fosforilata erano presenti esclusivamente nelle cellule del paziente PSP dopo differenziamento ed assenti nelle cellule indifferenziate del paziente e nelle MASC indifferenziate e differenziate dei soggetti sani. Pertanto, in collaborazione con il Centro di Malattie Rare dell'Azienda Sanitaria Universitaria Integrata di Udine (ASUIUD), si applicheranno i protocolli già ottimizzati nei laboratori di UniUD per isolare MASC da campioni di tessuti umani (Bergamin N, et al. Orphanet J Rare Dis. 2013;8:34). Presso UniUD è anche presente una biobanca di linee MASC ottenute da soggetti sani di diversa età che potranno essere utilizzate come controllo. Una volta ottenute le MASC si procederà al differenziamento neurale secondo protocolli ampiamente utilizzati presso UniUD, sia per MASC ottenute da soggetti sani che da pazienti (Domenis R, et al. PLoS One. 2014;9:e89232; Bergamin N, et al. Orphanet J Rare Dis. 2013;8:34; Cesselli D, et al. Circ Res. 2009;104:1225-34; Beltrami AP, et al. Blood. 2007;110:3438-46; Domenis R, et al. Stem Cell Res Ther. 2015;6:2; Bourkoula E, et al. Stem Cells. 2014;32:1239-53; Cesselli D, et al. Am J Pathol. 2011;179:349-66). Cellule indifferenziate e cellule differenziate saranno valutate mediante immunofluorescenza e microscopia confocale per evidenziare l'espressione di marcatori neuronali (MAP2, NeuN e sinaptofisina) e di tau, sia totale che fosforilata in specifici siti, per evidenziare l'eventuale presenza e localizzazione di aggregati neurofibrillari patognomonici di taupatia.

An application will be described to the diagnostics of neurodegenerative diseases choosing the area where the methods would be more beneficial (i.e. the area of rare diseases). We will consider a class of TAUopathies, known as Progressive Supranuclear Palsy (PSP), both in their main phenotype and in the rare PAGF phenotype i/e/ Pure Akinesia with Gait Freezing that has an "abundance" of 1 over 0.5 million individuals. Our approach involves the production of pluripotent stem cells from the adipose tissue of a well, clinically diagnosed, individual and their subsequent differentiation to neurons. If the latter result to be diseased then the road to the cure is possible and well planned. The main difficulty is in the complexity of the brain that sees, for example, the localization of the disease in the brain stem. The method has been, however, already proven to be effective in genetic diseases but our interest involves the more difficult case of sporadic diseases

Our goal is to ~~will need to~~ establish the level of production of protein TAU and compare it to the cells of a healthy individual from the adipose tissue of which the neurons will be produced following a similar protocol. Two complementary approaches will be followed: (i) if the cellular dilution factor (diseased neurons over the total number of neurons obtained) will not prove to be too limiting, we will be able to proceed directly for the molecule that may wash out the extra amount of TAU. Otherwise (ii), we will need to go to the second level looking to establish the presence of disease molecular fingerprint at the single cell level establishing, in such way, the heterogeneity of the disease. We plan to do that by means of nanopipettes that will have their interior coated with antibodies or nanobodies that recognize the TAU protein. When the nanopipette will suck out some of the cell fluid the TAU will stay inside the nanochannel of the pipette changing its ionic conductivity.



ALL OF MY MARVELLOUS TEAM FOR HELP ACKNOWLEDGEMENTS

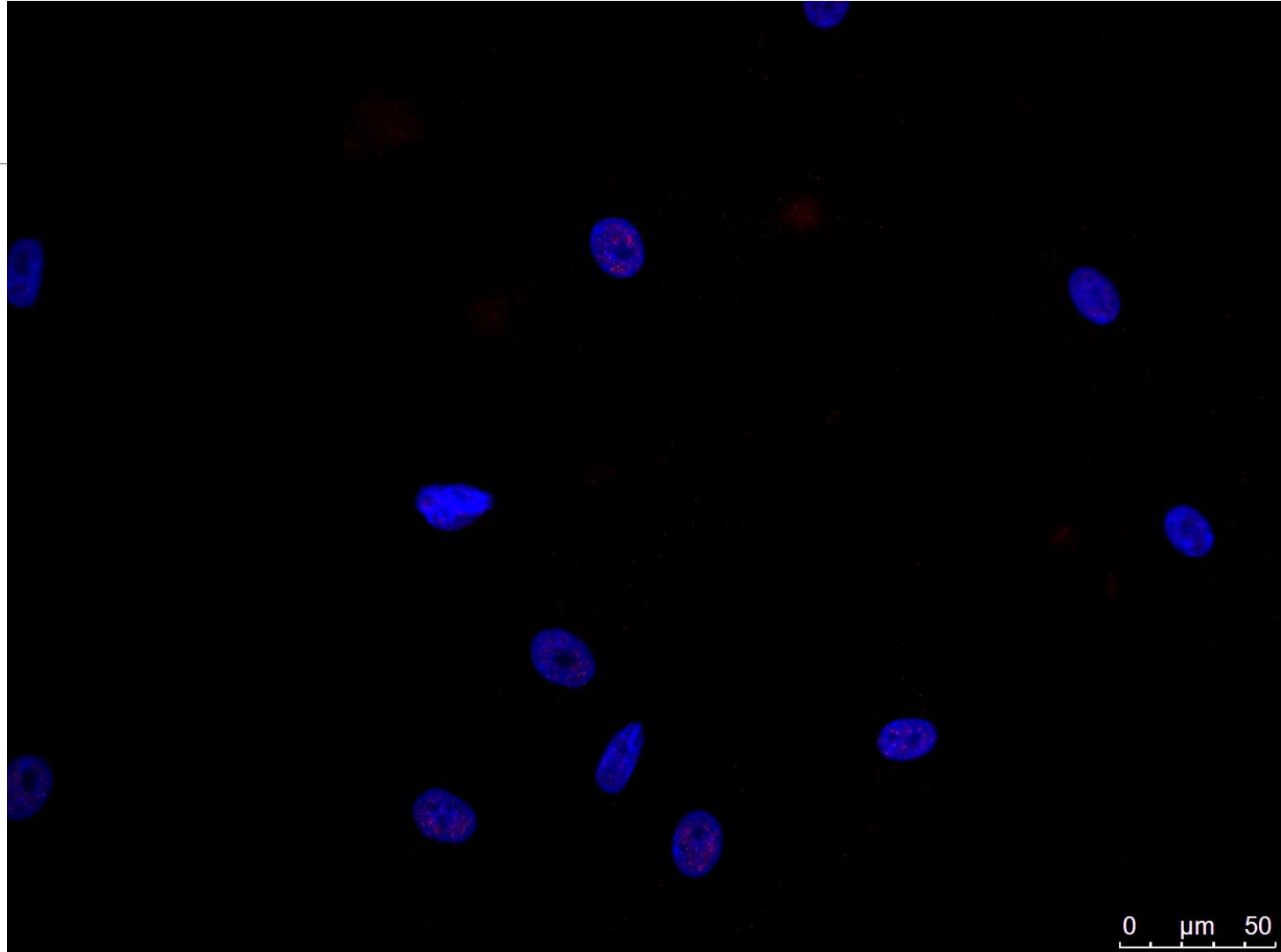
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AND MY MOST PATIENT WIFE GIOK-LAN



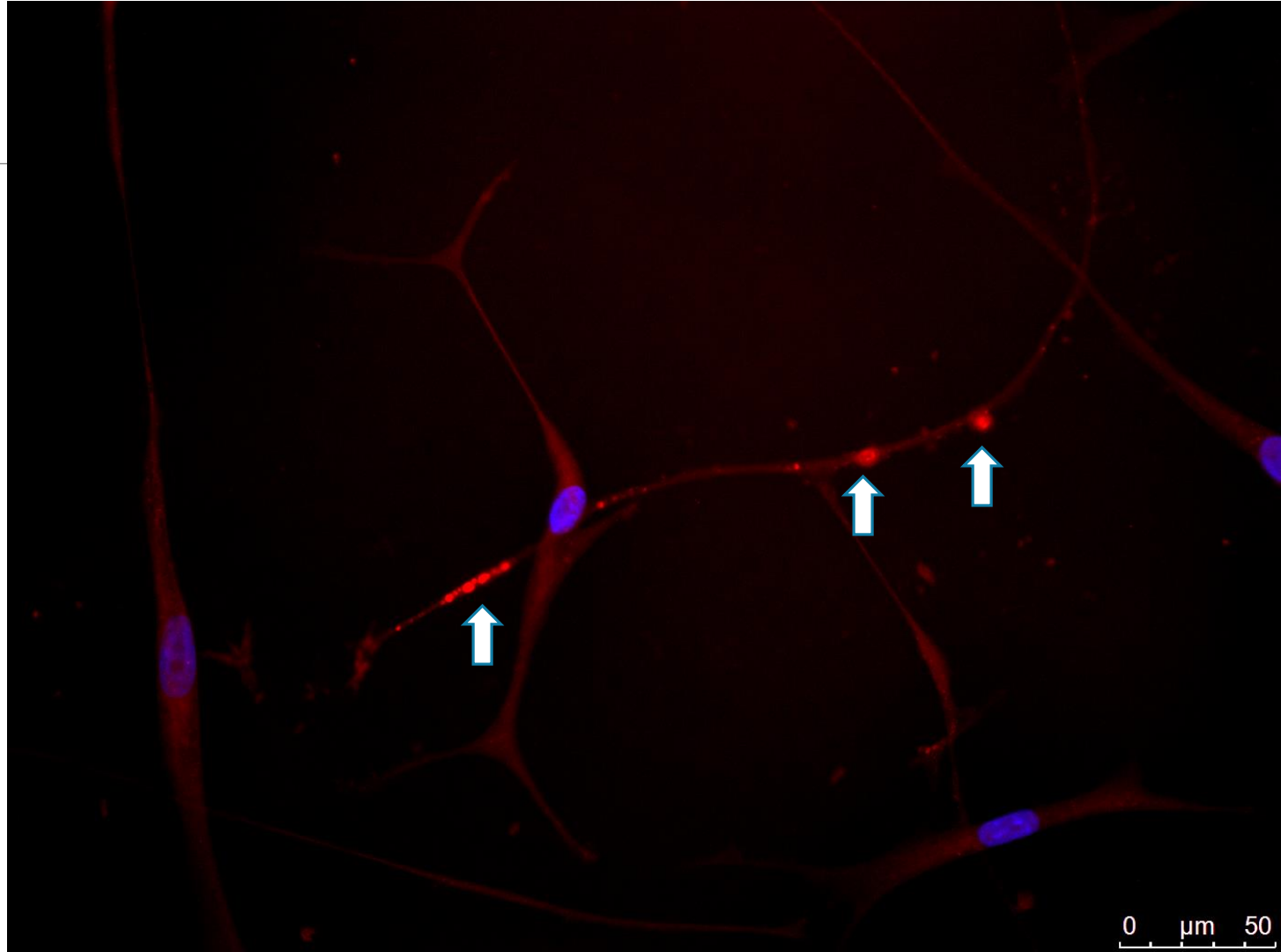
AT8: H-SLIM 121

Nota: si nota solo una
debole positività
nucleare puntinata



AT8: GS

Nota: il pattern è diverso.
C'è una debole positività
del citoplasma e la
presenza di alcuni
«accumuli»: vedi
MN1000/GS2









In summary:

1) I need to establish that my illness is a TAUopathy

a) to confirm the diagnosis and b) to decide to ask to participate in a phase 3 study conducted at U PENN with an antibody that is supposed to clear your brain from TAU. But the majority of participants have PSP-Richardson (level of TAU in CSF 200 times that in blood for healthy and diseased pers.

c) even in the case that I confirm the presence of tau in my peripheral blood it is not at all sure that I ask to participate in the study unless they show me that the class of patients with PSP-PAFG has been considered separately and the results have been found satisfactory including side effects.