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Utilizing Enhanced Green Fluorescent Protein (EGFP) as a Reporter Gene to Study the Nuclear/Cytoplasmic Localization of the Transcriptional Repressor ICER

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ABSTRACT

Cell division is a highly regulated, multi-complex system requiring an abundance of proteins working in conjunction with one another. This unity is what ensures proper development of the cell before entering a new phase in the cell cycle. Cells that infringe multiple cycle stages can become malignant, proliferating at an untamable rate. The transcriptional suppressor protein, Inducible cAMP Early Repressor (ICER), is present in some, but not all, cancer cells. ICER functions as a transcriptional repressor of the cAMP Response Element (*CRE*) gene in aberrant cells which could effectively prevent them from bypassing cell cycle checkpoints and proliferating unchecked. Downstream effects of *CRE* repression can prevent normal transcription of genes such as cyclin A; coding for a protein that is heavily involved in the S and G2 phases of the cell cycle. ICER has also been shown to have a role in secondary messenger pathways such as β-adrenoceptor pathways. β-adrenoceptors are involved in sympathetic regulation among several organ systems including the circulatory and respiratory systems.

Two enhanced green fluorescent protein (EGFP) constructs and Nikon DAPI-FITC-TRITC triple band excitation were used to determine the site of ICER-IIγ localization in human melanoma cells, SK-MEL-24. The constructs were designed to have EGFP on either the N-terminus or C-terminus of ICER-IIγ. We sought to establish if EGFP-hindered termini would affect nuclear localization of ICER-IIγ. Data demonstrated that the loci of EGFP tagging had no effect on nuclear localization. Interestingly, when EGFP was bound at the C-terminus, it was observed in the centrosomes of mitotic cells. Moreover, ICER-IIγ presenting cells with free N-termini were in higher abundance in nuclear cells than those with free C-termini. Our findings suggest that the N-terminus of ICER-IIγ may have a role in mitotic events.

MONTCLAIR STATE UNIVERSITY

Utilizing Enhanced Green Fluorescent Protein (EGFP) as a reporter gene to study the nuclear/cytoplasmic localization of the transcriptional repressor ICER.

by

Melissa Cabral

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UTILIZING ENHANCED GREEN FLUORESCENT PROTEIN (EGFP) AS A REPORTER GENE TO STUDY THE NUCLEAR/CYTOPLASMIC LOCALIZATION OF THE TRANSCRIPTIONAL REPRESSOR ICER

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ACKNOWLEDGEMENTS

In July of 2020, I approached Dr. Molina with my interest in being a part of his extensive laboratory. As a nervous graduate student I've spent a whole week before our initial meeting reading a majority of his papers. Each paper I've read, I was fascinated with *ICER*'s involvement in different pathways. For its commitment to potentially sabotage a cell's vitality was left unnoticed, especially with his papers that related to cancer. Who knew that a transcription factor that isn't widely known can carry so much weight as a regulator in those pathways. Although I was determined to work with Dr. Molina, I was prepared for the rejection that could come my way. I've never had Dr. Molina as a professor, nor did he know my dedication of being a life-long student. The unacquainted relationship we previously had truly had rejection waiting at my doorsteps. However, when we finally had our initial meeting, Dr. Molina had arrested any of my doubts that I had and graciously welcomed me to be part of his laboratory. There are many projects that Dr. Molina was working on, that I could have been involved in. However, he ensured that I would be a part of something that wasn't just going to be meaningful but it was also going to be feasible with the little amount of time that I had left here. For the times when our schedules had conflicted, he had always managed to make everything work. Thank you Dr. Molina for allowing me to be a part of your amazing team and for being such a great mentor. I am beyond grateful to have witnessed and have been able to be a part of something that will be profoundly known one day.

Next, I would like to thank Dr. Wu for all of her help when it came to using the fluorescent microscope. If it wasn't for her patience and her willingness to teach me. I would have never been able to obtain the beautiful confocal images that were taken for this project. I would also like to thank my two committee members Dr. Adams and Dr. Lee for taking the time to listen to my dissertation. They had left an everlasting impression on me that has ultimately motivated me to continue my education here at Montclair State University. Dr. Lee was one of my first professors that I've taken. I enjoyed her class so much that I've decided that I wanted to continue taking classes here. However it was after taking a few of Dr. Adams courses that I've decided that I truly wanted to get a master's. Thank you Dr. Wu, Dr. Lee and Dr. Adams for my memorable experiences of graduate school.

My thesis would not have been completed without the help of my colleague, scientist and friend Tyler Person. I needed a fourth eye for revision before my final submission and Tyler had helped me in every step of the way to perfect the paper. I am so grateful for everything you have done to make this paper even better than I anticipated it to be.

Lastly, I would like to thank my parents and sister for the constant support during my three years of graduate school at Montclair State University. I would have never been able to conclude my final chapter at Montclair State University if it wasn't for your encouragement and unconditional love.

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INTRODUCTION

Disparities arising from phenotypic changes in proliferating cancer cells stem from genetic and epigenetic alterations including genotypic inheritance, environmental carcinogens, DNA methylation, gene silencing, etc. (Mémin et al., 2011). The progressive state in a cancer cell is characterized by its unsupervised proliferation that occurs because it bypasses the regulatory cell cycle checkpoints. The cell cycle's machinery is the integrative point of information that is transduced through upstream cellular signaling pathways (Williams et al., 2012). Ultimately, the integrity of that sacred information is what separates a normal cell from a tumor cell.

ICER is a small transcription factor that originates from an alternative intronic promoter region within the cAMP Response Element Modulator (CREM) gene (Molina et al., 1993; Misund et al., 2007). This transcription factor has been observed to act as a tumor suppressor by its incorporation as an important mediator of cAMP anti-proliferative activity (Yehia et al., 2001). The cAMP proliferative pathway consists of phosphorylation of cAMP Response Element Binding Protein (CREB) and CREM which activates the transcription of cAMP Response Element (CRE) genes (Molina et al., 1993). ICER prevents the transcription of CRE-mediated genes by binding to CRE, which regulates cell division (Molina et al., 1993). This pathway is what makes ICER an interesting protein to study for cancer research.

The ICER-IIγ splice variant has been shown to arrest cells prior to and after DNA replication, demonstrating its regulatory action at the G1/S and G2/S stages of the cell cycle (Razavi et al., 1998). Moreover, ICER's tumor suppression abilities are believed to downregulate the expression of growth-related genes like cyclin A and c-fos (Mémin et al., 2011). Cyclins and cyclin-dependent kinases (CDKs) are two major protein groups responsible

for the progression of cells moving through various checkpoints in the cell cycle. Specifically, cyclin A is involved in both S phase and the G2/M checkpoint (Desdouets, et al., 1995). Likewise, c-fos is a gene known for activities related to oncogenesis due to its role in cell proliferation and differentiation (Güller et al., 2008; Sylvester et al., 1998) ICER is believed to bind to the promoter region of CRE, repressing transcription of the c-fos and cyclin A genes and stopping the unfavorable growth of cancer cells (Mémin et al., 2011).

The lack of nuclear abundance of ICER proteins in some cancers proposes a question of the significance of ICER localization in preventing cancer cell proliferation. Conservation of ICER mRNA in cells under expressing ICER and a lack of mutational evidence within the coding and promoter sequences of ICER presumes that abnormal expression may be related to posttranslational modifications, as opposed to being linked to transcriptional control. In prostate cancer, ICER has been shown to be underrepresented in the nucleus due to the Cdk1/cyclin B complex phosphorylation at Serine 35 and its subsequent monoubiquination, leading to its aberrant cytosolic localization. Due to its small size and lack of a nuclear export signal, it is believed that its nuclear localization signal is responsible for its compartmentalization and that monoubiquination masks this signal in prostate cancer cells, preventing its repressive action and allowing cells to proliferate unchecked (Mémin et al., 2011).

Posttranslational modifications can alter a protein's turnover rate. Studies have shown monoubiquitination linked to physiological functions such as membrane trafficking, histone function, transcription regulation, DNA repair and DNA replication (Parvatiyar et al., 2010). Histone phosphorylation, occurring predominately at the amino terminal tails of histones, can affect nucleosome formation by incorporating substantial negative charge to histones, thereby influencing chromatin structure (Bannister and Kouzarides, 2011). Nucleosomes are structural

units consisting of segments of DNA coiled around histone proteins which form a chromatin fiber when in association with each other through linker histones. The core histones that consist of H2A/H2B/H3/H4 form nucleosomes that are further packed by the linker histone, H1 (Chu et al., 2011). From the ten H1 isoforms that exist in humans, H1.4 is the most abundant (Izzo et al., 2008). A majority of the H1 families undergo a number of posttranslational modifications, like phosphorylation on the less conserved N-terminus and C-terminus tails (Nappel et al., 2009). Intrinsically, these phosphorylated H1 proteins are recognized as phosphoproteins and define an essential step in mitotic induction (Roth et al., 1992; Mémin et al., 2011; Nigg, 2001; Sarg et al., 2006).

H1 phosphorylation is linked to chromatin remodeling, heterochromatin formation, DNA replication, and DNA transcription (Alexandrow et al., 2005). Similar to the ICER phosphorylation locus, a study has revealed that the site-specific phosphorylation of the H1.4 N-terminal is associated with the cAMP-dependent pathway of protein kinase A (PKA). PKA phosphorylation of Ser-35 in H1.4 reduces the isoform's affinity for mitotic chromatin, which effectively causes histone dissociation and enables cells to continue with nuclear division (Chu et al., 2001).

Formation of the mitotic apparatus is a crucial step for the advancement of mitosis and involves both microtubule and non-microtubule components. Two proteins that are typically involved in mitosis are γ-Tubulin and centrin. γ-Tubulin is highly conserved in all eukaryotic cells and is essential for the initiation of microtubule assembly (Aylett et al., 2011). Located at centrosomes, also known as the initiation site, γ-Tubulin is associated in the ring-shaped complexes that are observed during mitosis, along with several other proteins (Aylett et al., 2011). In smaller complexes where initiation of microtubule assembly is not as large at the

initiation sites, γ-Tubulin can be found at the centrosome anchored minus ends of microtubules (Moritz et al., 1995). At those other locations, it is associated with what is known to be a mechanism of action of pause or rescue in microtubules (Aylett et al., 2011). The mechanism of action of pause or rescue microtubules refers to the depolymerization and restoration of microtubule elongation (Margolin et al., 2012). Centrin, on the other hand, is a small calcium binding protein that is known to be ubiquitous in centrosome components. These calmodulin-like calcium binding proteins are also present in all eukaryotic cells and are highly conserved members of the superfamily of calcium-binding proteins (Salisbury et al., 2002). Prior analysis of mutant yeast and Chlamydomonas has demonstrated that centrin is essential in the duplication of the centrosomes during the cell cycle and in microtubule expansion (Salisbury et al., 1995). In this project, fluorescence microscopy analysis of ICER-IIγ appeared as two fluorescent dots. We believed these ICER-IIγ cells were cells arrested in the mitotic phase. To further investigate this observation, centrin and γ-Tubulin antibodies were used for co-localization.

The purpose of this study was to examine the terminal ends of ICER-IIγ to determine if they affected the protein's localization. A pivotal study determined that translocation of nuclear ICER was induced by phosphorylation and subsequent monoubiquination of its Ser-35 residue, but the mechanism for cystolic localization was not determined (Mémin et al., 2011). To examine the localization of ICER-IIγ, we utilized two constructs with enhanced green fluorescent protein (EGFP) placed on either the N-terminus or C-terminus. Transcriptional reporter systems have become an invaluable tool for the study of gene expression and localization. There are many different assays that use divergent methods for reporting including the use of bioluminescence, fluorescence, enzymatic, etc. (Ultratna et al., 2012). Green Fluorescent Protein (GFP), a protein first isolated from the jellyfish Aequorea Victoria by Osamu

Shimomura, was the revolutionary bioluminescence tool that was solely used for the purpose of the experiment (Ultratna et al., 2012).

It is hypothesized that ICER-IIγ would reside in the nucleus despite the loci of EGFP labeling because of its tumor suppressor action necessary for proper cell cycle progression. Direct microscopy allowed for the observation of the N-terminus and C-terminus and showed that the C-terminus was incorporated into centrosomes of mitotic cells. This could indicate that the free N-terminus of ICER-IIγ may be involved in mitotic events.

MATERIALS & METHOD

Cell culture and DNA transfection

SK-MEL-24 (ATCC® HTB-71™) is a metastatic homosapien cell line derived from a skin lymph node purchased from ATCC™ (Manassas, VA). For the purpose of our experiment, SK-MEL-24 was used to generate clones that expressed ICER-IIγ in the plasmid enhanced green fluorescent protein (pEGFP). The growth medium had a base that consisted of the eagle's minimum essential medium with 15% fetal bovine serum (FBS) (ATCC 30-2020). The cells were maintained by medium renewal 2 to 3 times per week. The maintenance consisted of culture medium removal followed by brief rinsing of the cell layer with 0.25% trypsin-0.53 mM EDTA solution. 2.0 to 3.0 mL of Trypsin-EDTA solution was added to the flask until the cell layer was dispersed. 6.0 to 8.0 mL of growth medium was added to the cells followed by incubation at 37°C. The sub cultivation ratio was 1:2 and 1:4. DNA transfection was observed to be between 15-25% using the FuGene® HD Transfection reagent from Roche™ (Branchburg, NJ).

Plasmids

pEGFP-C1 and pEGFP-N1 were commercially purchased from Addagene™. An image of the two plasmids along with the direction of transcription is shown in Figure 1. Both plasmids were designed to have EGFP transcribed either before or after the multiple cloning sites, with ICER-IIγ as our gene of interest. The difference between the two plasmids was the location of insertion using the multiple cloning site in references to EGFP. The loci of gene insertion was on the C-terminus of EGFP in pEGFP-C1 or on the N-terminus in pEGFP-N1.

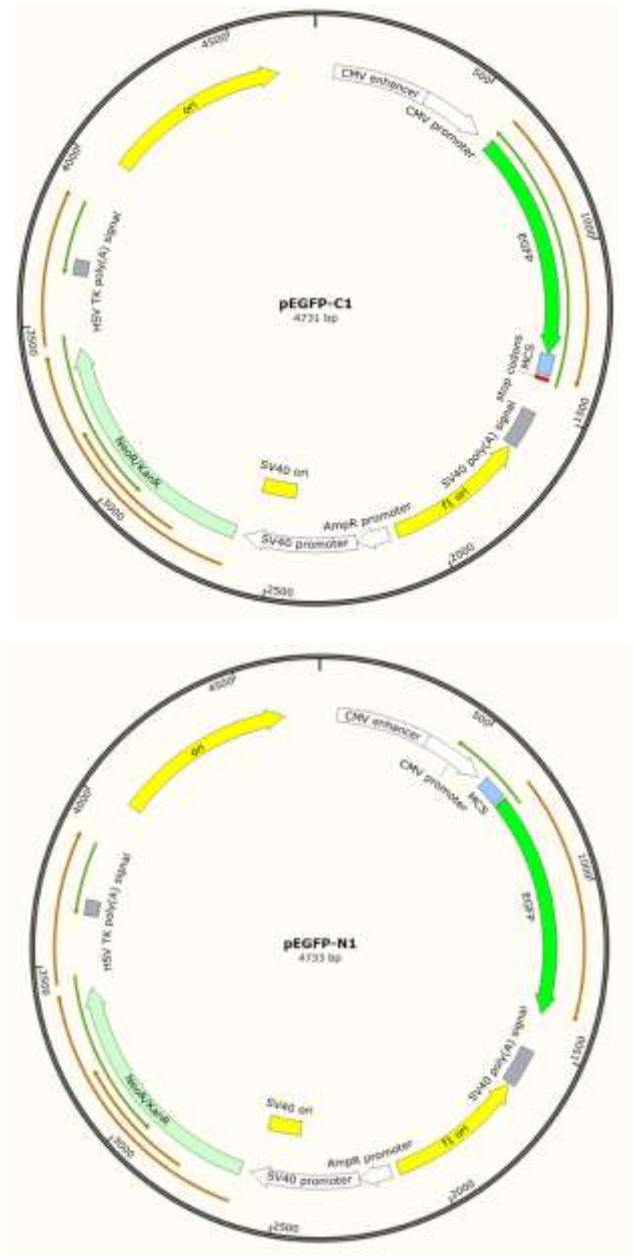

Figure 1: Map of pEGFP-C1 and pEGFP-N1 exhibiting the direction of transcription which is indicated in the mustard yellow colored arrows. Image is obtained from Snapgene™.

Subcloning methods were used to create pEGFP-C1-mICERIIγ. As shown in Figure 2, our ICERIIγ insert has BglII and EcoRI restriction sites at 49bp and 422bp. Enzyme digestion was then performed to have ICERIIγ inserted in the compatible areas of the multiple cloning sites at 1339 bp for Bg1II and 1359bp for EcoRI.

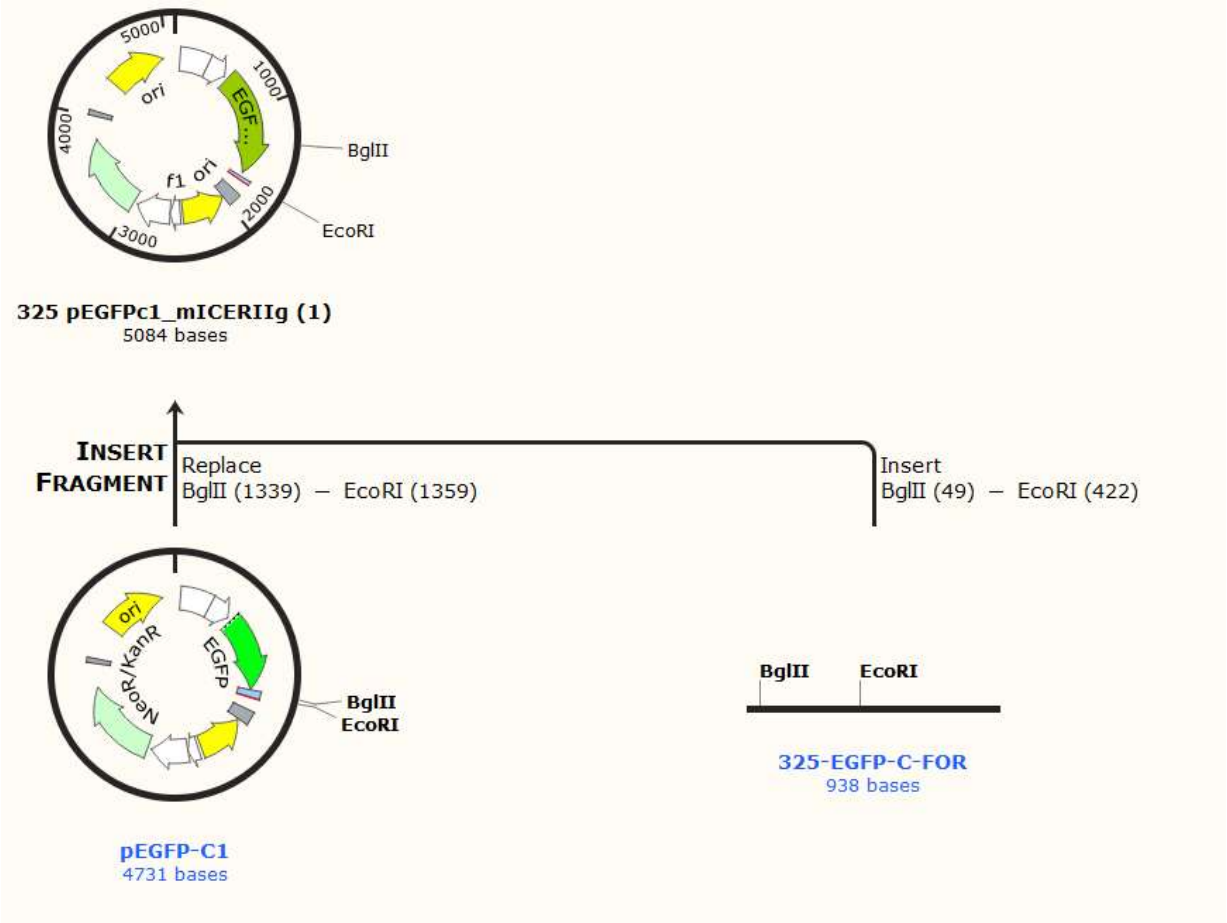

Figure 2: The insertion of ICER-IIγ in the pEGFP-C1, also known as pEGFP-C1-mICERIIγ. The subcloning for this plasmid was successful. Image is obtained from Snapgene™.

DNA transfection methods were also used to create pmICERIIγ-EGFP-N1. However, generating pmICERIIγ-EGFP-N1 had some challenges due to incompatible restriction sites of the insert to the multiple cloning sites. Figure 3, demonstrates the map of our attempts to create pmICERIIγ-EGFP-N1. ICER-IIγ was first amplified from pEGFP-C1-mICERIIγ by PCR. Unfortunately, our ICER-IIγ insert only had one restriction site that was common to the area it was going to be placed. Therefore, when enzyme digestion was performed, ICER-IIγ was inserted in the plasmid in reverse direction.

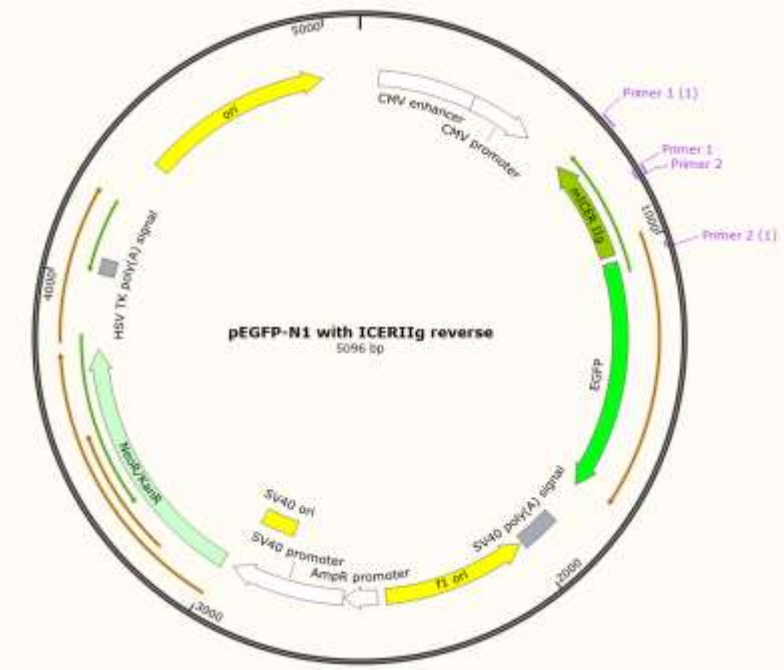

Figure 3: pmICERIIγ-EGFP-N1 with ICER-IIγ inserted in the reverse orientation (indicated with the dark green thin arrows) along with being partially transcribed. The direction of transcription indicated in the dark yellow arrows. Image is obtained from Snapgene™.

The solution to this problem was to find a way to flip ICER-IIγ and have it in frame with the restriction sites of the plasmid. As shown in Figure 4 it was decided to amplify ICER-IIγ from the pEGFP-C1-mICERIIγ by elongating our primers with the BamHI sequence. BamHI is a site that was found in both the N-terminus of ICER-IIγ and in the multiple cloning site. The primers used are listed below.

PRIMER I: AGTTCATAGTTAAATATTTCTACcggTtCTGTTTTGGGAGAG
PRIMER II: TGGCGACCGGTGGG

ICER-IIγ was amplified and flipped with PCR at AgeI site 22 bp and BamHI site 354 bp. Then followed by enzyme digestion, with ICER-IIγ inserted at BamHI site 660 bp and AgeI site 666 bp in the plasmid. This allowed for the successful transfection for pmICERIIγ-EGFP-N1.

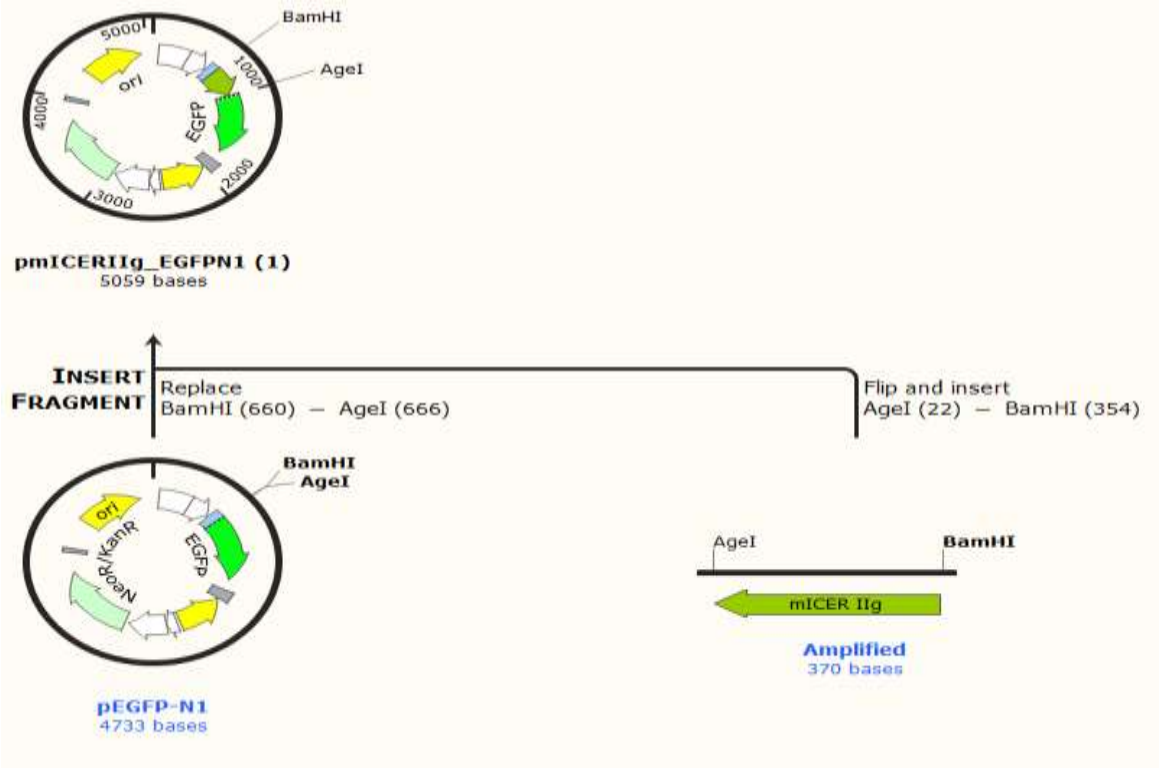

Figure 4: The insertion of ICER-IIγ in the pEGFP-C1, also known as pmICERIIγ-EGFP-N1 . The subcloning for this plasmid was successful. Image is obtained from Snapgene™.

Western-Blot

Protein extraction, SDS-PAGE and Western-Blot protocols were performed as described (Memin et al., 2002). The anti-ICER polyclonal antibody was produced from rabbit serum immunized against ICER-II-gamma protein (Memin et al., 2002). Anti-GFP rabbit polyclonal antibodies were purchased from Abcam™. Anti-gamma tubulin monoclonal antibody with Alexa Fluor 555 (Product # MA1850-A555) were purchased from ThermoFisher Scientific™. Anti-centrin rabbit polyclonal antibodies (ab156858) were purchased from Abam™. The secondary antibody, donkey anti-rabbit IgG (H+L) Highly Cross-adsorbed with Alexa Fluor Plus 555, was purchased from ThermoFisher Scientific™. Nocodazole treatments were used to block the cells in mitosis. Cells were treated with 100 ng/ml of nocodazole for 16-24 hours.

Immunocytochemistry for γ-Tubulin

SK-MEL-24 cells were transfected, the cultural medium was removed and washed two times with phosphate-buffered saline (PBS). The cells were then fixed by adding 4% formaldehyde in the PBS. Cells were washed with PBS, three times for 5 minutes, then stored for 2 days in 0.02% (w/v) sodium azide in PBS medium. Lastly, PBS was removed and 100µl of protein block was treated to the cells. The cells were incubated at room temperature for 1 hour, then it was washed with PBS. Staining of TUBG1 mouse monoclonal antibody occurred at a concentration of 20 µg/mL in the blocking buffer for 1 hour at room temperature. The cells were then washed with PBS four times afterwards. On the prepared slide a drop of the medium with DAPI was assembled along with a coverslip.

Immunocytochemistry for the detection of Centrin

SK-MEL-24 cells were transfected, and the cultural medium was removed and washed two times with PBS. The cells were then fixed by adding 4% formaldehyde in PBS for 20 minutes. Next, cells were washed with PBS, three times for 5 minutes. Cells were incubated in 0.1% Triton X-100 in PBS for 15 mins, then washed three times in PBS. PBS was removed and 100µl of protein block was treated to the cells. Cells were incubated for 1 hour at room temperature, followed by a PBS wash. 100µl of anti-centrin rabbit polyclonal antibody (ab156858) was incubated at 4°C overnight. The following day it was washed with PBS four times. 5µg/ml dilution of donkey anti-rabbit IgG (H+L) Highly Cross-adsorbed with Alexa Fluor Plus 555 at 1 µg/mL was incubated for 1 hour at room temperature. Then it was washed with PBS four times. On the prepared slide a drop of the medium with DAPI was assembled along with a coverslip.

Nikon C2 Plus Confocal Laser Scanning Microscope (CLSM) for Microscopy Images

Bifocal and confocal images of the cells were obtained from a fully automated Nikon Ti microscope that was entirely controlled by the NIS-Elements C software. Confocal scanning was performed at four different excitation channels: 405, 488, 561, and 640 nm. The 120 LED system was used for fluorescence and filters for DAPI, GFP and Tex Red. Images were obtained and magnified at 10x, 20x and 40x all with DIC capabilities. The detectors included the standard Photo-multiplier tubes, pco-edge 4.2 scientific CMOS camera, and transmitted detector.

RESULTS

Successful transfection of ICER-IIγ for pmICERIIγ-EGFP-N1

A recent study has demonstrated that the N-terminus of *ICER* is critical for its regulation and half-life (Molina et al., 2021). We sought to determine the localization of *ICER* by using EGFP on either termini to see if it truly would reside in its native nuclear position. Therefore, proper ICER-IIγ transfection for the pEGFP-N1 to generate pmICERIIγ-EGFP-N1 was crucial for this project. Prior attempts to subclone ICER-IIγ to the pEGFP-N1 had caused our protein to be incorporated reversely and remain partially transcribed. However, we incorporated a restriction site into our primers to ensure ICER-IIγ would be successfully transfected to pEGFP-N1 to create pmICERIIγ-EGFP-N1.

Successful transfection of our plasmids was observed in western blots, (Figure 5). In the western blot, WB:antiCREM/ICER, the anti-CREM-*ICER* antibody was used as a marker to recognize all CREM Tau and *ICER* in the plasmids. The columns were labelled as followed: ladder, pEGFP-N1, pEGFP-C1-mICER-IIγ, pmICER-IIγ-pEGFP-N1. pEGFP-N1, represented the plasmids that had the EGFP construct on the N-terminus followed by the multiple cloning site. This plasmid did not undergo transfection, indicated by the absence of the ICER-EGFP band in Lane 2 of the western blots, (Figure 5). pEGFP-C1-mICER-IIγ, Lane 3, represented EGFP on the N-terminus of *ICER*. pmICER-IIγ-pEGFP-N1, Lane 4, represented EGFP on the C-terminus. It is evident that successful transfection occurred for both plasmids as ICER-IIγ is typically about 18 kDa in size while EGFP is about 27 kDa. Therefore, ICER-EGFP is about 40 kDa, and this was observed in our Western blots, (Figure 5). ICER-IIγ expression was observed in pmICER-IIγ-pEGFP-N1 and pEGFP-C1-mICER-IIγ; seen as concentrated banding in Lane 4 and Lane 3, respectively, of WB:antiGFP, (Figure 5).

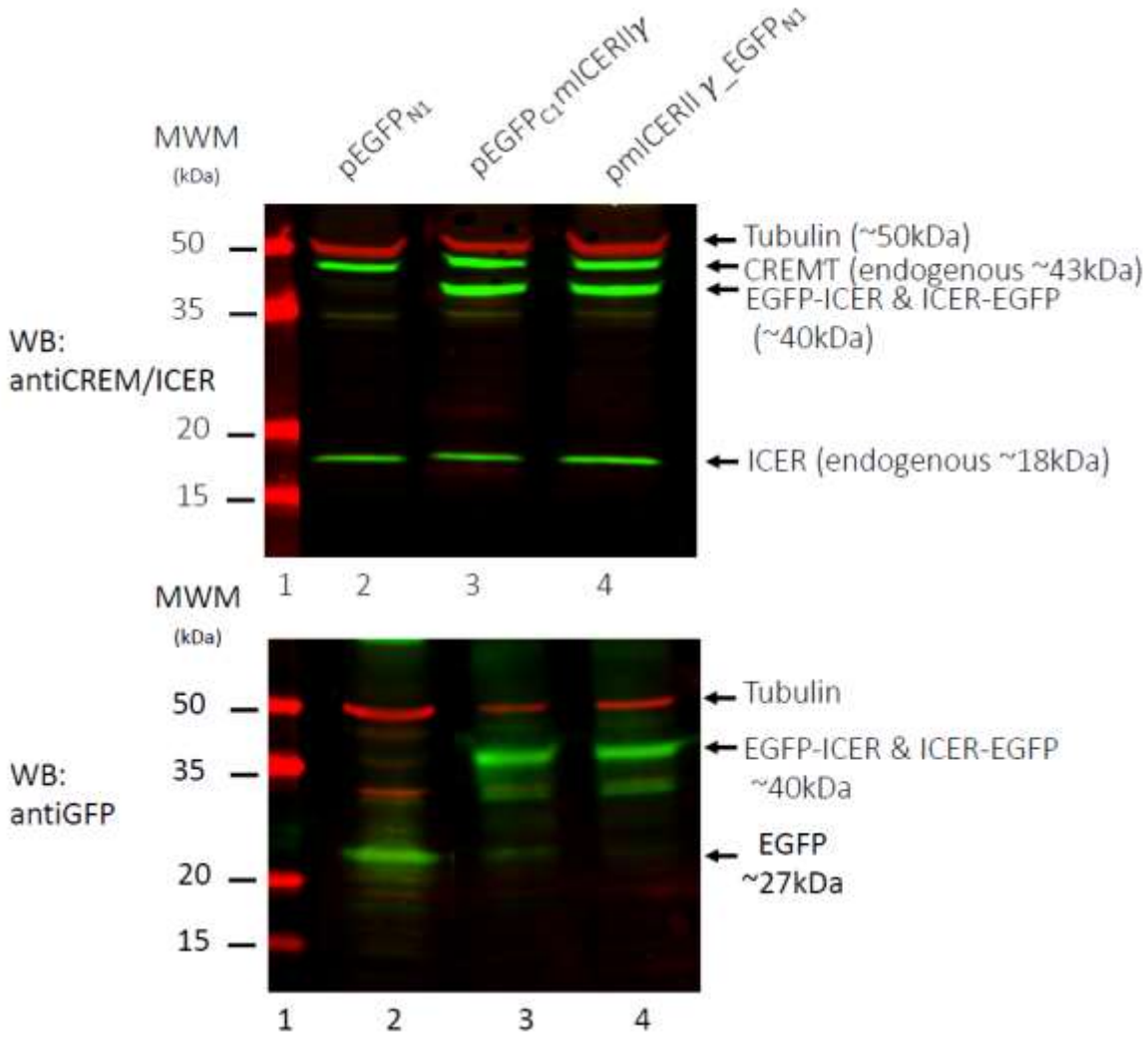

Figure 5: Western Blot for the transfection of ICER-IIγ for pEGFP-C1-mICER-IIγ and pmICER-IIγ-pEGFP-N1

Determining the localization of ICER-IIγ in pEGFP-C1-mICER-IIγ and pmICER-IIγ-pEGFP-N1

The Nikon C2 Plus CLS microscope was used to take bifocal images of pEGFP-C1-mICER-IIγ and pmICER-IIγ-pEGFP-N1. The SK-MEL-24 cell lines were stained with DAPI, a blue fluorescent DNA stain that allows proper viewing of the nucleus under a microscope. Since the cells were treated with nocodazole, the transfection efficiency would be between 15-25%. Meaning, out of 100 cells, only about 15-25 cells would express ICER-IIγ. By

using the FITC channel, cells that expressed ICER-IIγ fluoresced in a green color due to the EGFP incorporated into our insert.

The images that were taken from Nikon C2 Plus CLS microscope were recorded in an order that captured each channel separately, and the final image exhibited all of the individual channels together. The DAPI channel for pEGFP-C1-mICER-IIγ showed about 250 stained nuclear cells, (Figure 6A). The FITC channel for pEGFP-C1-mICER-IIγ showed roughly 40 cells expressing ICER-IIγ, (Figure 6B). When displayed together, (Figure 6C), cells expressing ICER-IIγ were shown to reside in the nucleus.

Nuclear localization was further demonstrated in the pmICER-IIγ-pEGFP-N1 snapshots, (Figures 7A, 7B, 7C). The nuclear SK-MEL-24 in the DAPI channel appeared to have about 100 distinguishable cells, (Figure 7A). A majority of these cells were highly expressed with ICER-IIγ, along with cells that appeared to be dotted, (Figure 7B). The "dotted cells" were not observed in the pEGFP-C1-mICER-IIγ, (Figure 6B). When the two channels were aligned, (Figure 7C), the dotted ICER-IIγ still resided in the nucleus. However, the DAPI staining was not as predominantly expressed as it was in pEGFP-C1-mICER-IIγ, (Figure 6C). (Figure 8C) provided a magnified look of the dotted ICER-IIγ in the pmICER-IIγ-pEGFP-N1.

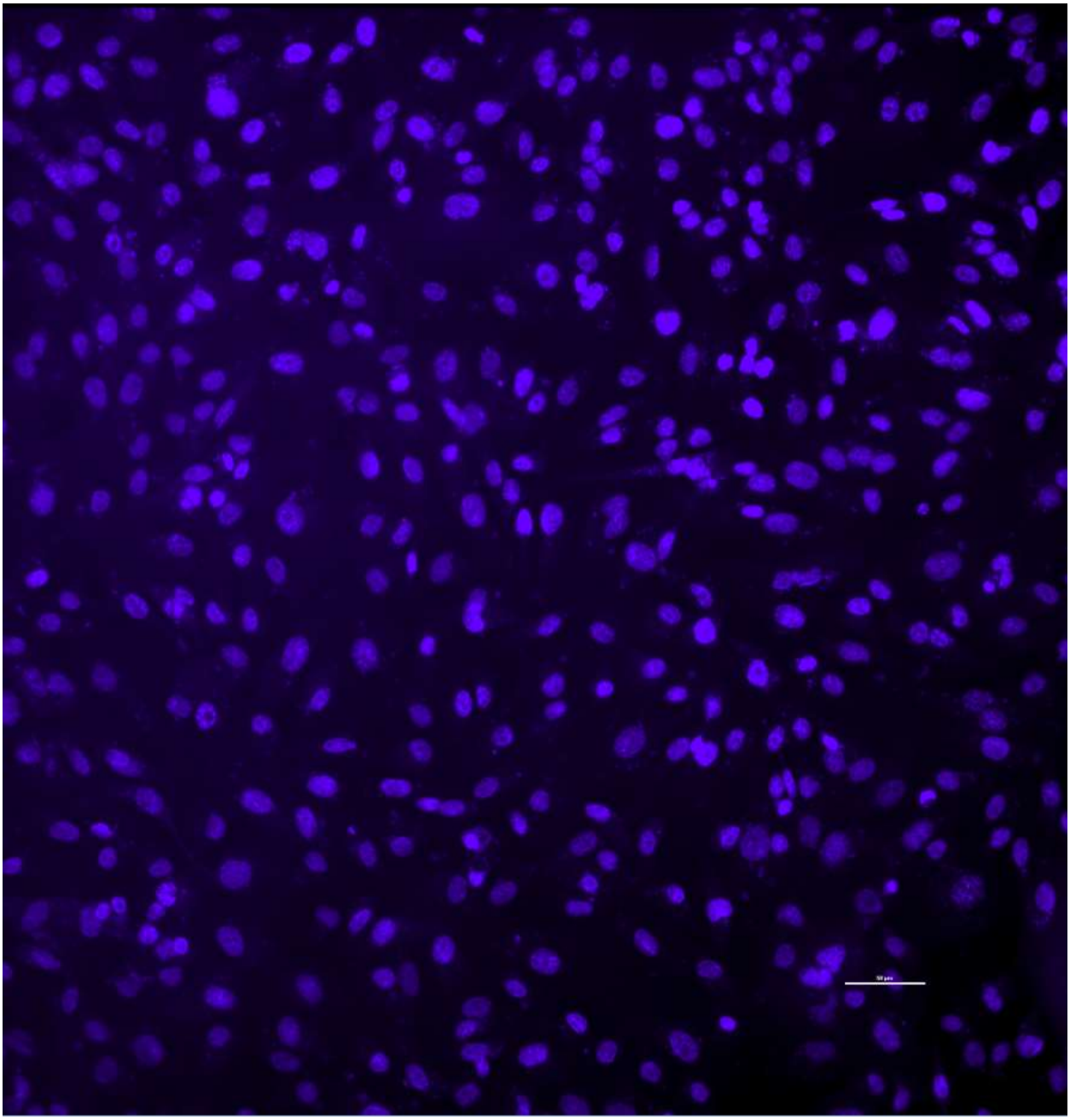

Figure 6A: pEGFP-C1-mICER-IIγ at 10X resolution using the DAPI channel from the Nikon C2 Plus CLS microscope.

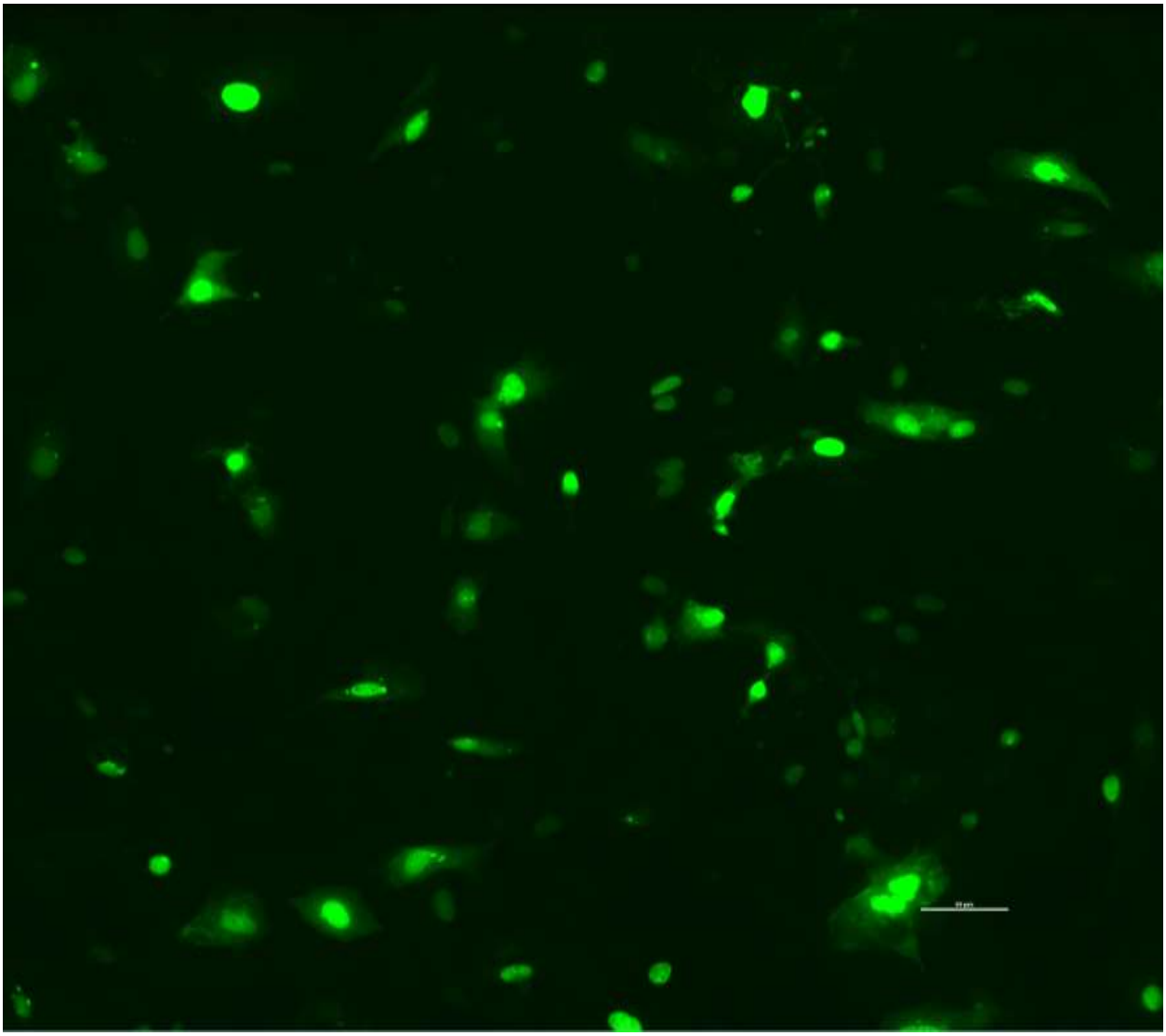

Figure 6B: pEGFP-C1-mICER-IIγ at 10X resolution using the FITC channel from the Nikon C2 Plus CLS microscope.

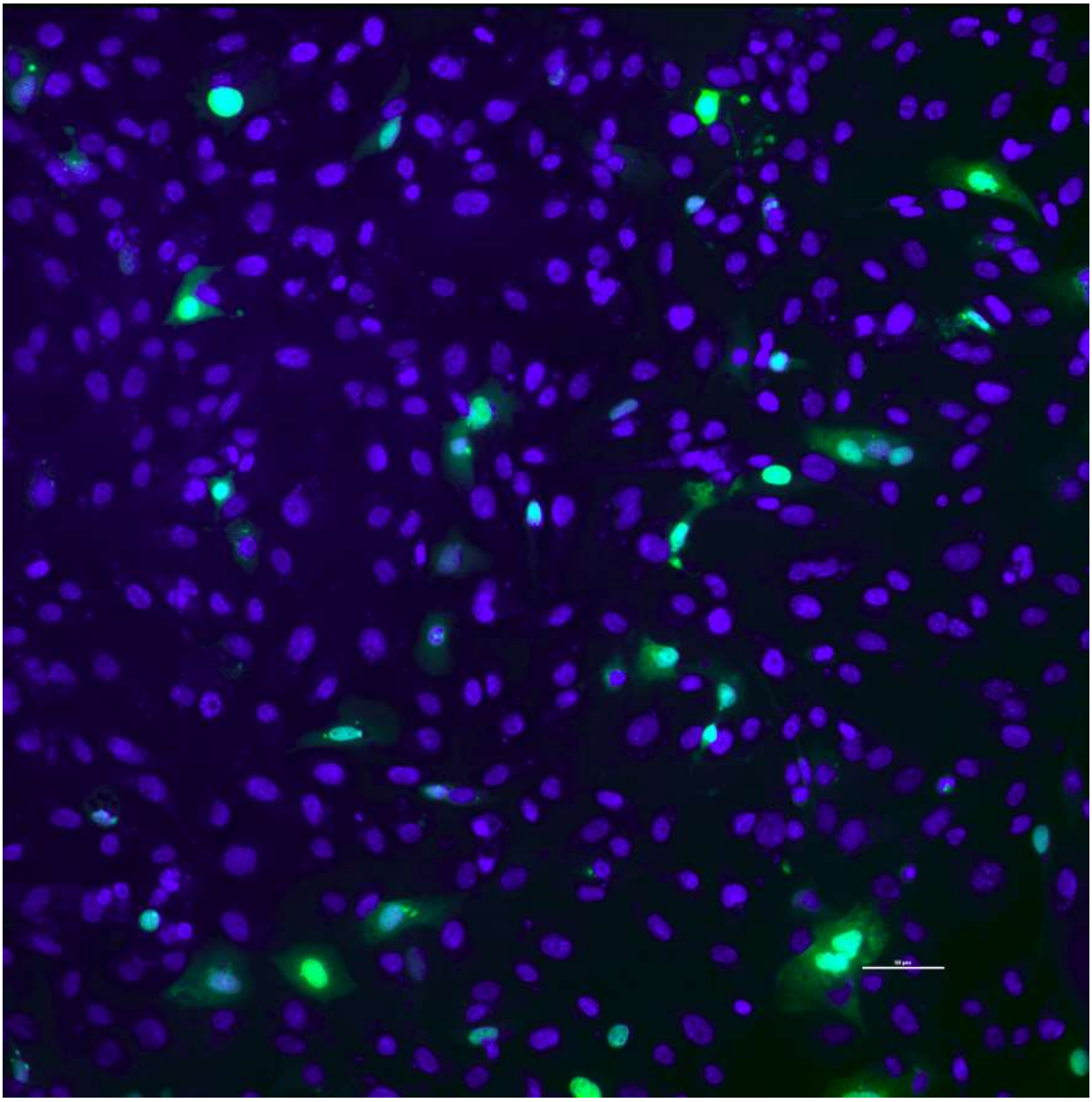

Figure 6C: pEGFP-C1-mICER-IIγ at 10X resolution overlapping the DAPI and FITC channel from the Nikon C2 Plus CLS microscope.

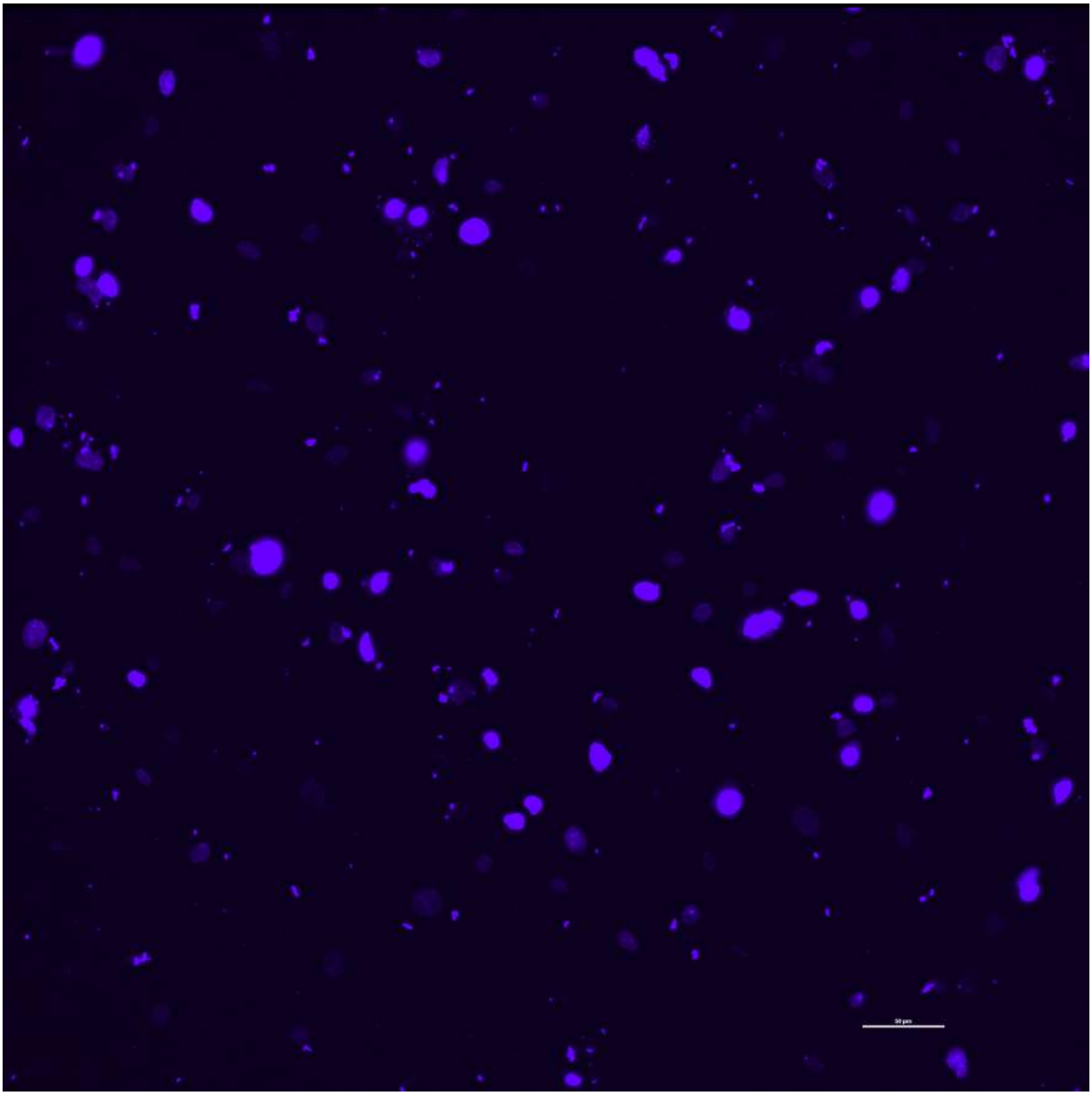

Figure 7A: pmICER-IIγ-pEGFP-N1 at 10X resolution using the DAPI channel from the Nikon C2 Plus CLS microscope.

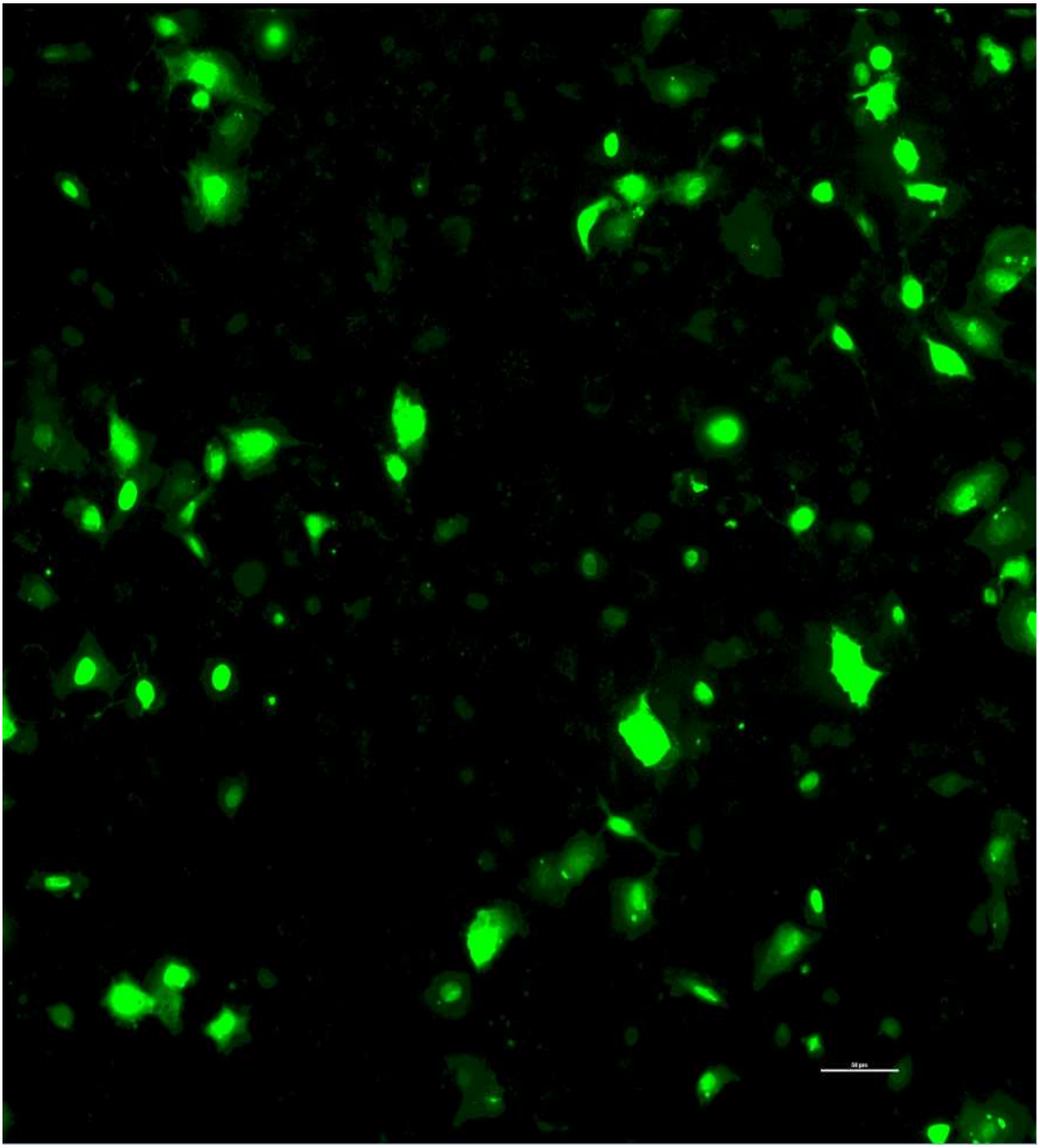

Figure 7B: pmICER-IIγ-pEGFP-N1 at 10X resolution using the FITC channel from the Nikon C2 Plus CLS microscope.

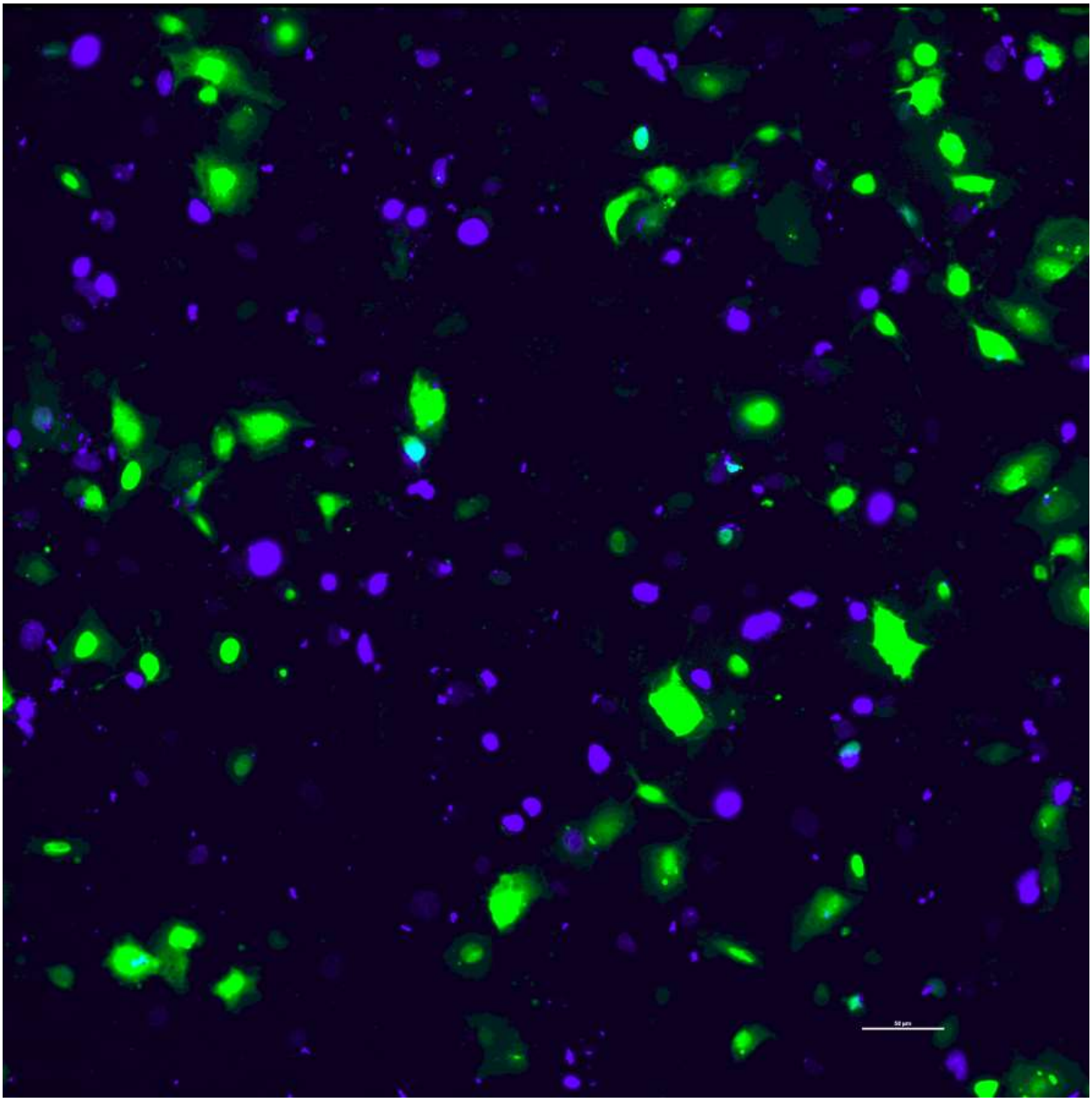

Figure 7C: pmICER-IIγ-pEGFP-N1 at 10X resolution overlapping the DAPI and FITC channel from the Nikon C2 Plus CLS microscope.

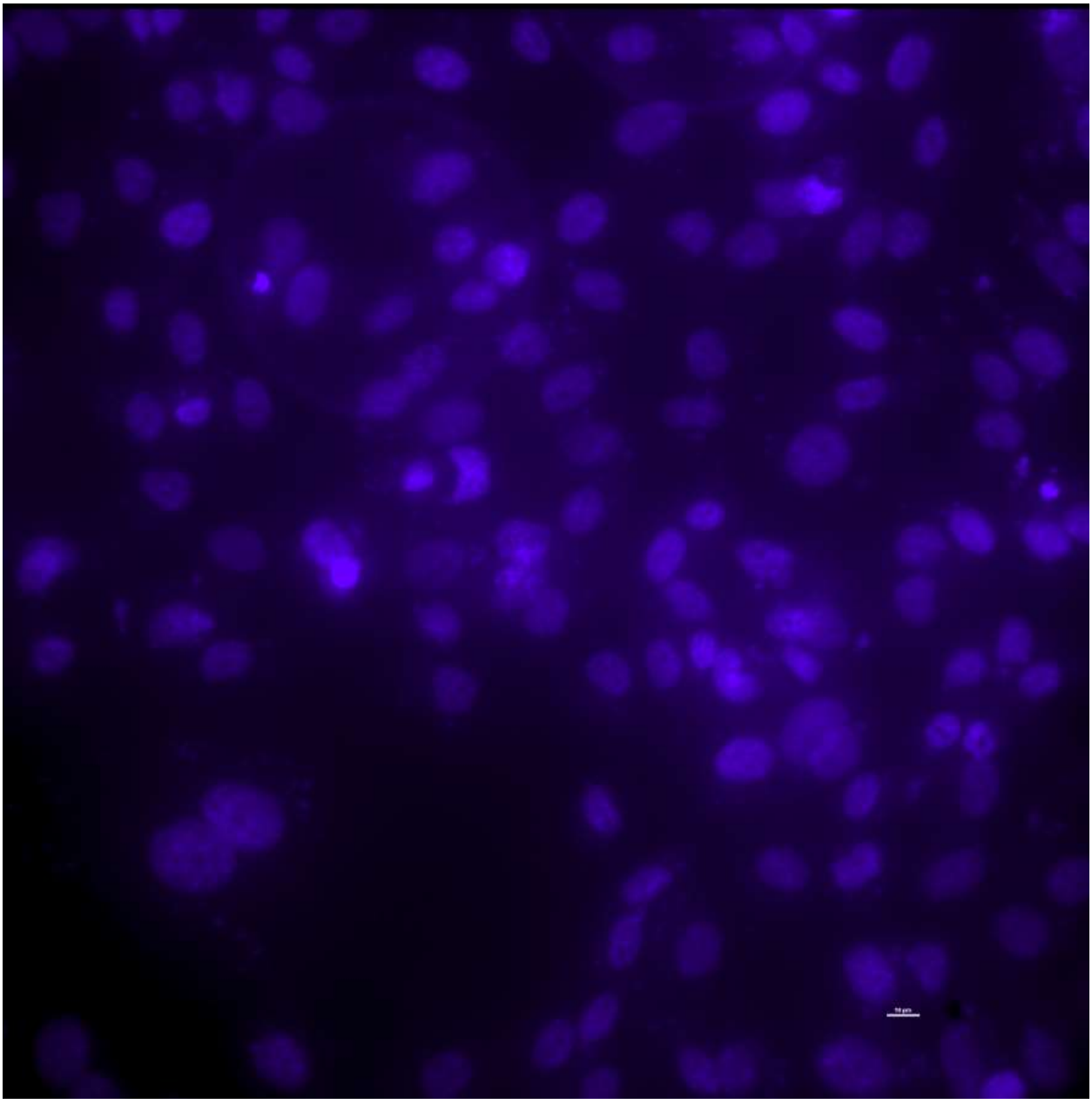

Figure 8A: pmICER-IIγ-pEGFP-N1 at 40X resolution using the DAPI channel from the Nikon C2 Plus CLS microscope.

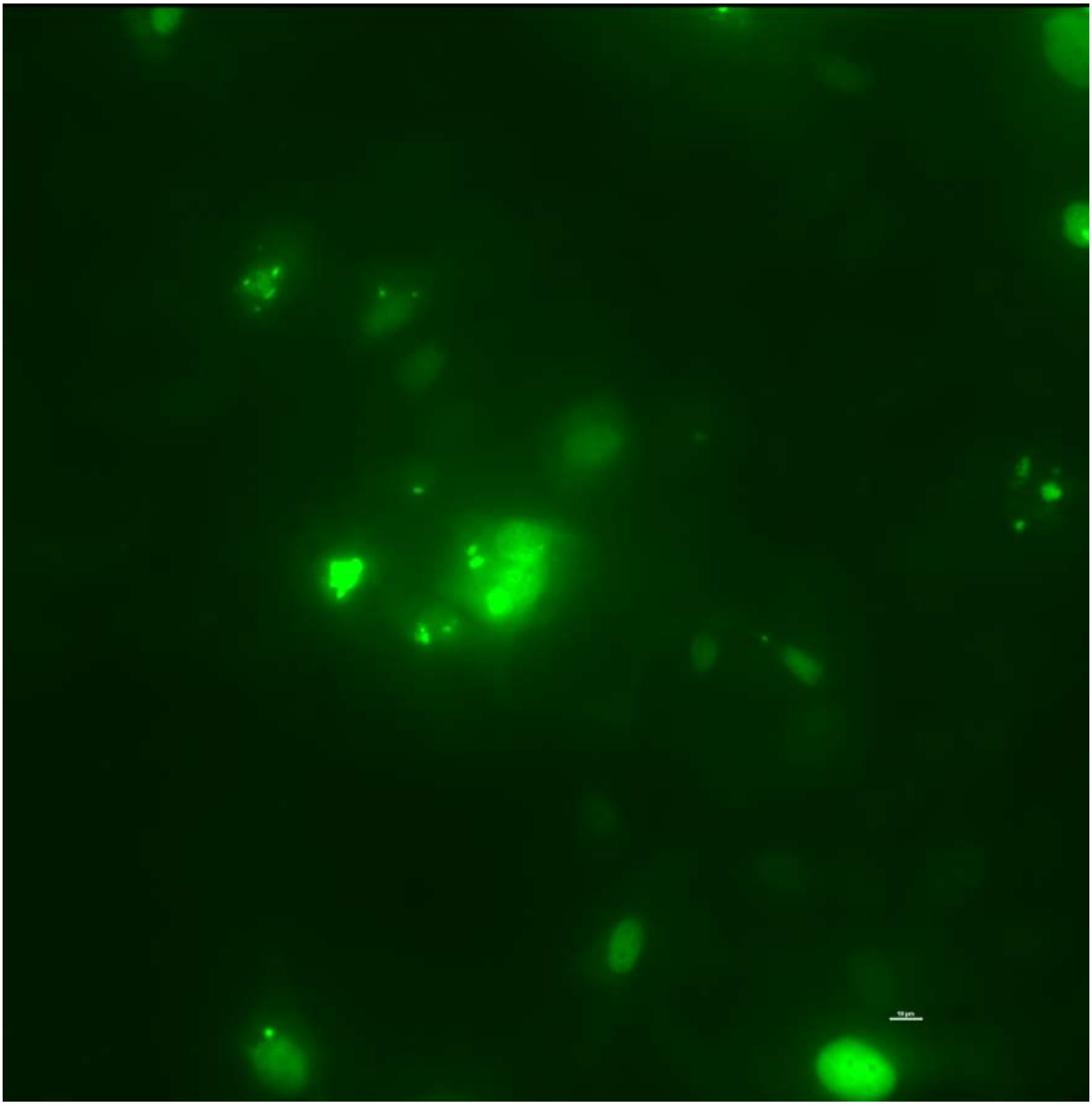

Figure 8B: pmICER-IIγ-pEGFP-N1 at 40X resolution using the FITC channel from the Nikon C2 Plus CLS microscope.

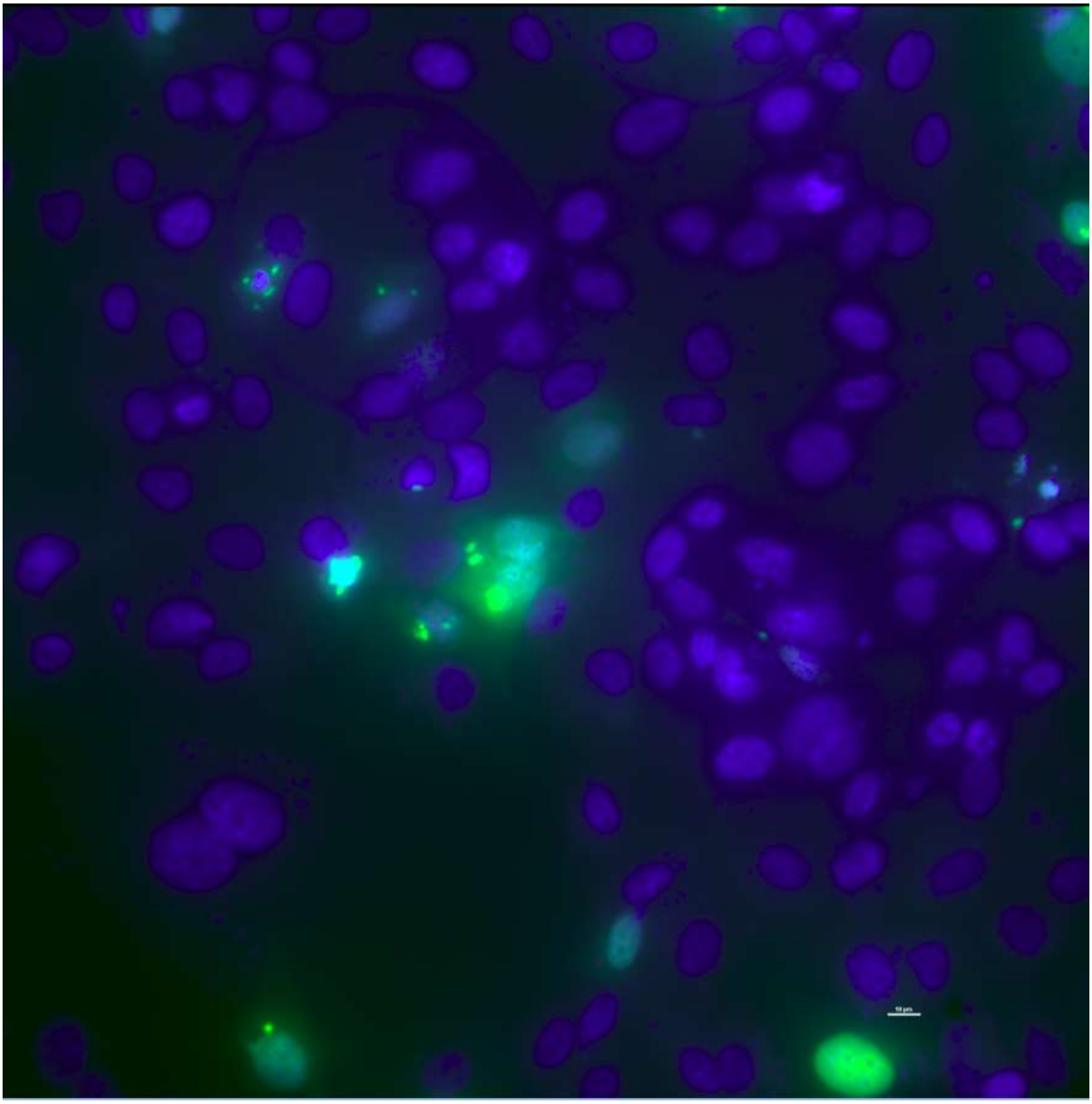

Figure 8C: pmICER-IIγ-pEGFP-N1 at 40X resolution overlapping with the DAPI and FITC channel from the Nikon C2 Plus CLS microscope. The two dotted ICER-IIγ is what we are interested in.

Anti-Gamma Tubulin antibodies used for the tracing of possible mitotic cells for ICER-IIγ-pEGFP-N1

The dotted ICER-IIγ in the pmICER-IIγ-pEGFP-N1, (Figure 8C), were presumed to be arrested mitotic cells due to treatment with nocodazole. These cells seemed to be heavily expressed at what appeared to be mitotic spindles. To further investigate, anti γ-Tubulin antibodies were utilized. γ-Tubulin provides centrosomes the ability to initiate microtubule growth, making it a viable candidate for mitotic cell targeting (O'Toole et al., 2012).

The Nikon C2 Plus CLS microscope was used to take confocal images of pmICER-IIγ-pEGFP-N1 after undergoing immunocytochemistry for the selection of γ-Tubulin. The antibody used to express γ-Tubulin fluorescence was viewed under the TRITC channel due to the probe's excitation wavelength. pmICER-IIγ-pEGFP-N1 was viewed under the DAPI channel, (Figure 9A), and FITC channel, (Figure 9B). These figures showed the continued presence of mitotic cells. γ-Tubulin was observed under the TRITC channel, (Figure 9C). A composite of the DAPI, FITC and TRITC channels, (Figure 9D), exhibited multiple areas of white fluorescence. Those areas revealed a strong signal of colocalization for DAPI, FITC and TRITC. In those white fluorescent areas, the dotted ICER-IIγ cells were observed. This colocalization insinuates that ICER-IIγ is expressed and associated with γ-Tubulin in mitotic cells. A higher magnification image of nuclear ICER-IIγ aligned with γ-Tubulin in mitotic cells can be seen in (Figure 10D).

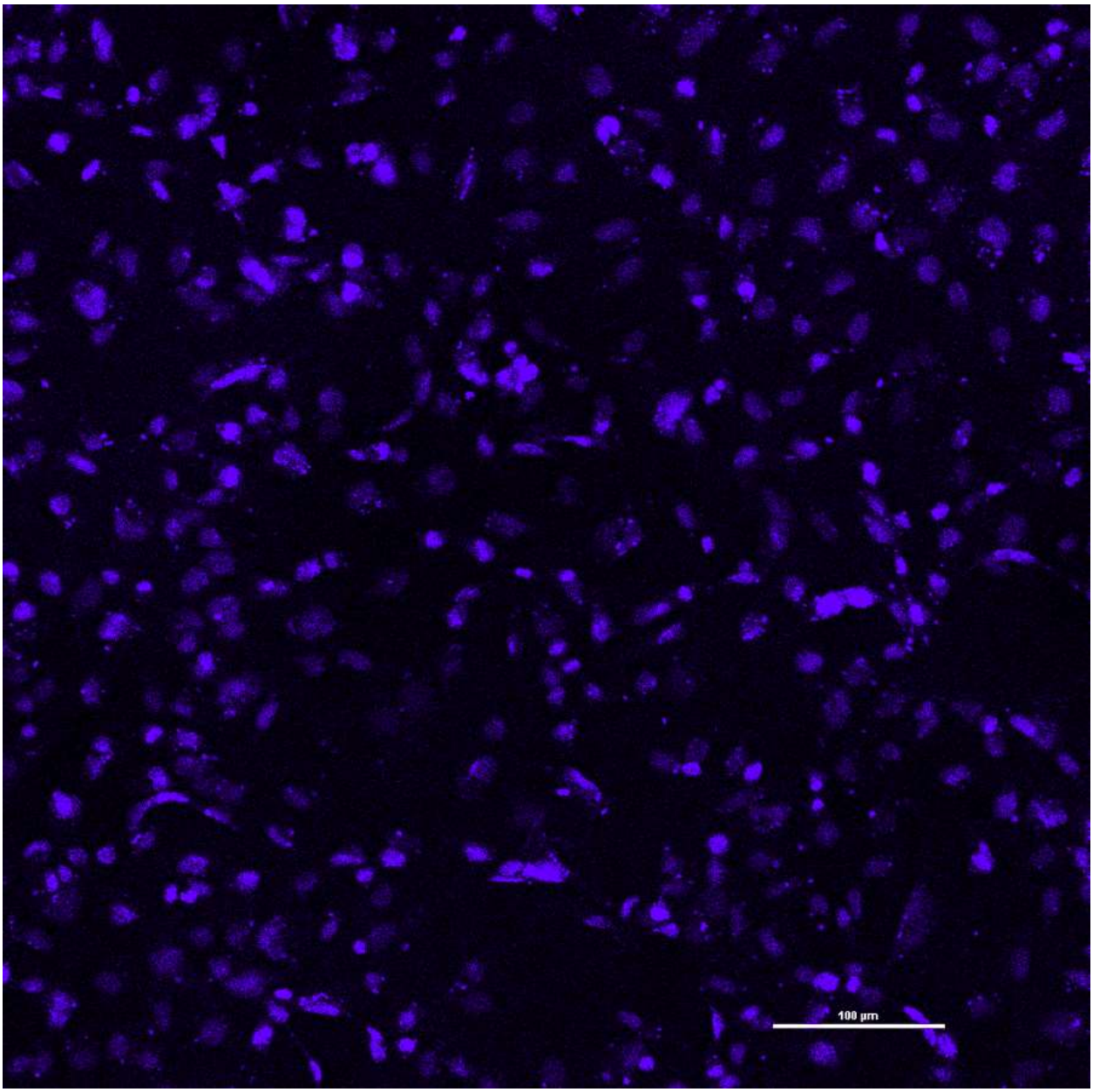

Figure 9A: pmICER-IIγ-pEGFP-N1 at 10X resolution with the DAPI channel from the Nikon C2 Plus CLS microscope. These cells have undergone immunocytochemistry to select ANTI-TUBULIN.

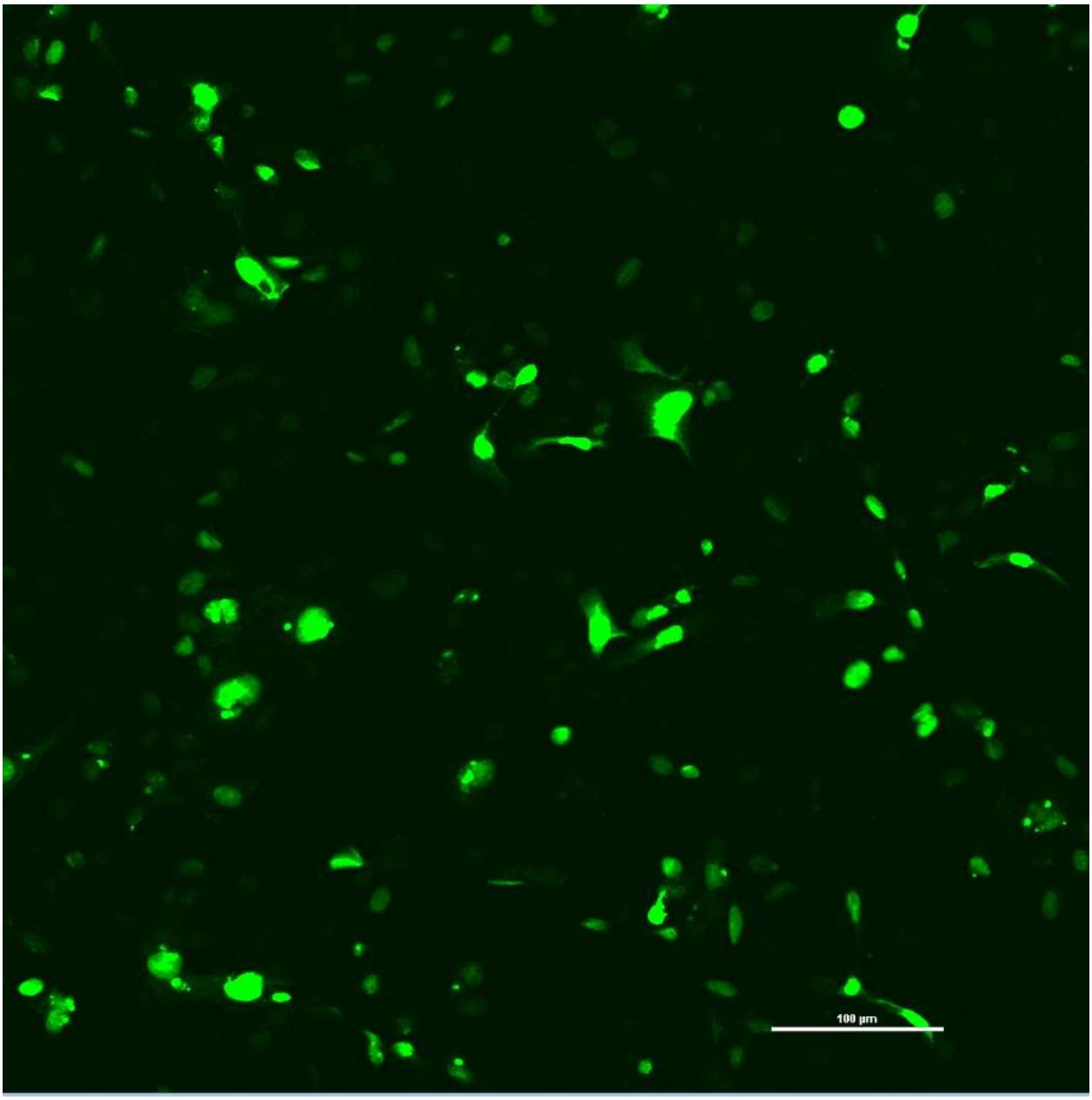

Figure 9B: pmICER-IIγ-pEGFP-N1 at 10X resolution with the FITC channel from the Nikon C2 Plus CLS microscope. These cells have undergone immunocytochemistry to select ANTI-TUBULIN.

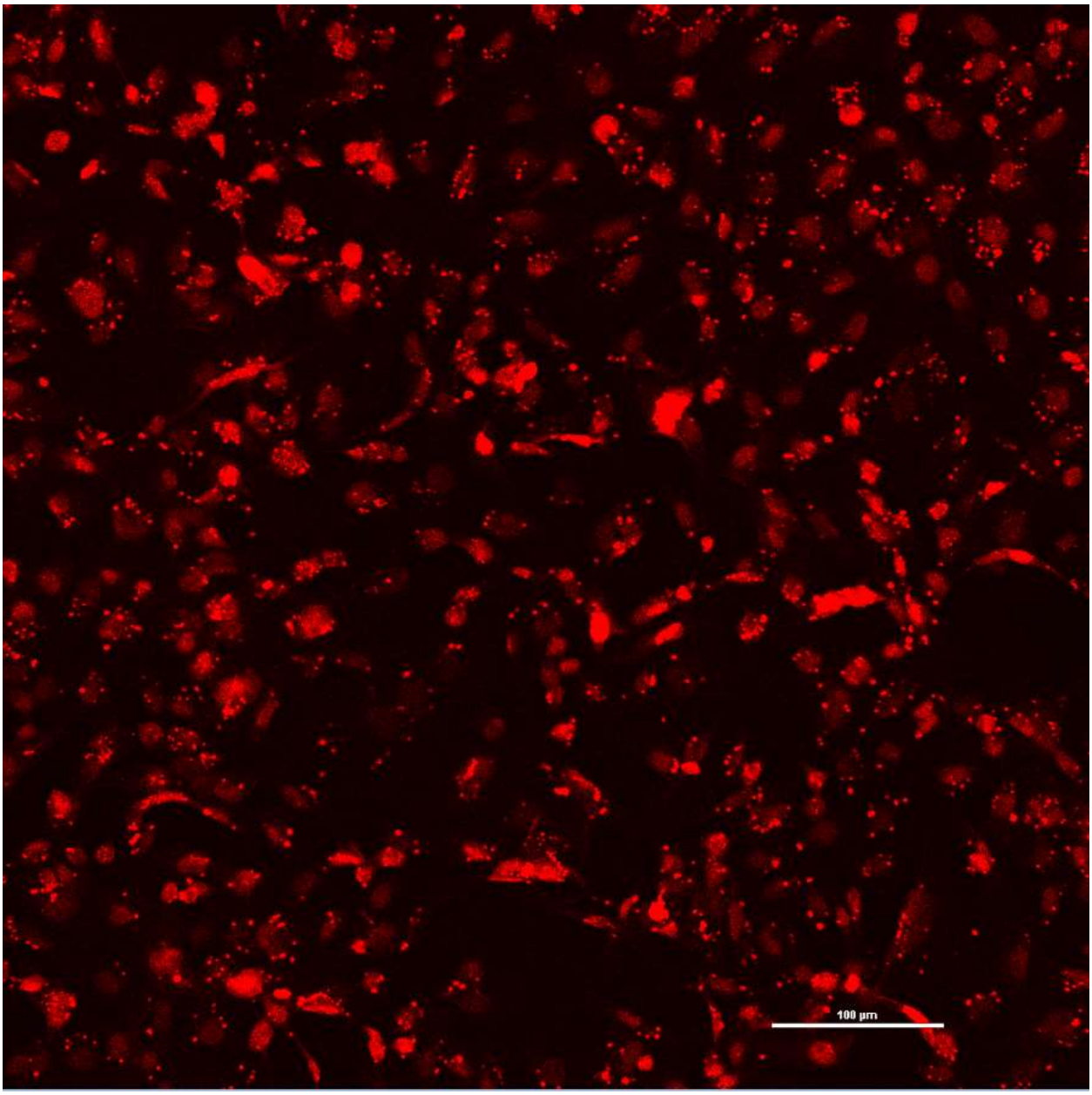

Figure 9C: pmICER-IIγ-pEGFP-N1 at 10X resolution with the TRITC channel from the Nikon C2 Plus CLS microscope. These cells have undergone immunocytochemistry to select ANTI-TUBULIN.

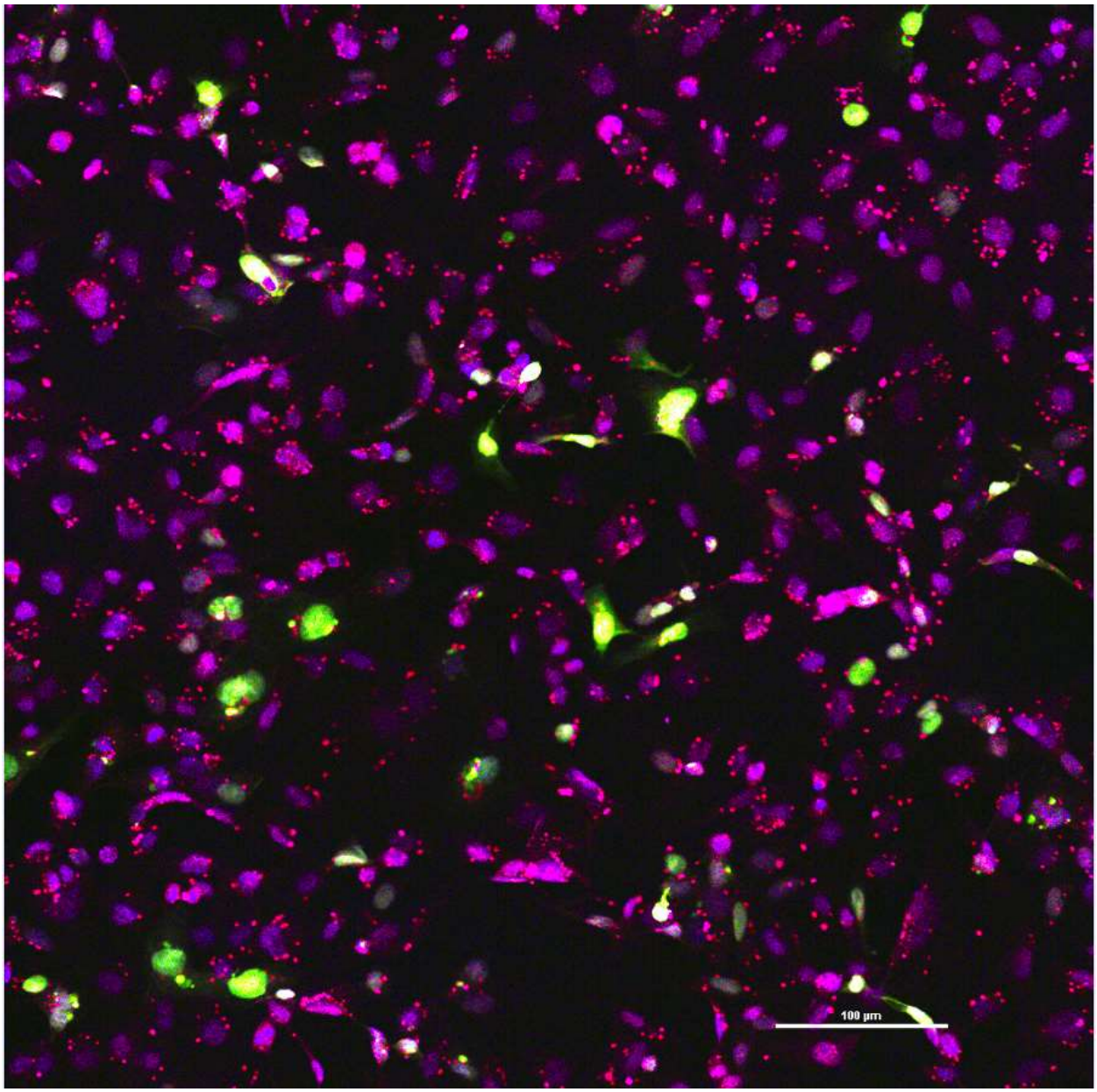

Figure 9D: pmICER-IIγ-pEGFP-N1 at 10X resolution with the DAPI, FITC and TRITC channels from the Nikon C2 Plus CLS microscope. These cells have undergone immunocytochemistry to select ANTI-TUBULIN.

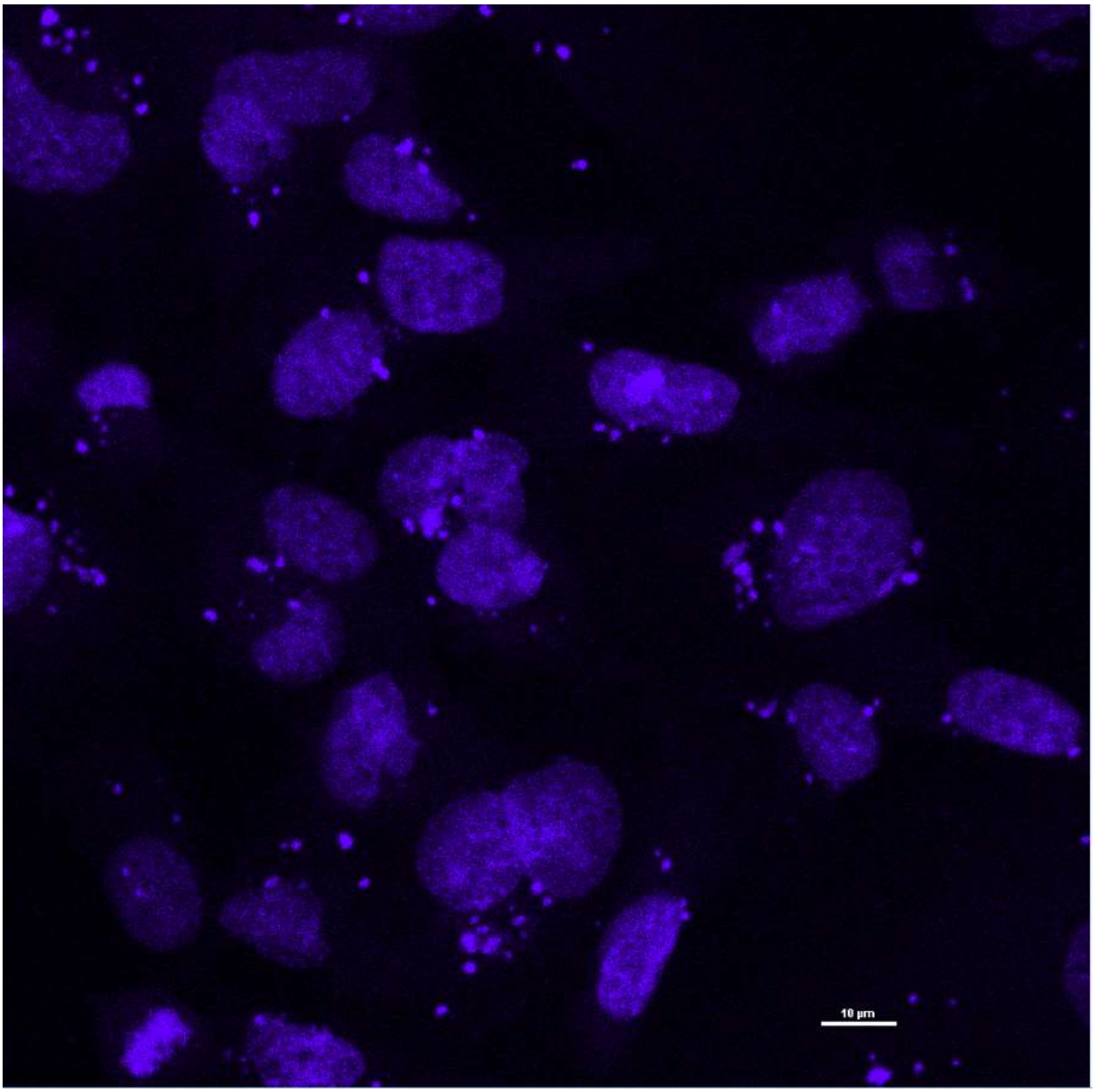

Figure 10A: pmICER-IIγ-pEGFP-N1 at 40X resolution with channels from the Nikon C2 Plus CLS microscope. These cells have undergone immunocytochemistry to select ANTI-TUBULIN.

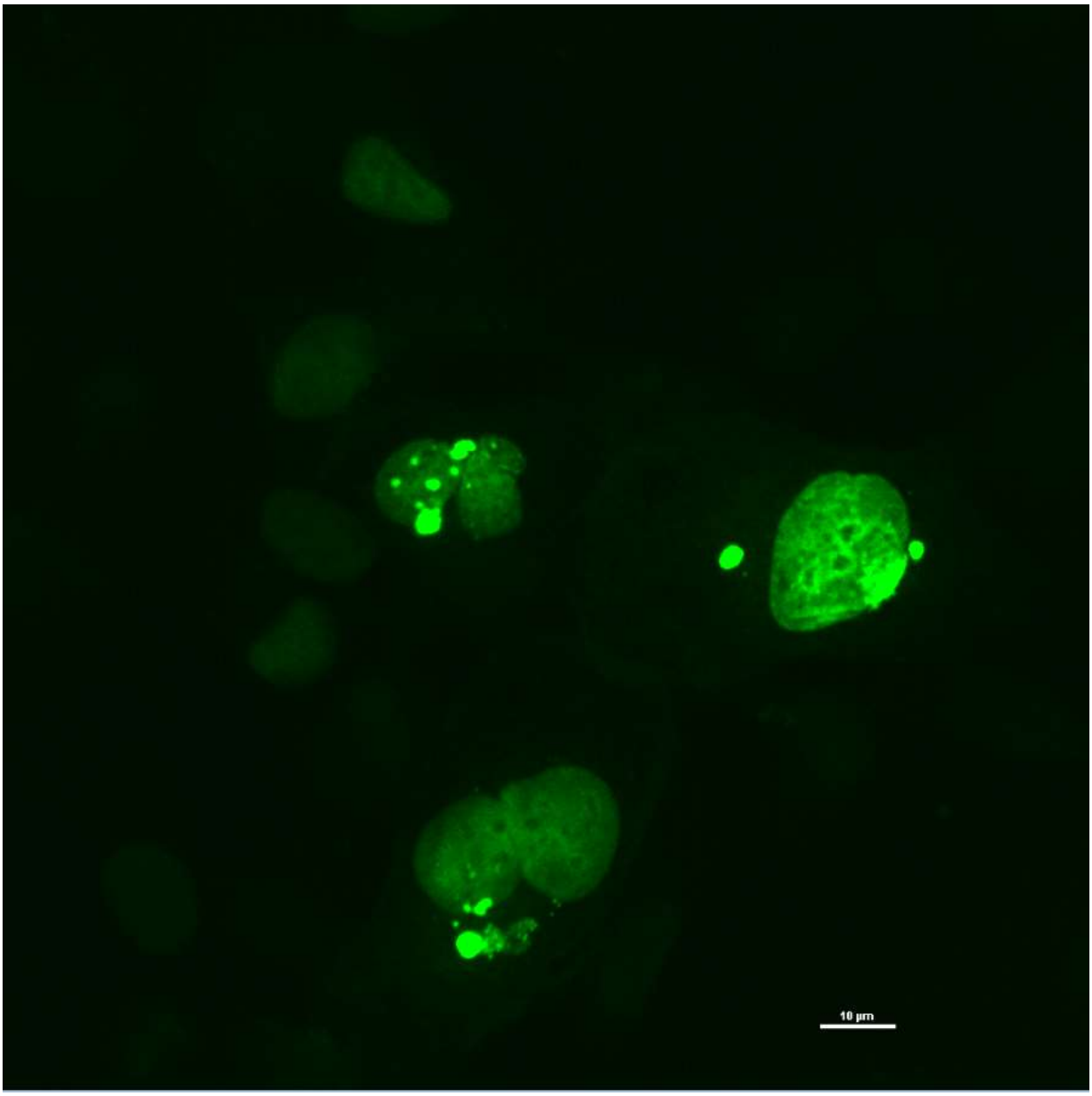

Figure 10B: pmICER-IIγ-pEGFP-N1 at 40X resolution with the FITC channels from the Nikon C2 Plus CLS microscope. These cells have undergone immunocytochemistry to select ANTI-TUBULIN.

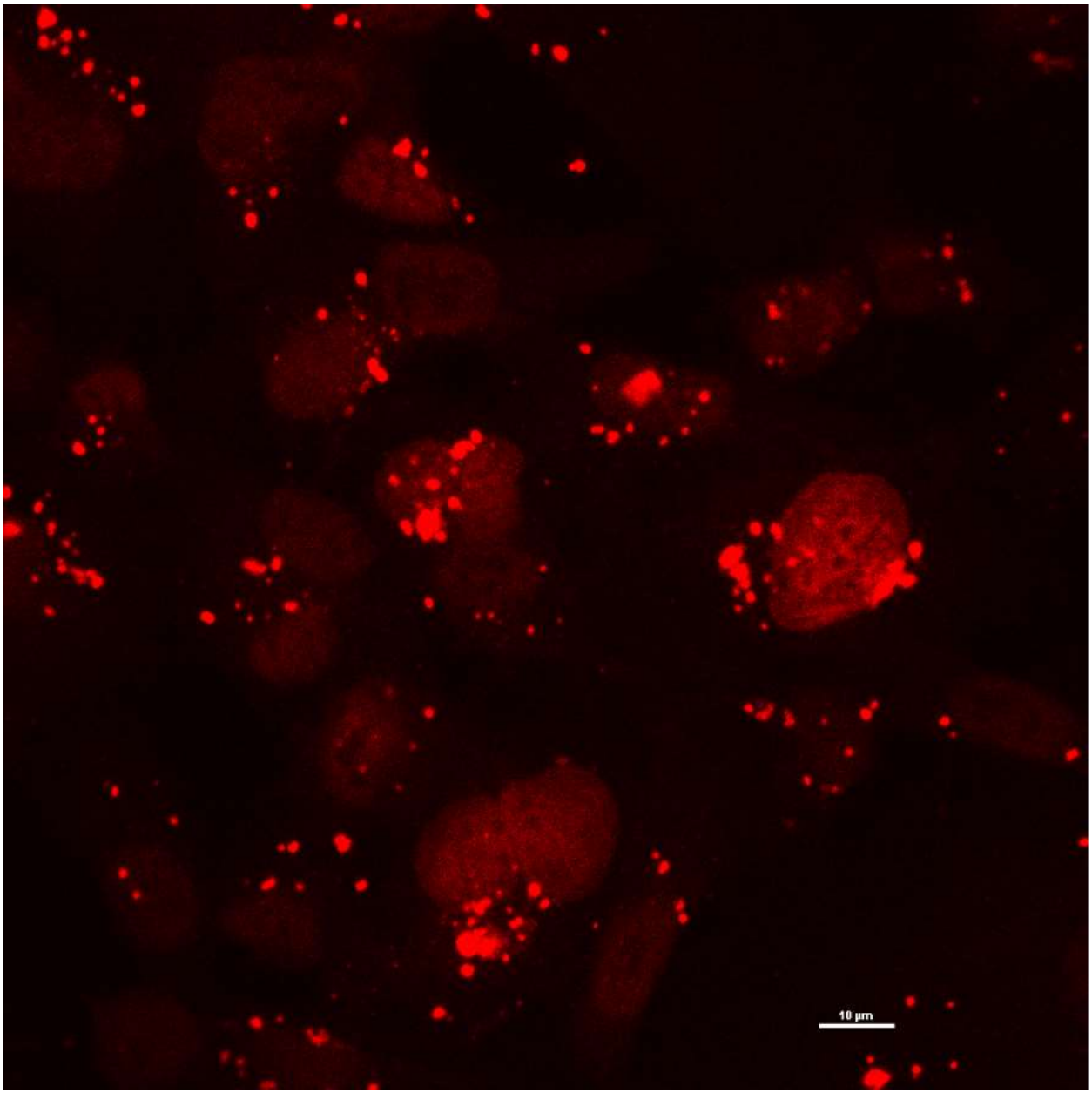

Figure 10C: pmICER-IIγ-pEGFP-N1 at 40X resolution with the TRITC channels from the Nikon C2 Plus CLS microscope. These cells have undergone immunocytochemistry to select ANTI-TUBULIN.

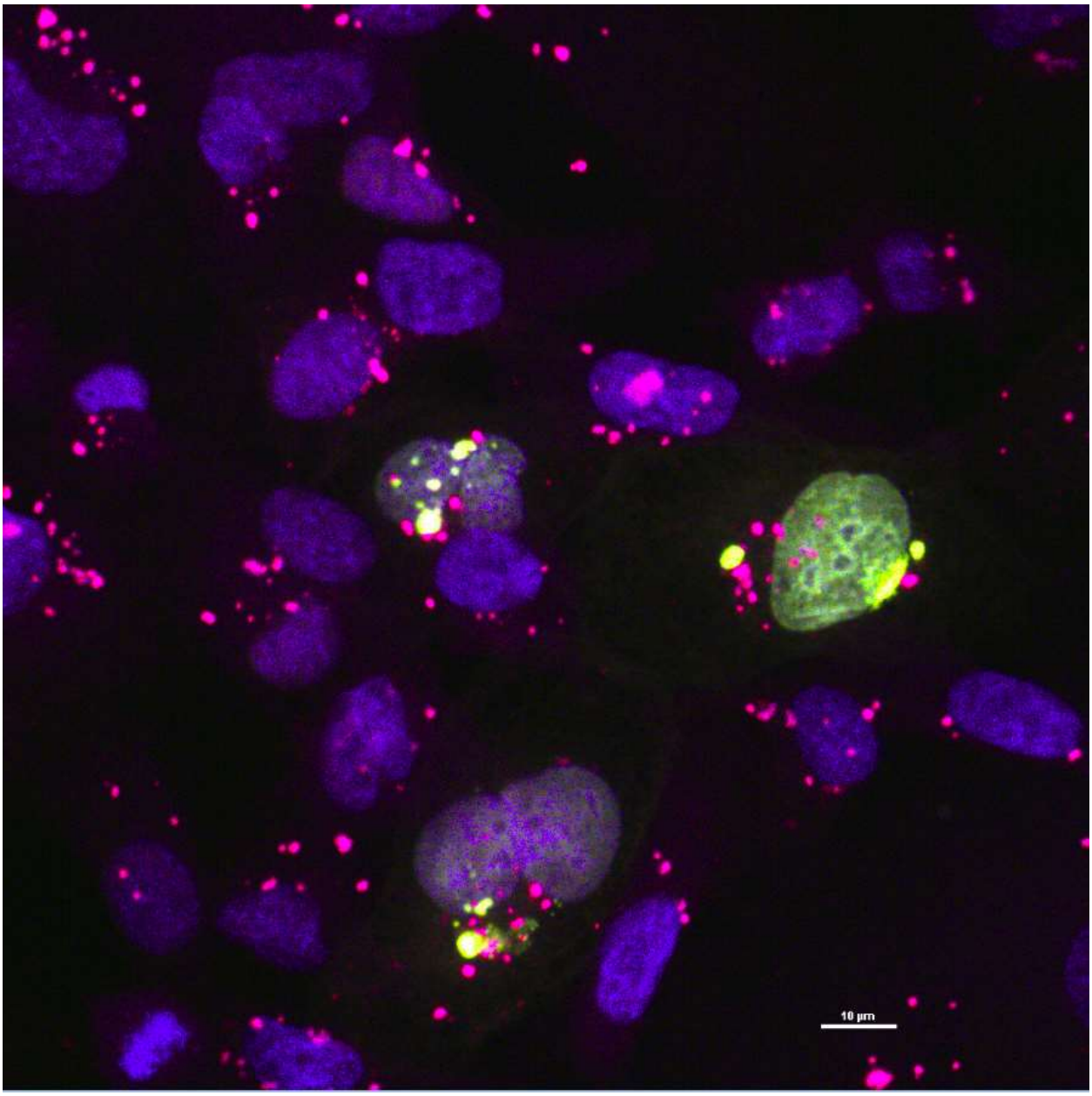

Figure 10D: pmICER-IIγ-pEGFP-N1 at 40X resolution with the DAPI, FITC and TRITC channels from the Nikon C2 Plus CLS microscope. These cells have undergone immunocytochemistry to select ANTI-TUBULIN. The white areas indicate a strong signal of all 3 channels.

Mitotic ICER-IIγ found in highly expressed centrin areas

To further investigate the prevalence of ICER-IIγ in mitotic cells, anti centrin antibodies were employed. Centrin is a highly conserved protein found in all eukaryotic cells. It is established in the initiation sites of centrosomes making centrin important for mitosis (Salisbury et al., 1995). Similar to the fluorescence of the γ-Tubulin antibody, centrin's antibody will fluoresces in the TRITC channel. The Nikon C2 Plus CLS microscope was used to take confocal images of pmICER-IIγ-pEGFP-N1 after undergoing immunocytochemistry for the selection of centrin. The DAPI channel displayed the nucleus of the pmICER-IIγ-pEGFP-N1 cells, (Figure 11A). Mitotic cells were observed expressing ICER-IIγ in the FITC channel, (Figure 11B), and centrin was viewed under the TRITC channel, (Figure 11C). When examining the alignment of all three channels, cells that were excited in yellow indicated colocalization, (Figure 11D). A closer image of mitotic ICER-IIγ aligned with centrin, in the nucleus, is shown in (Figures 12D and 13D).

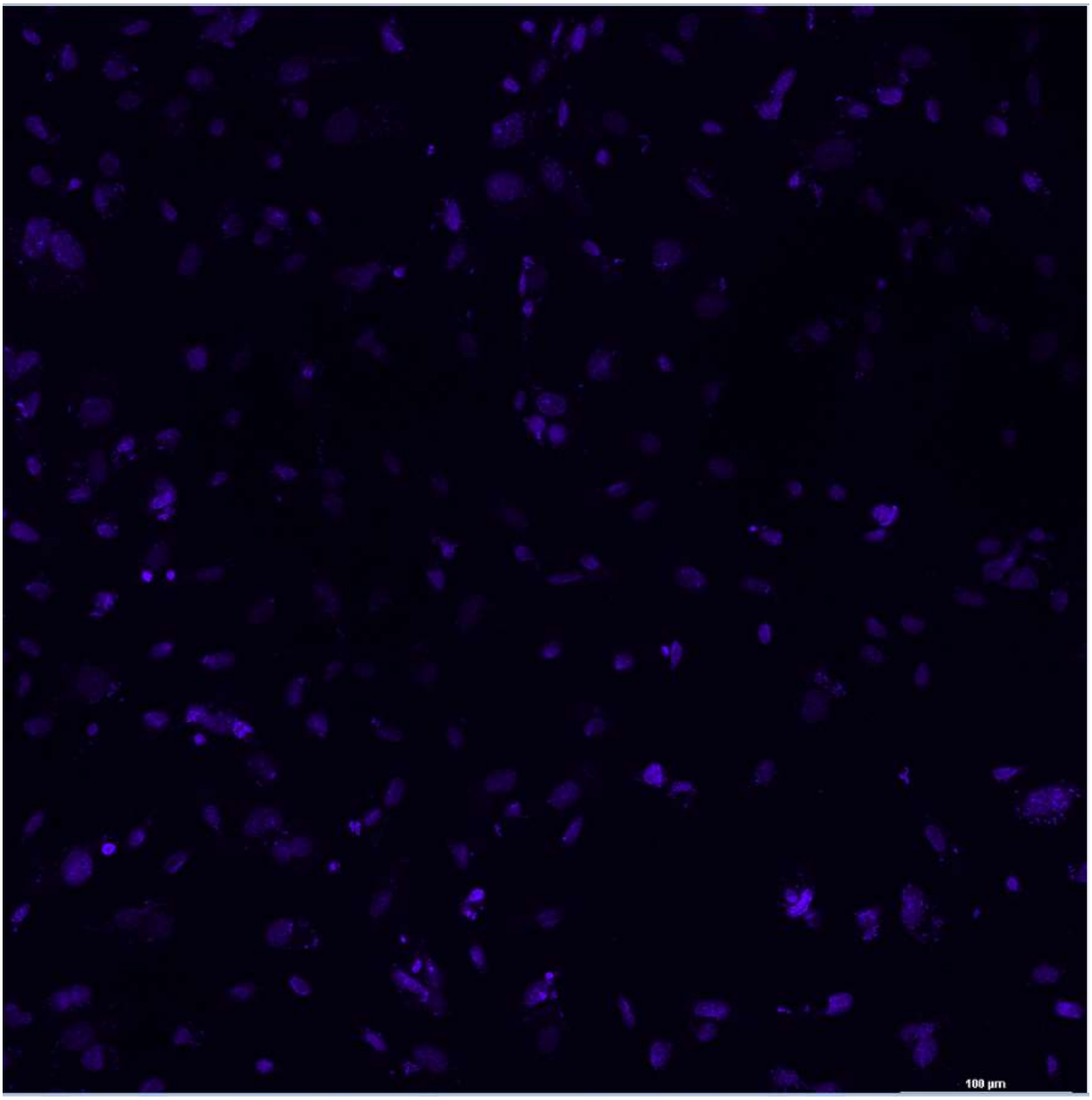

Figure 11A: pmICER-IIγ-pEGFP-N1 at 10X resolution with the DAPI channel from the Nikon C2 Plus CLS microscope. These cells have undergone immunocytochemistry to select ANTI-CENTRIN.

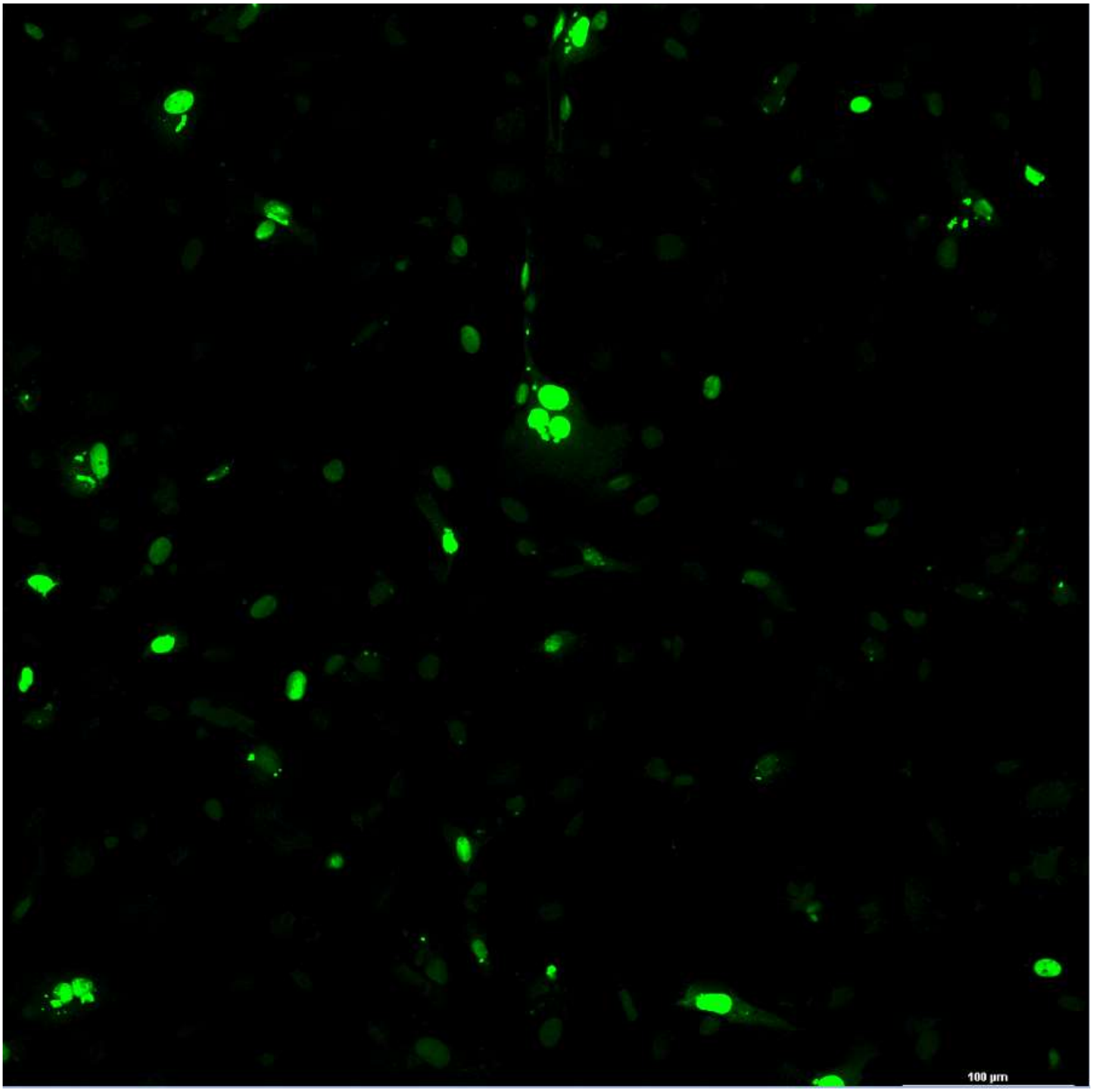

Figure 11B: pmICER-IIγ-pEGFP-N1 at 10X resolution with the FITC channel from the Nikon C2 Plus CLS microscope. These cells have undergone immunocytochemistry to select ANTI-CENTRIN.

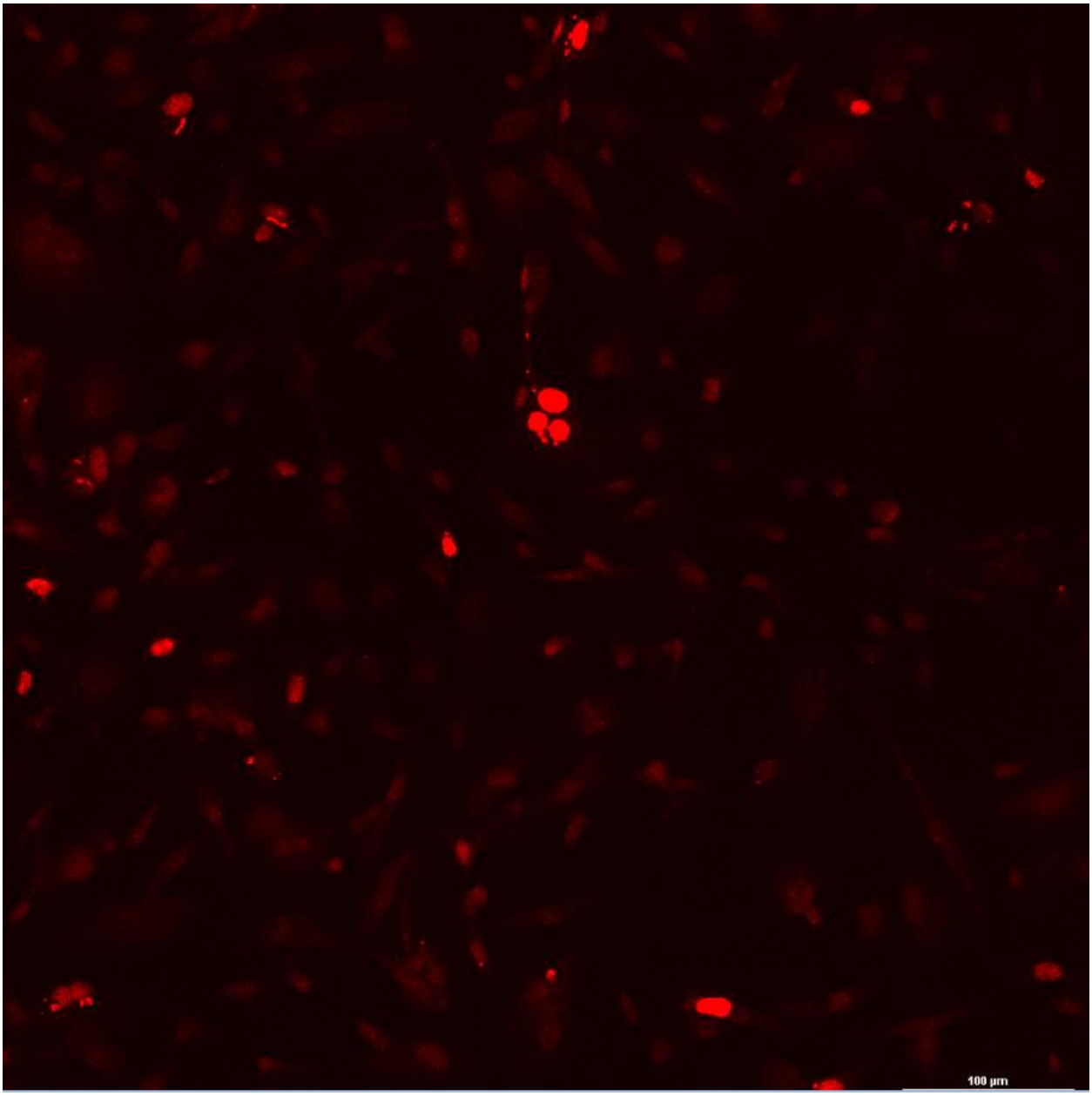

Figure 11C: pmICER-IIγ-pEGFP-N1 at 10X resolution with the TRITC channel from the Nikon C2 Plus CLS microscope. These cells have undergone immunocytochemistry to select ANTI-CENTRIN.

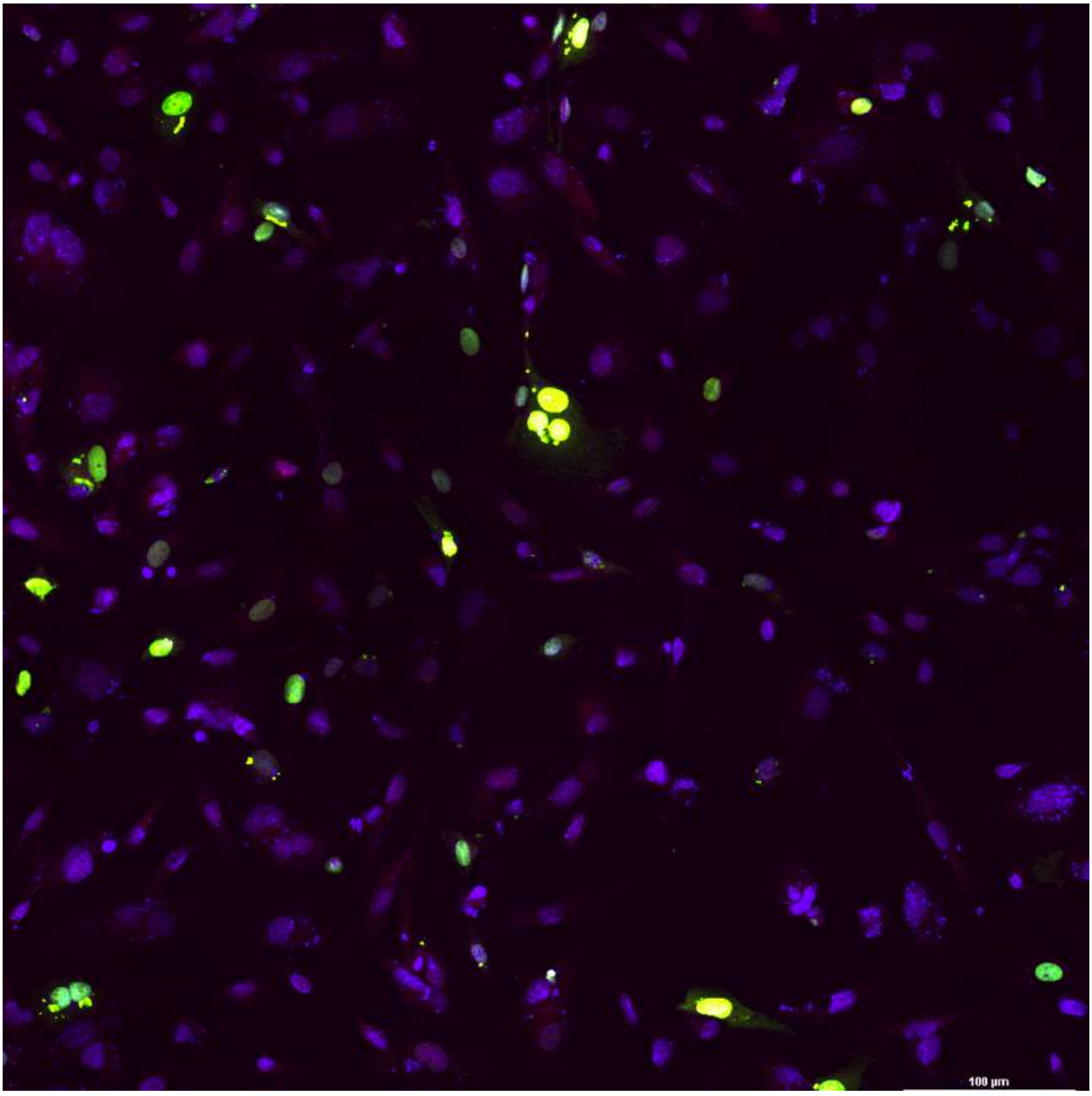

Figure 11D: pmICER-IIγ-pEGFP-N1 at 10X resolution with the DAPI, FITC, and TRITC channel from the Nikon C2 Plus CLS microscope. These cells have undergone immunocytochemistry to select ANTI-CENTRIN. The yellow areas indicate a strong signal of all 3 channels.

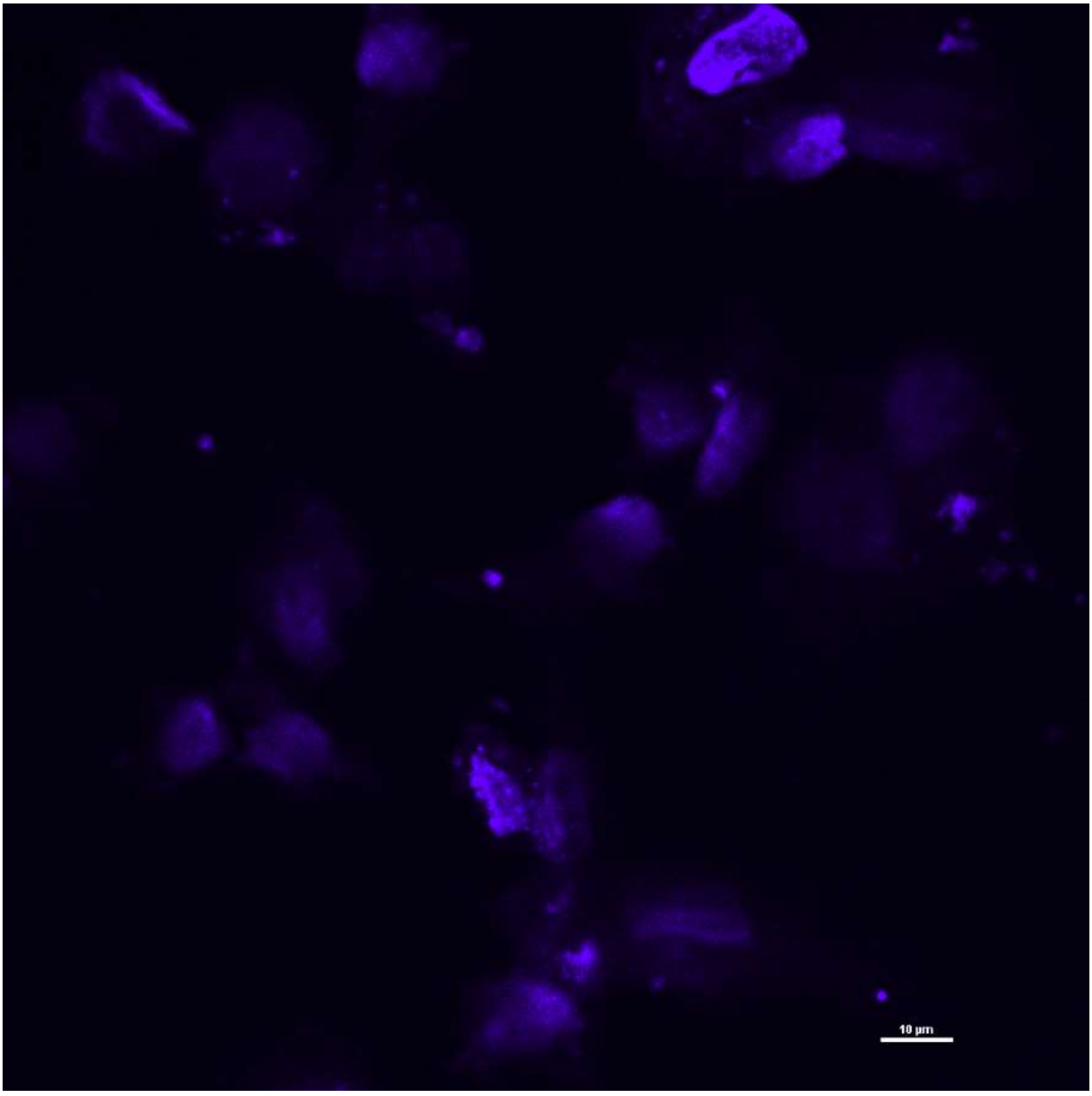

Figure 12A: pmICER-IIγ-pEGFP-N1 at 40X resolution with the DAPI channel from the Nikon C2 Plus CLS microscope. These cells have undergone immunocytochemistry to select ANTI-CENTRIN.

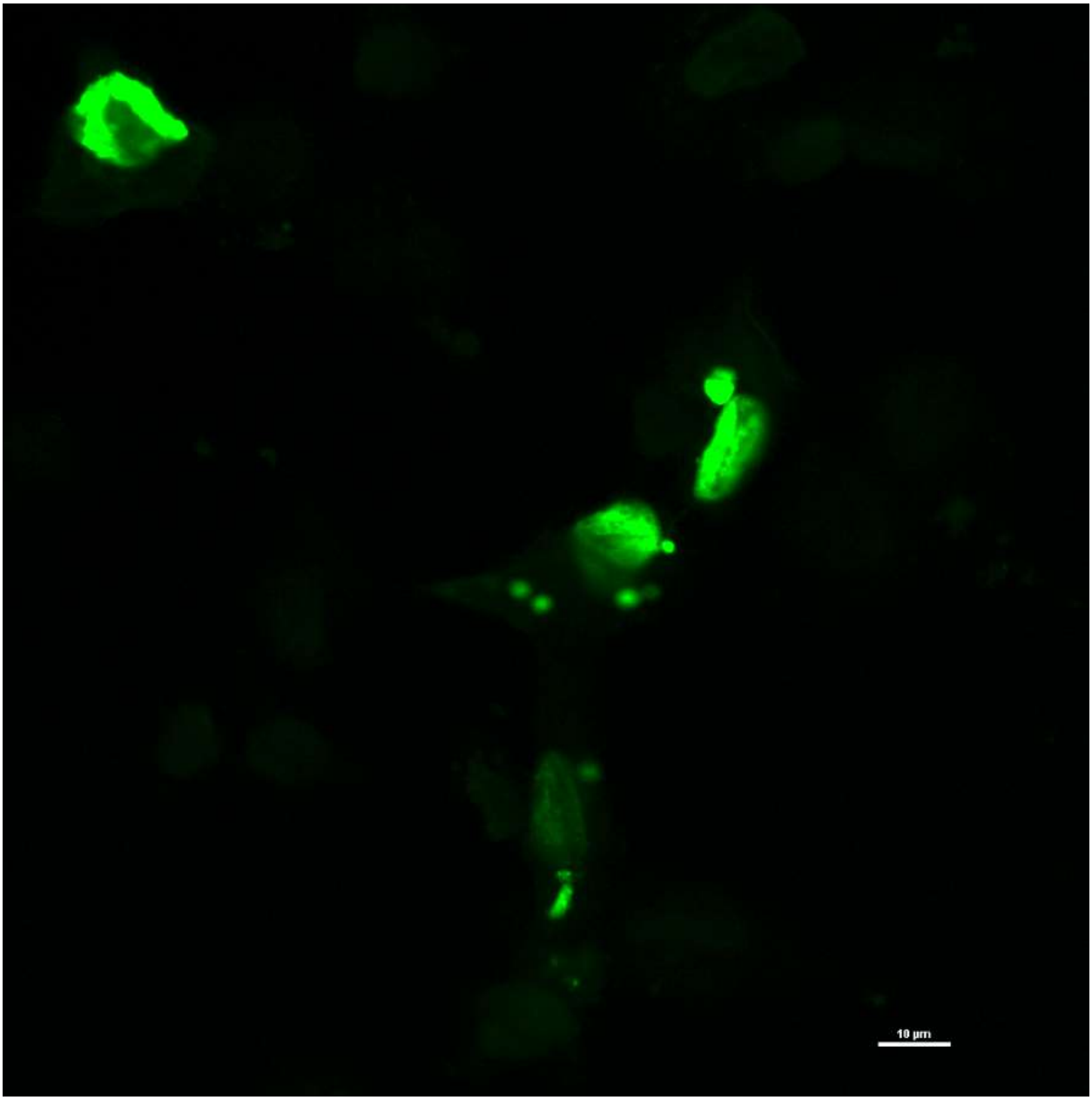

Figure 12B: pmICER-IIγ-pEGFP-N1 at 40X resolution with the FITC channel from the Nikon C2 Plus CLS microscope. These cells have undergone immunocytochemistry to select ANTI-CENTRIN.

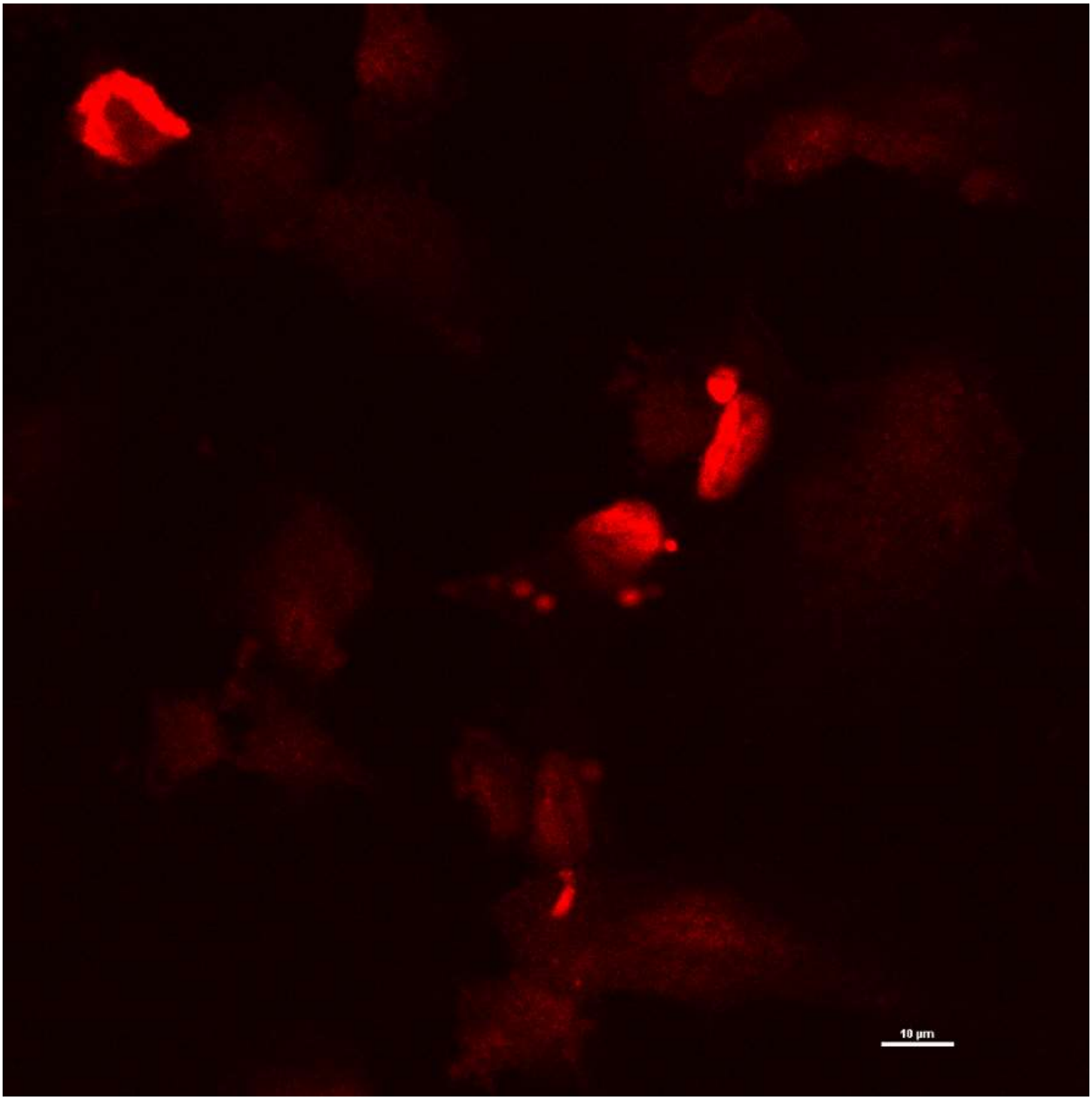

Figure 12C: pmICER-IIγ-pEGFP-N1 at 40X resolution with the TRITC channel from the Nikon C2 Plus CLS microscope. These cells have undergone immunocytochemistry to select ANTI-CENTRIN.

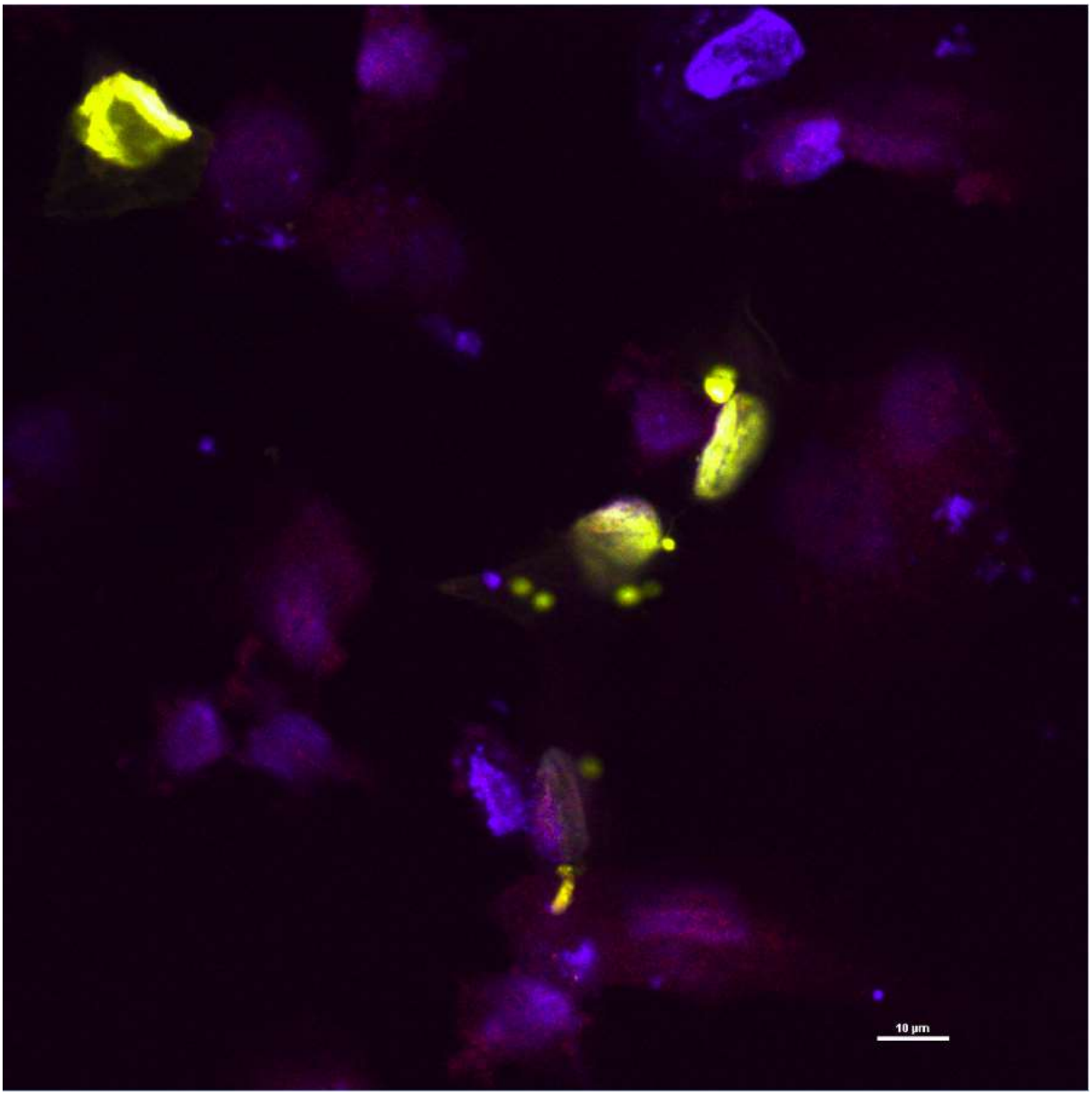

Figure 12D: pmICER-IIγ-pEGFP-N1 at 40X resolution with the DAPI, FITC and TRITC channel from the Nikon C2 Plus CLS microscope. These cells have undergone immunocytochemistry to select ANTI-CENTRIN. The yellow areas indicate a strong signal of all 3 channels.

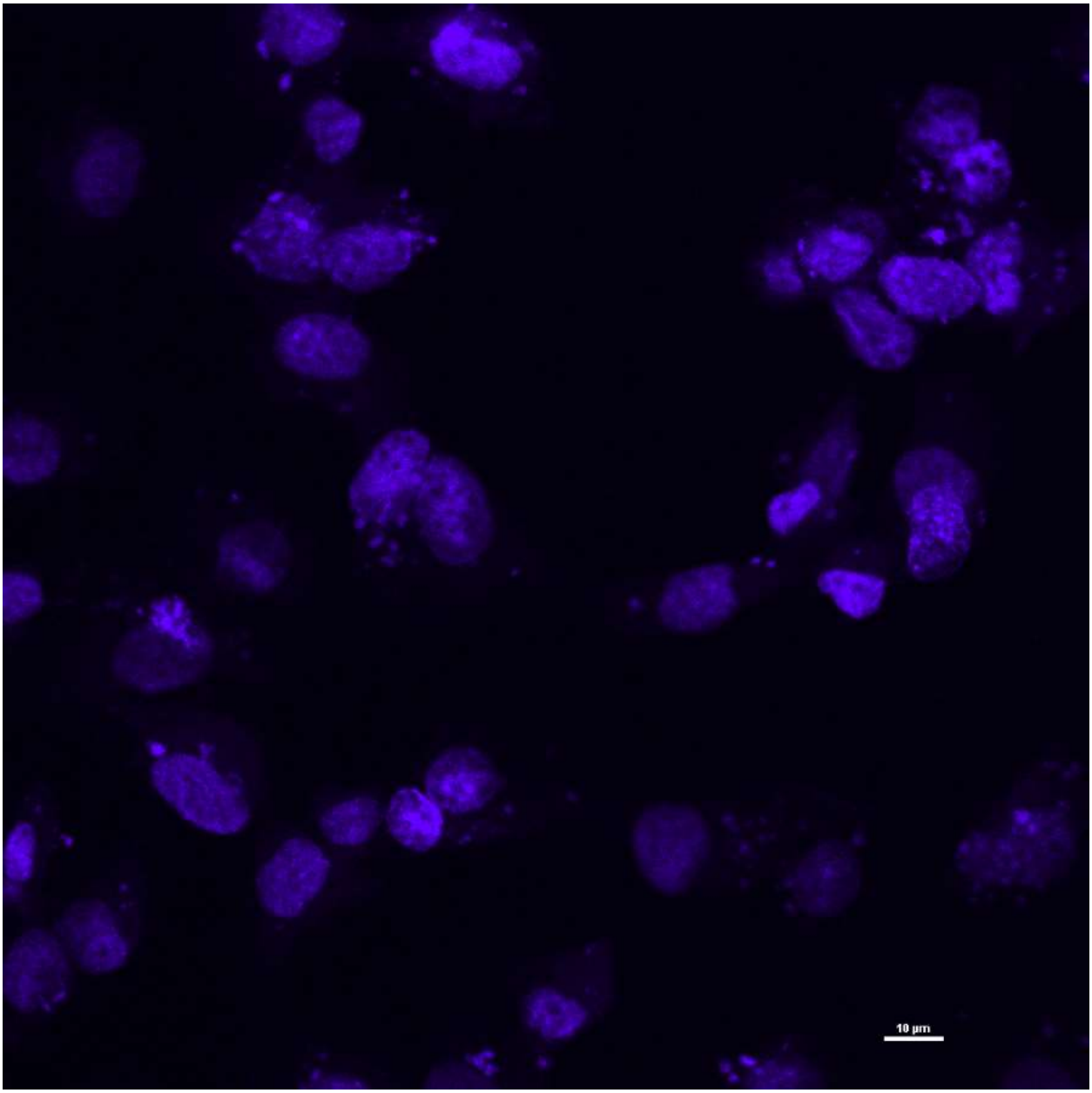

Figure 13A: pmICER-IIγ-pEGFP-N1 at 40X resolution with the DAPI channel from the Nikon C2 Plus CLS microscope. These cells have undergone immunocytochemistry to select ANTI-CENTRIN.

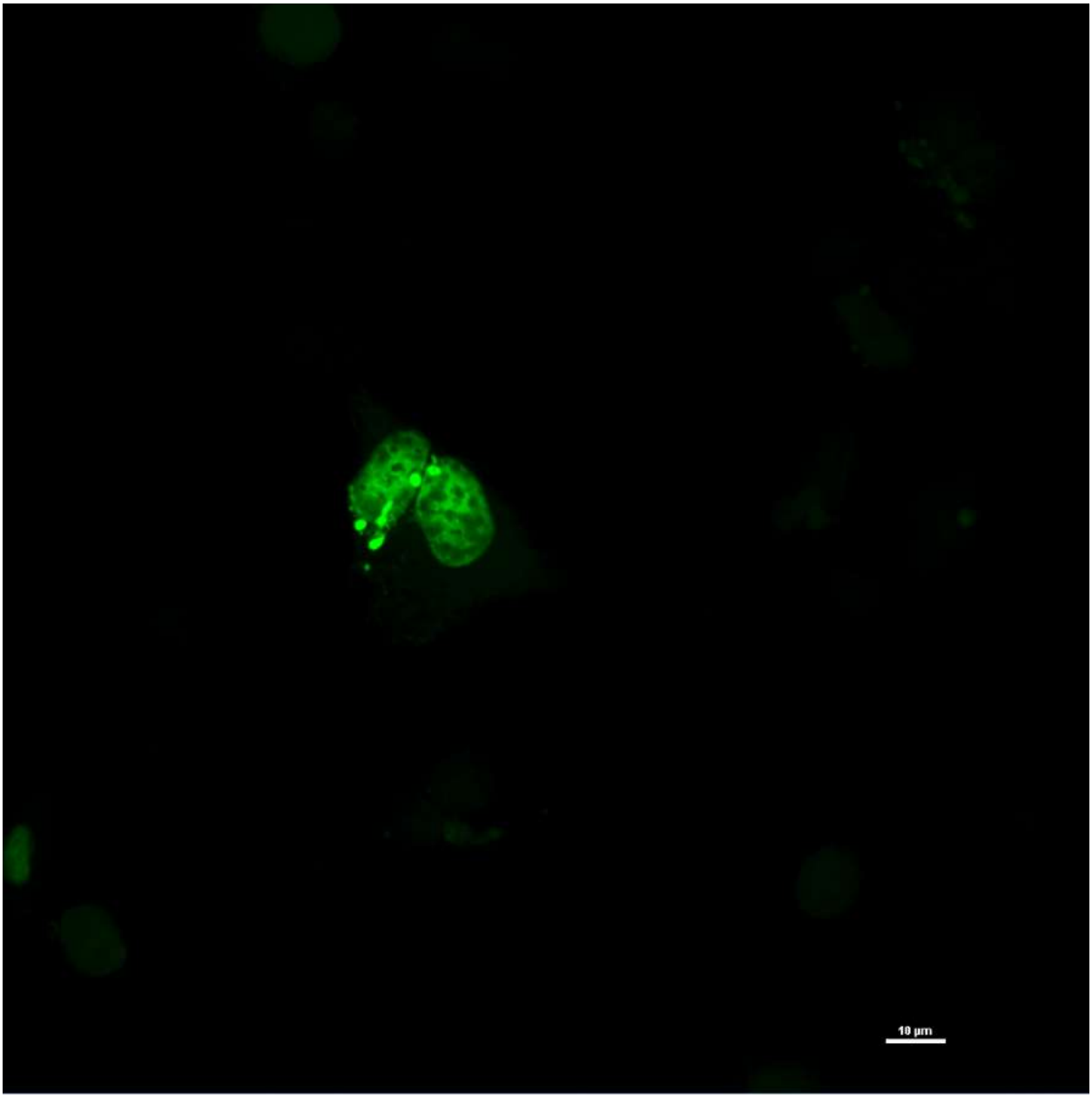

Figure 13B: pmICER-IIγ-pEGFP-N1 at 40X resolution with the FITC channel from the Nikon C2 Plus CLS microscope. These cells have undergone immunocytochemistry to select ANTI-CENTRIN.

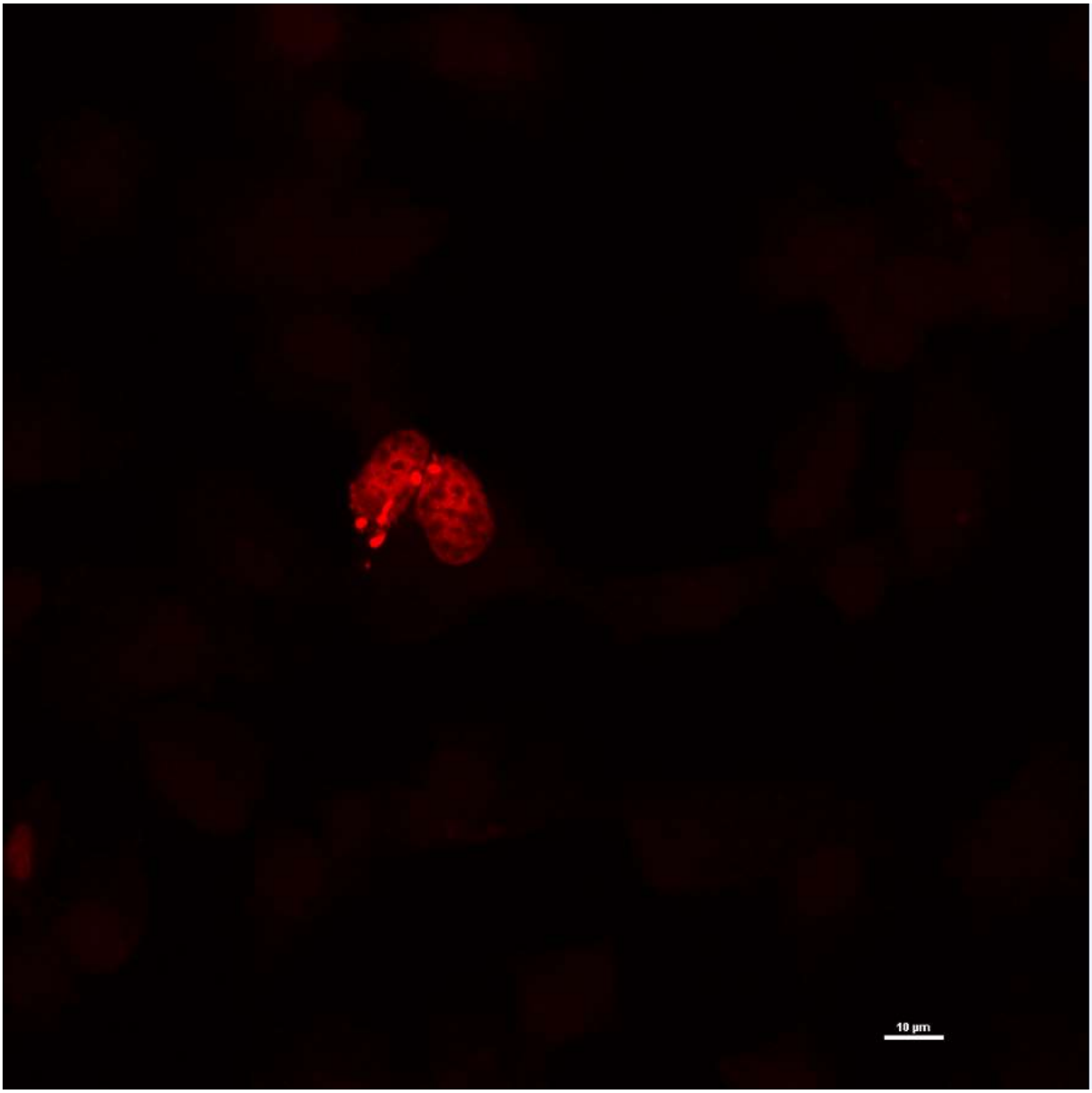

Figure 13C: pmICER-IIγ-pEGFP-N1 at 40X resolution with the TRITC channel from the Nikon C2 Plus CLS microscope. These cells have undergone immunocytochemistry to select ANTI-CENTRIN.

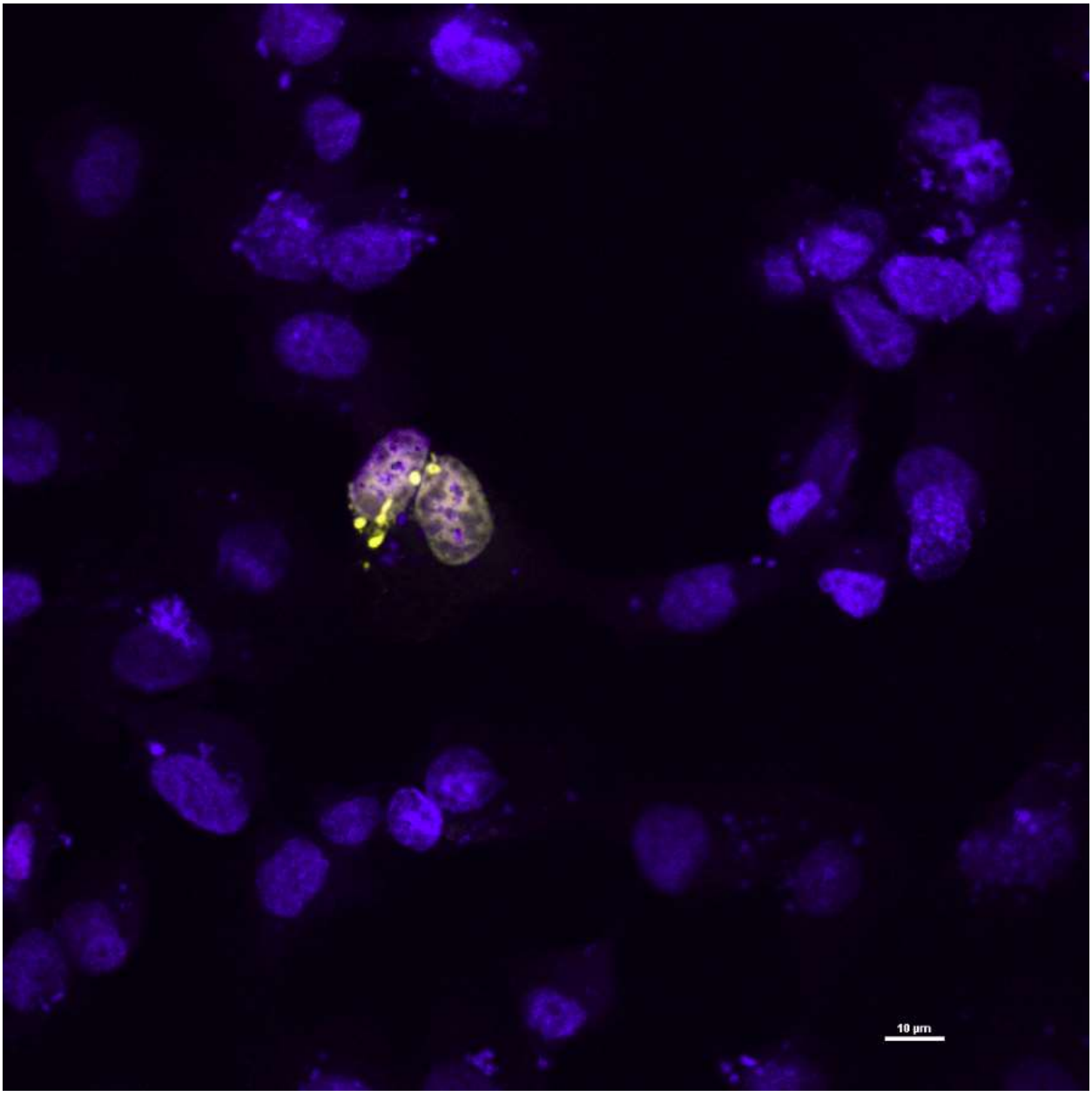

Figure 13D: pmICER-IIγ-pEGFP-N1 at 40X resolution with the DAPI, FITC and TRITC channel from the Nikon C2 Plus CLS microscope. These cells have undergone immunocytochemistry to select ANTI-CENTRIN. The yellow areas indicate a strong signal of all 3 channels.

DISCUSSION

Our analyses may indicate mitotic activity of the ICER-IIγ N-terminal. Prior studies identified nuclear localization of ICER, however, posttranslational monoubiquination of an ICER residue was shown to cause protein translocation to the cytosol (Mémin et al., 2011). ICER is known as a small transcription factor that originated within an intron promoter region of the *CREM* gene. One of its roles is to act as a putative tumor suppressor by mediating cAMP anti-proliferative activity. Once ICER is transcribed it negatively regulates *CRE* gene transcription by binding to its promoter region, repressing the activity of *CRE*'s in cell division (Mémin et al., 2002).

In this study, two constructs differing in loci of EGFP tagging were used to conduct our analysis of ICER-IIγ localization. pEGFP-C1-mICERIIγ had EGFP attached to the N- terminus of ICER-IIγ, allowing the carboxyl end of the protein to freely associate. pmICERIIγ-EGFP-N1 had EGFP attached to the C- terminus, allowing the amino end of ICER-IIγ to be unhindered. It was hypothesized that EGFP attachment to either end of ICER-IIγ would not affect localization in the nucleus since stressors were not added to the cell line for it to undergo any unwanted activities.

We have concurred nuclear localization of both ICER-IIγ-pEGFP clones which was observed in DAPI and FITC channels overlay images (Figure 6C, Figure 7C). DAPI staining proposes the visibility of the nucleus of our SK-MEL-24 cells while the FITC channel reports EGFP with ICER-IIγ. During the initial direct microscopy analysis of the two plasmids, it was found that a strong signal of ICER-IIγ appeared to be two small dots in opposing directions within the nucleus. When comparing free C-terminal ICER-IIγ, pEGFP-C1-mICER-IIγ, to free N-Terminal ICERII-γ, pmICER-IIγ-pEGFP-N, there was a much lower percentage of free C-

terminal ICER-IIγ expressing cells in relation to cell density (Figure 6C, Figure 7C). This suggests that the ICER-IIγ N-terminus could not only have additional mechanisms within the nucleus, but that its role is more significant than that of the C-terminus. Additionally, since expression of free N-terminal ICER-IIγ cells was observed to be much higher, this suggests that they underwent more mitotic events compared to those cells with free C-terminals (Figure 6C, Figure 7C). Furthermore, pmICER-IIγ-pEGFP-N cells expressed dots that may indicate the occurrence of mitotic events (Figure 8C). These findings led to the use of antibodies against proteins prevalent in the mitotic apparatus. Antibodies selected for centrin and γ-Tubulin were incorporated and colocalization was still prominent in our pmICER-IIγ-pEGFP-N1 expressing cells (Figure 9D, Figure 11D). This suggests that ICER-IIγ's N-terminus could possibly be involved in the initiation process of mitosis.

In many cases, proteins that enter the nucleus have a sequence of amino acids recognized by the nuclear import mechanism. This mechanism allows proteins to be transported across the nuclear membrane through a signal known as a nuclear localization signal (NLS). Prior studies have shown that ICER is phosphorylated and ubiquitinated during the cell cycle. The mitotic kinase complex Cdk1/cyclin B can phosphorylate ICER in two distinct serine residues, serine 35 and 41, leading to monoubiquination or polyubiquination, respectively (Mémin et al..2011). In this study, they showed that phosphorylation on Ser-35 was associated with the monoubiquitination of ICER, and that monoubiquitinated ICER resided in the cytosol. When NLS is blocked, recognition from nuclear export can be caused by other transcription factors or histones causing chromosome remodeling.

N-terminal phosphorylation of the histone H1 at Ser-35 has been known to accumulate at mitosis after histone H3 phosphorylation has occurred at Ser-10. Histone H1 binds to the

entry/exit sites of DNA on the surface of the nucleosome core particle and completes nucleosome formation. PKA is found to be a kinase for the phosphorylation of Ser-35 for H1. These results suggest that PKA contributes to mitotic regulation with peaks at mitosis that regulates spindle formation (Izzo et al., 2008), CDC2/cyclin B activity, and anaphase-promoting complex activity (Chu et al., 2011). This ultimately suggests the significance of contribution of Ser-35 and PKA phosphorylated proteins to the continuation or repression mitosis. Linker H1when phosphorylated on Ser-35 is involved in chromatin remodeling, allowing DNA replication to occur. While ICER when it is phosphorylated on Ser-35 is re-located in the cytosol, which also allows replication to occur. We have hypothesized that the same phosphorylation events repressing ICER and signaling H1 to remodel to undergo cell division may be a pathway that is involving these two proteins to continue DNA replication. In particular, it may involve with the N-terminus of ICER.

Another activity/pathway that ICER is involved in is the stimulation of β-adrenoceptors which regulates the sympathetic responses in cardiovascular, pulmonary, metabolic and central nervous systems. ICER has been linked to the formation of another isoform of the protein known as the smICER (Seidl et al..2020). Just how ICER is transcribed by the *CREM* gene, smICER is produced from an isoform of CREM. An unknown activity involving ICER is the to induce production of smICER to saturation, allowing the manufacture of gene transcription and cardiac remodeling from prolonged β-adrenoceptor stimulation (Seidl et al..2020). This suggests that there are multiple CRE elements found in the genome. A study had identified that there are about 6800 binding sites occupied by CREM isoforms (Martianov et al., 2010). These studies may suggest that an unknown isoform of CREM may relate to a production of specialized N-terminus ICER that will be involved in cell division.

Further investigation is needed to evaluate the signaling pathway of the N-terminus of ICER. A future study could include using binding assays of different CRE isoforms for ICER in the mitotic phase. Additionally, an investigation could be conducted on phosphorylation events of the N-terminus of ICER. Since our findings show that the N-terminus of ICER is involved in mitosis, this may help to aid in studies that can allow the incorporated of future strategies for cancer therapies.

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