# Highly selective behaviour of colon adenoma after administration of EMR composition and its HCT116-based model.

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**Article**

**Keywords:** Adenoma, Adenomatous Polyps, Endoscopic Mucosal Resection, HCT 116, alpha-amylases

**Posted Date:** August 12th, 2021

**DOI:** https://doi.org/10.21203/rs.3.rs-631801/v2

# Abstract

When applying the improved composition of solution used during endoscopic mucosal resection (EMR), we have observed unexpectedly large and quantitatively significant differences in adenoma response vs. healthy tissue of surrounding GIT tract, namely the selective reaction enhancing the adenoma volume and differentiated colour. The *in vitro* experiments on model neoplasia cell line HCT116 suggest that the robust differences in the response of starving cells can be traced down principally to the tetrastarch digestion and enhanced metabolic rate of neoplastic cells. The neoplastic tissue grows into several intestine layers so that submucosal injection of iso-oncotic tetrastarch compound leads to degradation of starch and production of oncotic molecules in submucosa transported by facilitated transport into the neoplastic tissue. The colour distinction is due to concentration differences of the reporting dye between three separated compartments, further enhancing the utility of the contrasting mixture. The diffusion dynamics shall be tuneable by optimizing starch composition, improving desirable pharmacokinetics.


# Introduction

Neoplasms of the gastrointestinal tract are tumour-transformed tissues that can gradually progress into malignant tumours. The therapy of such neoplasms is usually radical, by surgical procedure-incision of detected neoplasms. The position, shape, and size of the neoplasm are typically corroborated using the endoscopic method. The endoscopist uses an endoscopic optical probe to localize the position of such polyps along the gastrointestinal tract.

The neoplasia which is not embedded into the deeper layers of the tissue can be removed during the same endoscopic examination session by using endoscopic mucosal resection (EMR). By resection through the middle or deeper part of the submucosal layer, EMR allows complete and curative resection of the diseased mucosa. For indicated early stages of progression, the EMR can be accomplished with minimal cost, morbidity, and mortality-and can improve the long-term quality of life of patients[1,2].

Injection of a suitable solution is used to separate the neoplasm from muscularis propria. If the lesion is distinct visually, it usually means that there is no deep submucosal invasion. On the other hand, the “non-lifting sign” has been found to have 100% sensitivity, 99% specificity, and 83% positive predictive value for invasive carcinoma[3].

Successful elevation of neoplasm allows the application of polypectomic loop and control of incision process. The composition of the injection solution for EMR use is not standardized[4], but efforts have been made to improve the coagulant and colouring properties of such compositions. In literature, using a highly concentrated salt solution as a coagulant with the addition of adrenalin or epinephrine is reported[5]. Alternatively, the physiological solution with the addition of the derivatives of cellulose, succinyl gelatine, glycerol, and fibrinogen have been formulated to slow down diffusion[4]. The physiological solution with the addition of methylene blue or sodium salt of indigotindisulfonate was applied as a visual tool staining the neoplasm[5].

The disadvantage of currently utilised solutions for diagnostics and surgical treatment of neoplasm of the GI tract is mainly the short time of the elevation of the lesion (separation of adenoma) and only moderate visual distinction from surrounding tissue. The lifetime of such raised adenoma is determined by fast diffusion of injected solution, which leads to the disappearance of adenoma without its colour distinction. When an auxiliary colouring agent is used, the colour boundary between the adenoma and healthy tissue is dispersed due to the rapid diffusion of the colouring agent into both volumes.

During our search for the optimal solution composition, we have found the very pronounced effect when a specific injection solution - composed from components approved for systemic administration and administered under valid law - was applied as a submucosal injection into the neighbourhood of suspected neoplastic tissue as a part of EMR procedure. Several polyps raised above the injected tissue, lasting several minutes and forming pronounced stem allowing for comfortable application of polypectomic loop. The colouring compound formed three differently coloured volumes - injected tissue, neoplastic tissue, and the thin boundary between them, further helping to diagnose the extent of neoplastic tissue and its level of embeddedness. The new empirically found composition clearly and repeatedly improved the EMR procedure compared with the actual clinical practice at the hospital and compared with literature.

In our attempt to elucidate the behaviour observed during EMR, we have performed the *in vitro* experiments on the HCT116 (human colorectal carcinoma) cell line subjected to the conditions as present during endoscopy. In the paper, we report our observations and suggest the mechanism of action based on our limited experiments and known facts.

# Materials And Methods

*Preparation of EMR solution.* The EMR solution we used consists of three-components: the physiological (saline) solution, in which the visual contrasting aid - the sodium salt of [4-(alpha-(4-diethylaminophenyl)-5-hydroxy-2,4disulphophenyl-methylidene)-2,5cyclohexadiene-1-ylidene]diethyl-ammonium hydroxide inner salt (PATENTEBLAU V sol. inj. 2ml/50mg, GUERBET, France)[6] and the colloid modulator of velocity - a Hydroxyethyl starch (HES) (VOLUVEN® sol. inf. 1x500ml, Fresenius Kabi, Bad Homburg, Germany)[7] was dissolved. We prepared a mixture of EMR composition by mixing 500 ml of isotonic saline solution with 1 ml of colour constituent (PATENTEBLAU V) followed by diluting with a HES drawn up into the syringe with Combi-Stopper (syringe bung) in the ratio 3:7 under aseptic conditions and apyrogenic (dilution closed path) in a laminar flow hood.

*The selection of suitable patients and administration protocol.* From March 1$^{st}$ to June 30$^{th}$, 2014, 62 patients (19 females and 43 males of average age 56,8 and 61,1 years respectively) were indicated for the EMR at the Department of Gastroenterology of the Central Military Hospital.

The patients were selected for the EMR either directly at the Gastroenterology Department or following doctor referral at another department of gastroenterology in Slovakia due to a preventive examination.

The patients were diagnosed during routine colonoscopy with sessile or semisessile adenoma of colon and rectum (diagnoses group ICD-10 codes D 12.0, D12.2-D12.8). All 62 patients undergoing the polypectomy at The Department of Gastroenterology of the Central Military Hospital during this period were administered the new EMR solution. Exclusion criteria (EMR contraindication) were thrombocytes (PLT) less than $50x10^9$/l, prothrombin time ratio (INR) more than 1.4, discontinuation of anticoagulant or dual antiaggregant therapy less than seven days before EMR. The number of patients (sample size n=62) who underwent polypectomy with EMR intervention is typical for 4-month period at the hospital. There were no other selection and/or exclusion criteria applied. In other words, all patients within the period were administered the new EMR composition, with probability p=1.0.

Informed consent was obtained from all patients. The procedure was approved by the Ethical Committee of the Central Military Hospital SNP Ružomberok, Slovakia according to the valid Slovak law, pursuant to §18a, No. 140/1998 Coll.[8] of 3 April 1998 on Medicines and Medical Devices, on the Amendment of Act No. 455/1991 Coll. on Trade Licensing (Trade Licensing Act) as amended and on the amendment of the Act of the National Council of the Slovak Republic no. 220/1996 Coll. about advertising for the non-interventional clinical trials.

The administration protocol followed the routinely used procedure. The only change in the application protocol was replacing the EMR composition consisting of a physiological (saline) solution, PATENTEBLAU V, and adrenaline with the new composition.

During the endoscopic session, the close neighbourhood of suspect tissues was injected by submucosal injection via the endoscopic channel in 1-20 ml volume (depending on neoplasm size), leading to the bolus and subsequent volume and colour changes. The elevated polyps were removed using polypectomic loop and removed tissue was analysed for histology. As a rule, several polyps became visible with formed stem lasting several minutes and removed in a single step during the same endoscopic session.

*Cell line model.* The human cancer cell line HCT116 (large intestine) was purchased from American Type Culture Collection (ATCC, CCL-247™) and cultured in RPMI 1640 growth medium (Biosera, Kansas City, MO, United States). The growth medium was supplemented with a 10% foetal bovine serum (FBS), 1x HyClone™ Antibiotic/Antimycotic solution (GE Healthcare, Little Chalfont, UK) and maintained in an atmosphere containing 5% $CO_2$ in humidified air at 37 °C. Before experiments, the viability of cells was analyzed by trypan blue assay. The cell line was authenticated by the ATCC Laboratory Authentication Service using Sanger sequencing. ATCC declared no Mycoplasma contamination. Before experiments, cells specimens were tested again on Mycoplasma contamination by DNA staining and fluorescence microscopy visualisation with negative results.

For experiments, cells were seeded in 96-well low-density plates and maintained in a complete culture medium for 24 hours. The cells were then starved in saline solution without nutrients for 24 hours,

mimicking the patient preparation before EMR surgery.

The initial cell culture was grown to 10 thousand cells per well, forming plaques. During the starving, part of the cells detached and floated freely in the medium. We removed free-floating cells with the part of liquid media, and the removed volume was then restored by adding the same volume of the saline solution. Even though the saline solution detaches part of the starving cells, a significant fraction of the starving cells stays attached to the density plate after adding the saline solution.

*Adding the contrasting composition.* The EMR composition was applied for two groups of cells – nonstarving and after 24 hours starving in saline solution. After 24 hours, the non-starving cells were adherent to the density plate, while in starving cells, the free-floating cells had to be removed and media replenished by saline solution first. In both cases, the last step of the procedure consisted of replacing the saline medium with an EMR composition. For starving cells, about half of the volume of saline was replaced by EMR composition. Subsequently, we have prepared a set of the substance without a colour constituent. In all cases, the cells exposed to the solution were followed by 10 minutes of live video flow on a Cytation 3 Cell Imaging multimode sensor (BioTek Instruments, Inc.) and evaluated visually for cell count, cell volume, and cell shape change.

# Results

*EMR use.* Under the conditions specified above, the EMR composition was applied to 62 patients with adenoma qualifying for EMR. The typical reaction to the administration of contrasting composition by submucosal injection of adenoma larger than approximately 20 mm is depicted in Figs. 1 - 4. EMR composition provides colour contrasted differences between the tissues. The normal tissue is light blue. The thin boundary of dark blue colour is formed between the adenoma and the healthy tissue, while the adenoma is not coloured. By injecting this EMR composition into the submucosa layer, the adenomatous polyps increase their volume noticeably and elevate above the surface for 10-25 minutes, prolonging the time window for the resection. At the same time, sharp colour differences between the healthy and the neoplastic tissue and the boundary between them can be observed. Colour distinction and the increased volume of the elevated polyp thus improve the precision and quality of polypectomic surgery. The observed reaction of adenomatous polyps to new EMR composition differs from reaction to commonly used solutions where the volume changes are in the submucosa and the colour contrast – if colouring component is used – is more diffuse and not so sharply pronounced.

*In vitro model.* For non-starving cells, we observed no significant changes when the saline solution was replaced by EMR composition. This is in sharp contrast with the reaction of starving HCT116 cells. In repeated experiments, the HCT116 cells starved by 24 hours in saline solution detached entirely from the density plate. Fig. 5 and Fig. 6, show rare cases where the group of detached cells remains partially attached so that the cells could still be localised and recognised. In other cases, the reaction of cells was so pronounced that the cells completely disappeared from the visual field of the microscope and were not identified. For the cells on both Fig. 5 and Fig. 6, we estimated cell volume changes about 4%. Still,

for most experiments, the expected volume expansion leading to the separation of cells must be higher but was not quantified.

The experiments repeated after two weeks with different HCT116 cultures confirmed the same results.

# Discussion

Our original attempt to improve the EMR composition led us to observe significant differences in the response exclusively for starving cells. Starvation of *in vitro* cell culture was achieved by leaving the cells 24 hours in saline only (i.e., no standard fasting medium). In clinical practice, the starvation of the cells is accomplished by the patient abstaining from oral food and fluid intake for 24 hours (*nill per os*) before EMR.

The only component of EMR composition capable of triggering such reaction is HES. The colon and rectum are not involved in starch digestion, so the differences must be due to the starch processing capabilities of neoplastic tissue.

The administration of EMR composition into submucosa exposes the embedded part of the adenomatous polyp to the HES - chemically modified starch. HES as a large macromolecule (average molecular weight 130 kDa)[9] is expected to diffuse slowly in the submucosa. Indeed, this can be seen by comparison of Fig. 2 and Fig. 3 separated in time by 150 seconds.

We expect to find alpha-amylases (or functionally equivalent enzymes) present in the submucosa and released to the environment by starving neoplastic cells. While we found no direct data supporting the expression of starch-processing alpha-amylases in colon adenomas, they are reported in similar and thus related lung adenocarcinomas[10]. Under the conditions indicated for EMR, the adenoma polyps of size about 20 mm are still mostly benign – less than 10% are further progressed along known adenoma-carcinoma sequence[11]. The probability of 62 EMR resections being all carcinoma is thus very low, and we shall assume that adenomas already express alpha-amylases in sufficient amounts. Due to the applied protocol, we can assume that the alpha-amylases are present even when part of the liquid (the saline solution used for starving) is removed - in the time scale of experiments, the *de novo* synthesis of alpha-amylases should not manifest within tens of seconds of HCT116 exposition to EMR composition.

If alpha-amylases (or their functional equivalents) are present in the submucosa, the initial volume of iso-oncotic HES can be degraded, and progressively smaller hydrolysis fragments formed in the submucosa. In contrast to blood plasma, even the gradual hydrolysis to fragments below the renal threshold (45-60 kDa)[12] remain available in submucosa for further hydrolysis. As a result, a continuous supply of glucose and hydroxyethyl glucose (hydroxyethylated at C2/C6 ratio 9.05:1)[9] is delivered into the submucosa. The dynamic mixture of fragments, including the final monosaccharide product of alpha-amylases, is produced in the submucosa, contributing to the rise of oncotic pressure.

Without the transport of monosaccharides, the oncotic pressure would manifest as a volume increase of submucosa. In our observation, though, the dominant volume changes are observed in the volume of neoplastic tissue. A similar volume change is also observable *in vitro* at adherent 2D plaques formed by model HCT116 line. While carcinomas are known for enhanced expression of glucose transporters, adenomas must gradually acquire the capability in the early stage of adenoma-carcinoma transition. It seems thus reasonable that polyps of approximately 20 mm size, indicated for EMR, already possess an enhanced amount of glucose transporters, particularly GLUT1[13,14].

In healthy tissue, the transport of monosaccharides proceeds in the direction from the lumen to the serosa, and the transport for an excessive concentration of monosaccharides is facilitated. The adenomas expose the same surface present to the lumen to the submucosa so that the direction of facilitated transport of monosaccharides is reverted.

The facilitated transport of sugars into cells is specific because different saccharides are transported with different efficiency. Also, the hydroxyethyl glucoses are expected to be transported into the cells less efficiently than anhydrous glucose. Thus, the depletion of the pool of oncotic pressure generating saccharides in the submucosa volume follows complex kinetics, well beyond our current focus.

Nevertheless, the *in vitro* HCT116 model cells eagerly transport oncotic molecules, as demonstrated by violent volume changes leading to loss of cell adherence. This kinetics can be why the polyp rather than the submucosa increases the volume in clinical observation/application.

*EMR administration details.* The EMR protocol which was applied corresponds to the submucosal injection of the EMR composition, where the actual composition is delivered into the submucosa.

The resection starts by administration of the EMR composition below the adenoma - into the healthy submucosa. The preparation of the patient includes 24 hours of fasting.

HCT116 cell line forming 2D cell plaque is often utilised as an imperfect model of human colon cancer cells. The adenomatous polyps larger than 20 mm have an increased probability of being malignant[15]. Thus, HCT116 should be reasonably representative of the most numerous and voluminous cell composition of the expected stages of adenoma progression during reported EMR.

# Conclusion

On the practical side, the modification of EMR composition and the application protocol lower the application barrier for EMR by improving the comfort and precision of the EMR. By clearly delineated polyp boundary and the volume changes, lasting a longer time, the EMR can be done in less time-related stress and lower risk of unwanted complications.

Moreover, our work indicates - somewhat surprisingly – that those adenomas in the early stage of transition adenoma-carcinoma already express alpha-amylases and exhibit elevated glucose transport

responsible for volume changes. This provides the opportunity for functional diagnostics similar in spirit to fluorodeoxyglucose contrasting in PET[2].

The explanation we present suggests modifying the application protocol by taking advantage of different pharmacokinetics based on controlled and tuneable development of oncotic pressure. This can probably be used to develop more selective drug delivery to more specifically characterised target tissues.

# Declarations

# Conflict of interest

We declare that none of the authors have competing financial or non-financial interests as defined by Nature Portfolio.

# Data availability

The authors declare that the data supporting the findings of this study are available within the paper. The additional data that support the results of this study are available from the corresponding author upon request.

# Financial support

The authors would like to acknowledge the following grants and project contributions:

FP7-REGPOT-2012-2013-1 call - Proposal 316310 - CELIM "Fostering excellence in multiscale imaging", Faculty of Science, Pavol Jozef Šafárik University in Košice

Open scientific community for modern interdisciplinary research in medicine (OPENMED), ITMS2014+: 313011V455 supported by the Operational Programme Integrated Infrastructure, funded by the ERDF Charles University grant (SVV 260 551)

Center of Clinical and Preclinical Research MEDIPARK, Faculty of Medicine, Pavol Jozef Šafárik University in Košice

**Role of authors:**

**Patrik Jakabčin** conceived and elaborated the idea, clarified the legislative conditions and prepared the EMR composition, performed the cell -line *in vitro* experiments, participated in writing paper. **Martin Kello** cultivated the cell-lines and performed the *in-vitro* experiments. **Jozef Záň** performed the EMR resections. **Jozef Kolář** advised the clinical pharmacology aspects. **Jozef Uličný** conceptual design of the paper, supervising, assisted during *in-vitro* experiments, writing-review and editing.

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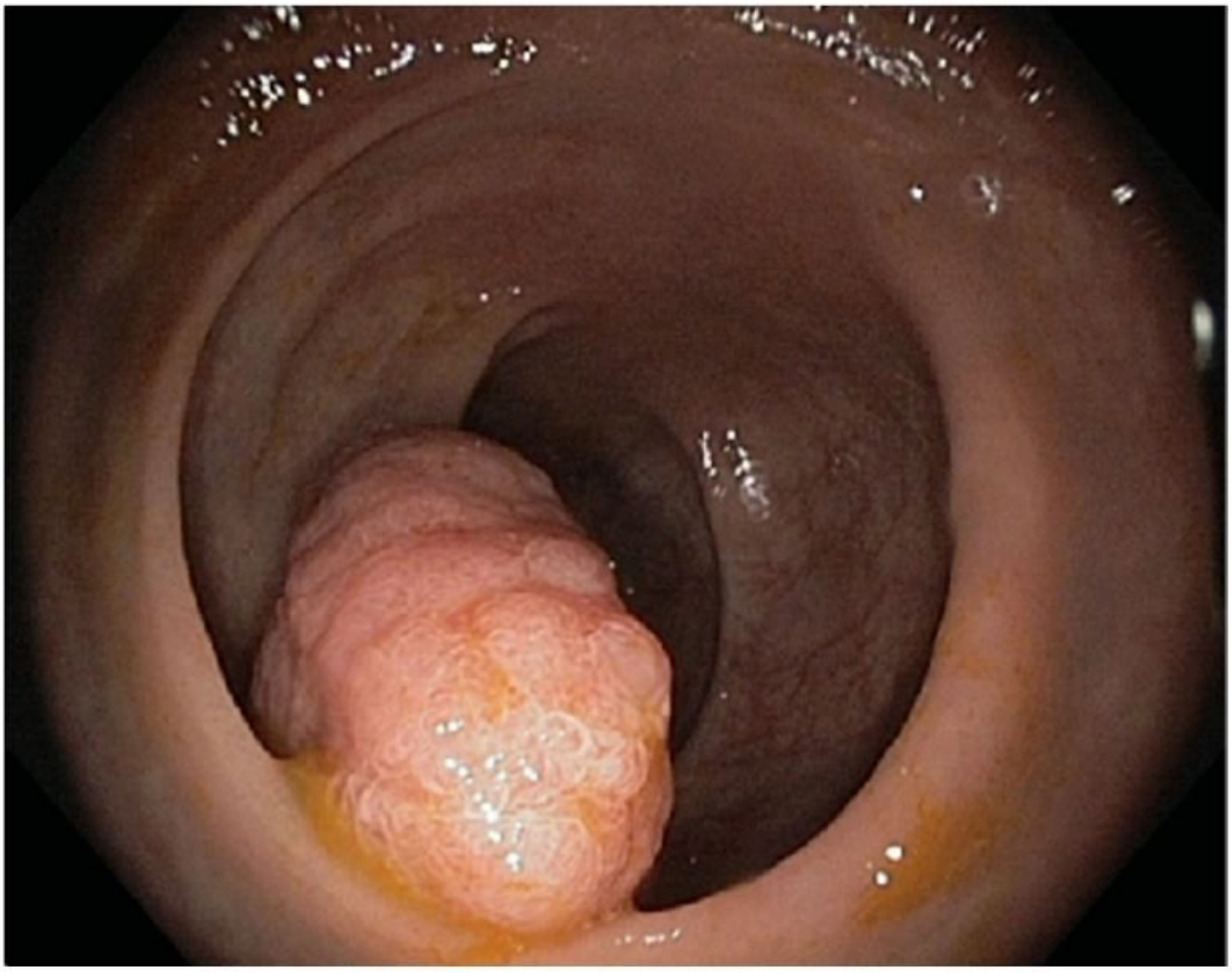
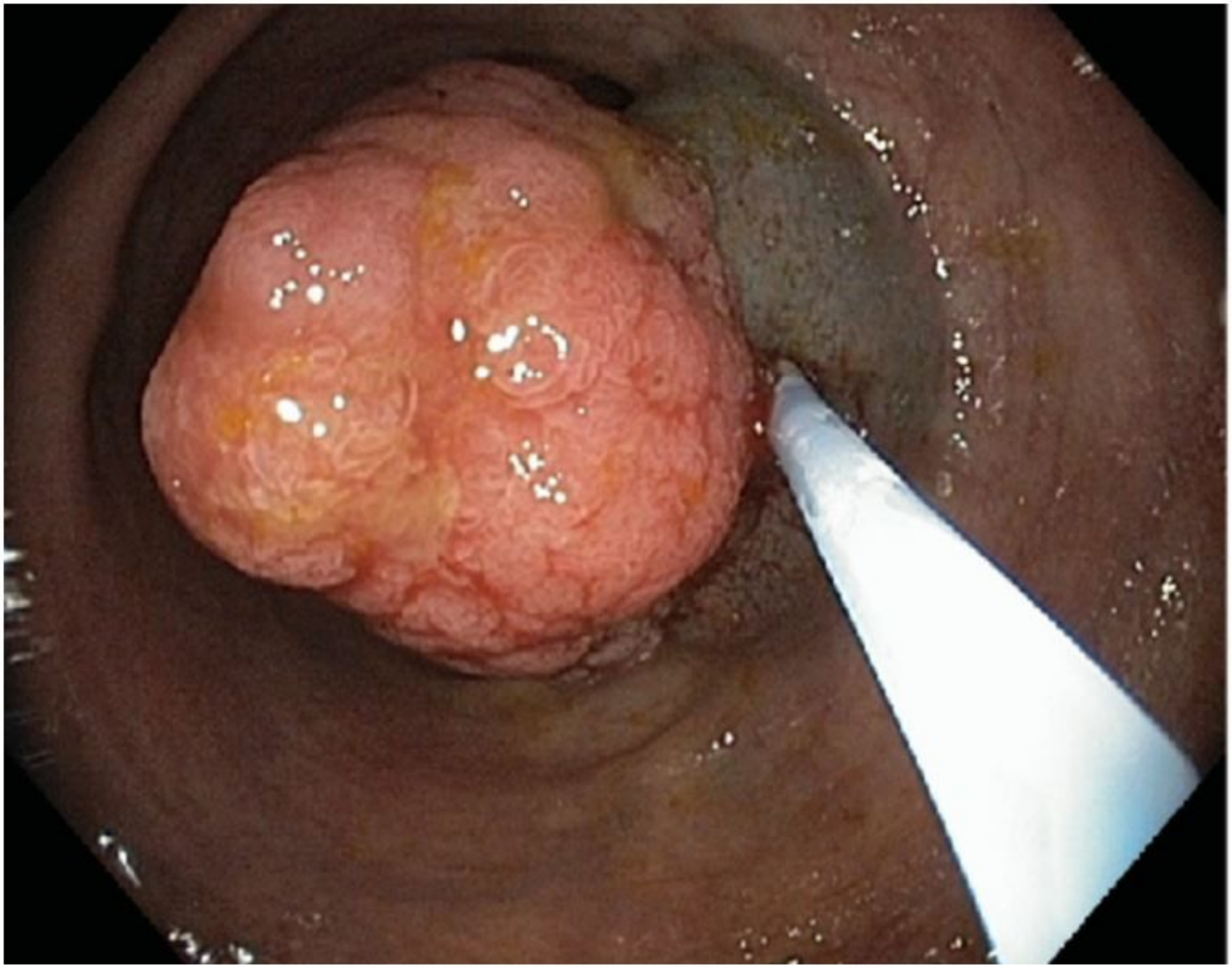
# Figures



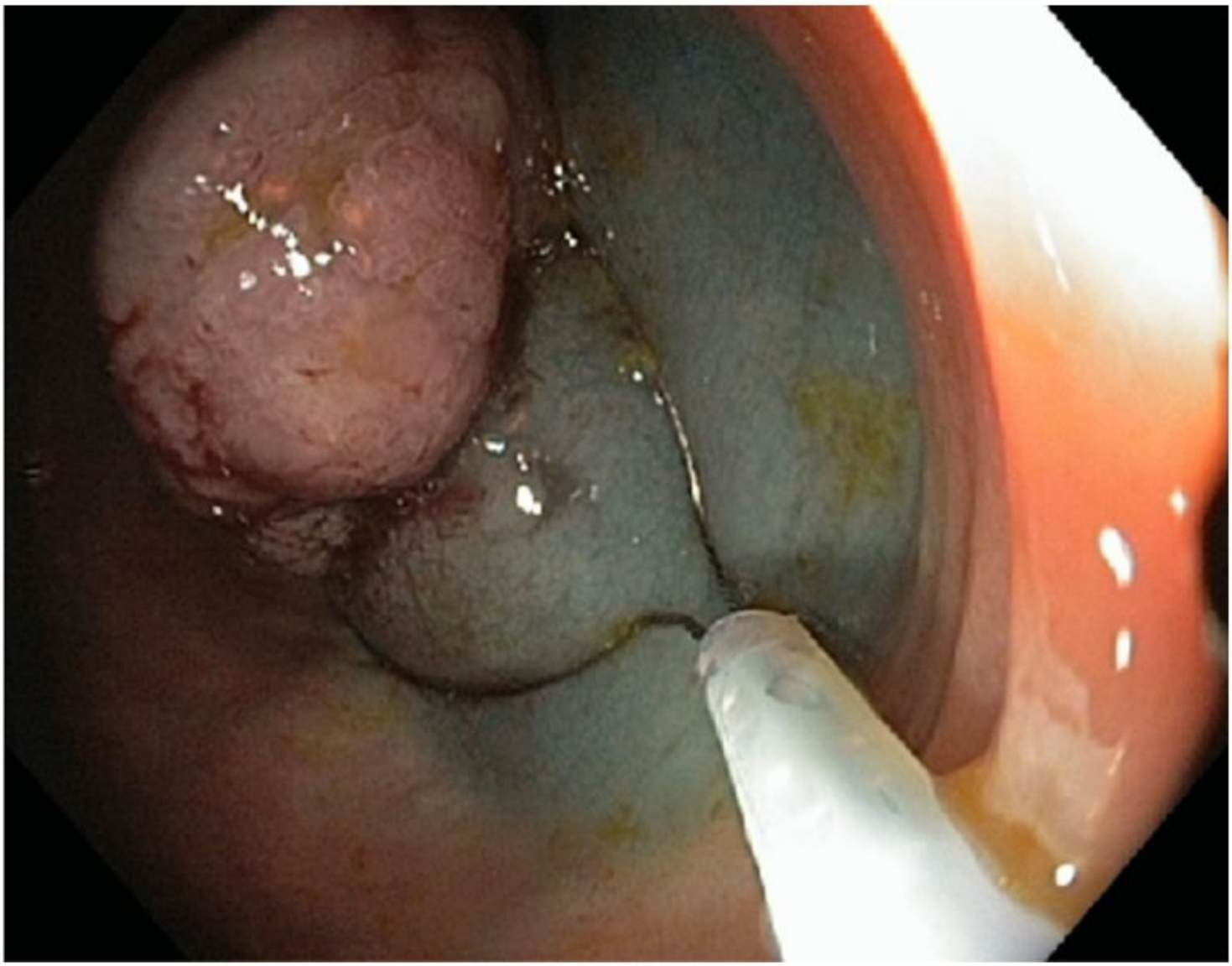
## Figure 1

Endoscopic findings before submucosal injection of the EMR composition. The size of the polyp is about 4.5 cm. (Time T+0 seconds immediately before submucosal injection)



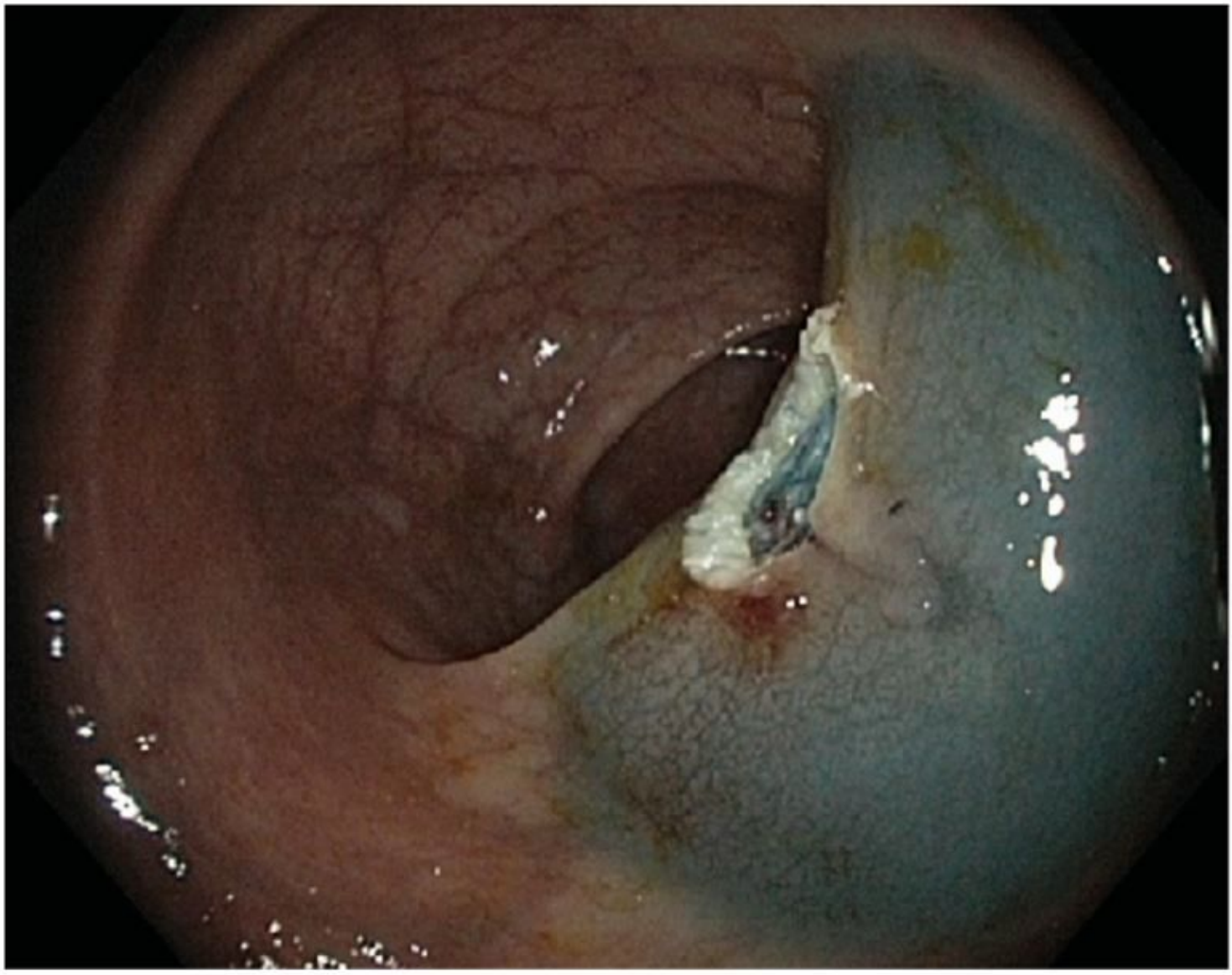
## Figure 2

Submucosal injection of EMR composition below the adenoma. The surrounding healthy tissue is of light blue colour, the adenoma colour is nearly unchanged. Note the rise of volume of the adenoma. (Time T+150 seconds after submucosal injection of EMR composition)



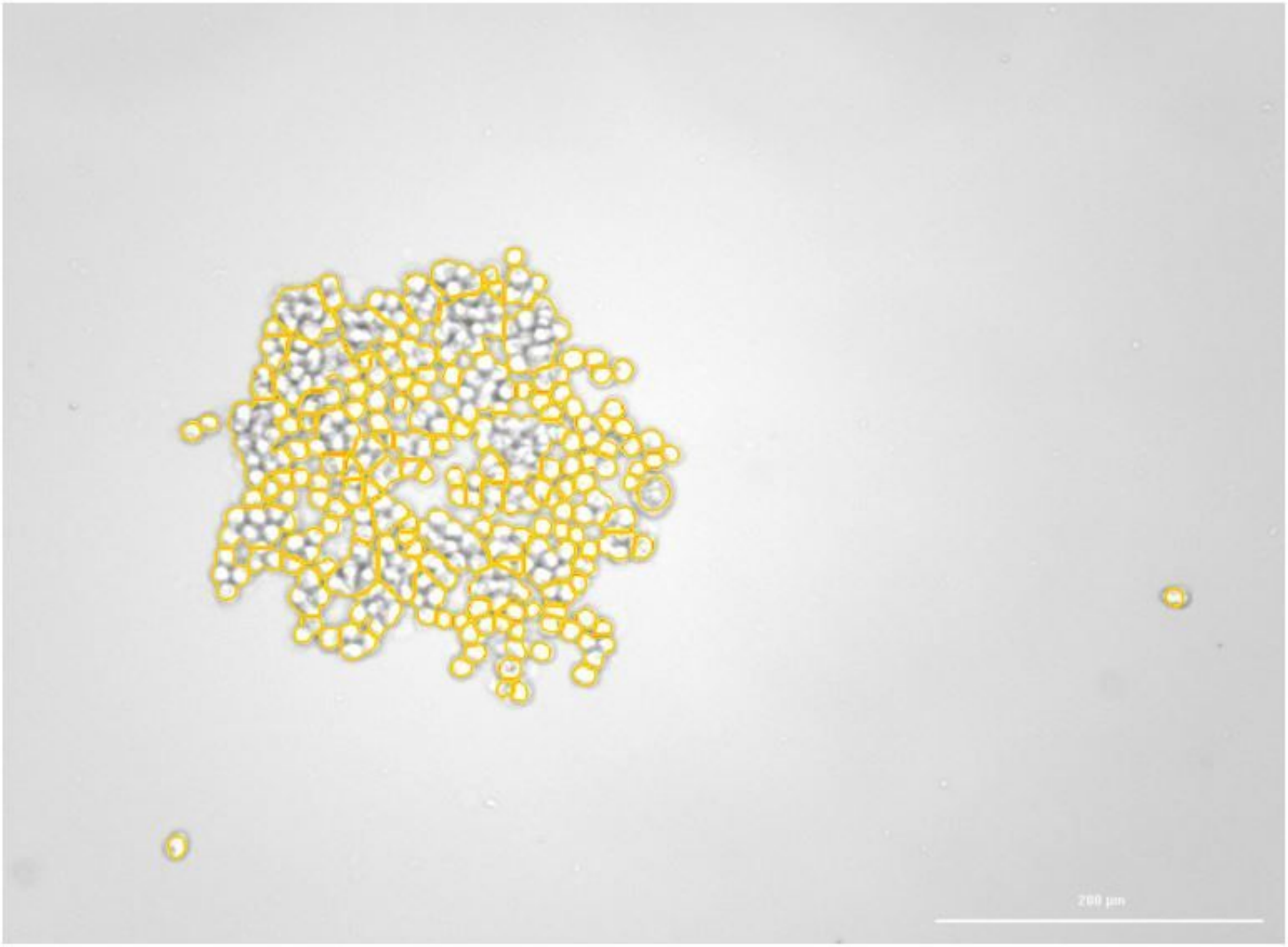
## Figure 3

The thin dark blue boundary is formed between the adenoma and surrounding healthy tissue. Polypectomic loop is inserted. Note the visible progress of diffusion of the colour in the healthy tissue. (Time T+300 seconds after submucosal injection of EMR composition)



## Figure 4

The resection and complete removal of the GI adenoma. The mucosa and light blue submucosa below are visible after resection. (Time+ 330 seconds after submucosal injection of EMR composition)



## Figure 5

Group of starving cells HCT 116 with free-floating cells removed (bright-field image)

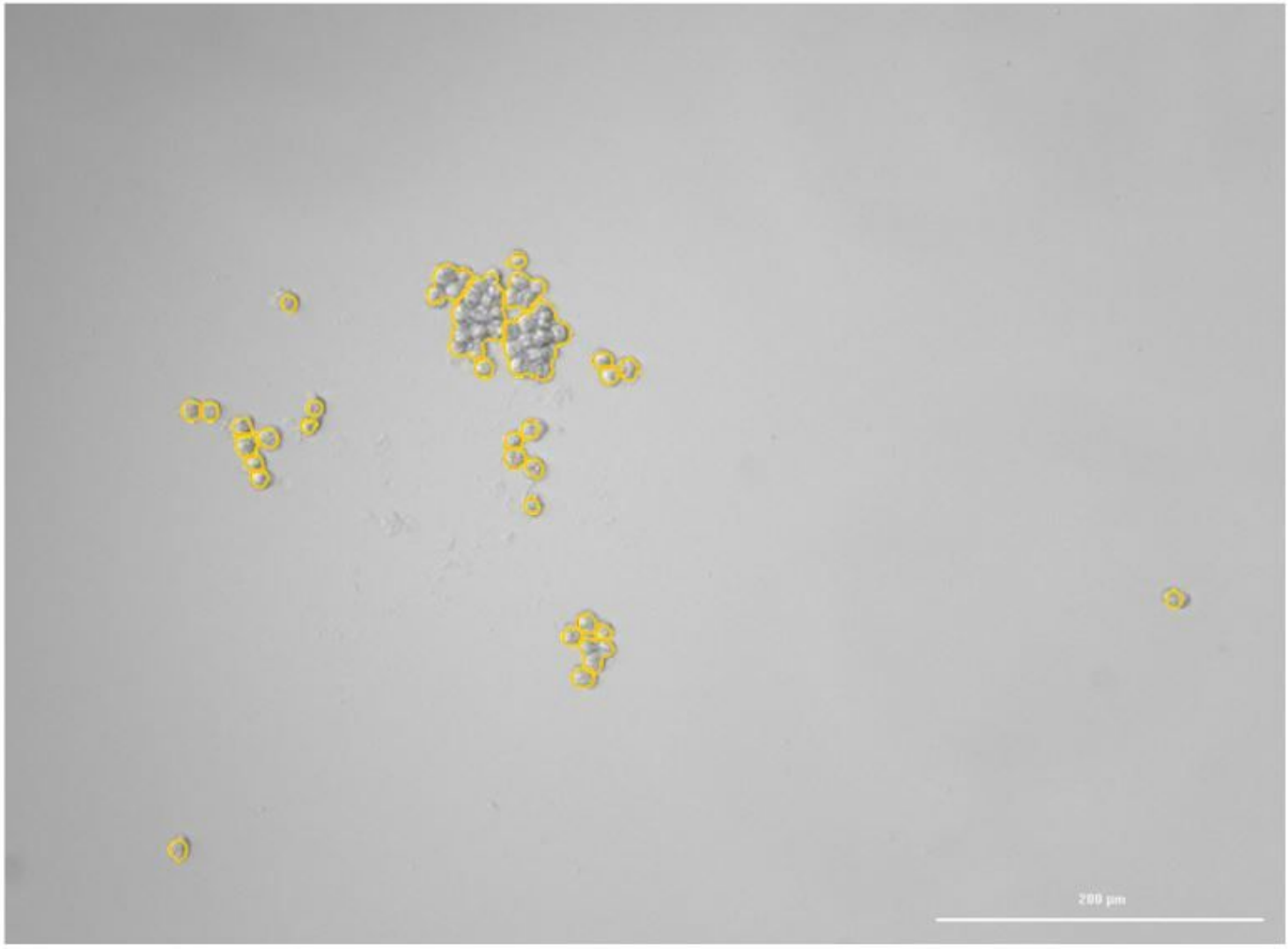



**Figure 6**

The same field of view as in Fig. 5 after the cells were exposed to combination saline solution and HES in a ratio 3:7. After T+30 seconds the cells detach and for most part float away from the field of view (bright field image)