Analysing the Use of Fluorescent Cellular Imaging and Lentiviral Biosensors for Autophagosome Formation

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In the last 15 years analysis of subcellular structure dynamics has been revolutionised by the refinement of genetically-encoded fusions between fluorescent proteins and cellular structural proteins. By using fluorescence microscopy, such fusion proteins incorporate into the structure of interest without disturbing its function, and permit visualisation of the structure in live cells and in real time [1]. Traditionally, the cDNAs encoding the fusion proteins have been delivered into cells by chemical transfection or electroporation. Such transfection procedures have shortcomings, including variable expression levels and low efficiencies for transfecting primary cells.

Lentiviral and adenoviral vectors are recently-developed viral gene delivery vectors which permit transduction of virtually any cell type, at more tightly controlled expression levels. Although viral vectors have been successfully utilised for expressing genetically encoded subcellular markers, prepackaged vectors have not been widely available, leaving researchers to perform packaging procedures themselves.

Here we present the advantages of using prepackaged lentiviral particles, which were developed to encode fluorescent fusion proteins with subcellular markers for cell fate, cytoskeletal structures and adhesion. The lentivirus particles are packaged with technology that produces pseudoviral particles with low probabilities of pathogenicity [2]. The fluorescent proteins employed are TagGFP2 and TagRFP, which have been demonstrated to be monomeric for minimal interference with the function of the fusion partner proteins, and have quantum yields comparable to fluorescent proteins from other species [3,4].

In this article we highlight biosensors to assay for autophagy, in which TagGFP2 and TagRFP are fused to the autophagosome marker LC3. LC3 precursors in the cytosol are proteolytically processed to form LC3-I. Upon initiation of autophagy, the C-terminal glycine is modified by addition of a phosphatidylethanolamine to form LC3-II, which translocates rapidly to nascent autophagosomes in a punctate distribution [5]. DNA constructs encoding fluorescent proteins fused to LC3 are widely introduced into cells for monitoring autophagosome formation. GFP- and RFP-LC3 lentiviral particles provide bright fluorescence and precise localisation of LC3 to the autophagosome to enable live cell analysis of autophagy.

Materials and Methods

Construction of lentiviral vectors encoding fluorescent protein fusions

LentiBrite™ Lentiviral Biosensors (EMD Millipore) were constructed as follows. The cDNAs encoding TagGFP2 and TagRFP were obtained from Evrogen, and the cDNA encoding human LC3A residues 1-120 was cloned. The resulting fusion proteins, TagGFP2-LC3 and TagRFP-LC3, leave the C-terminal glycine (Gly120) of LC3 available for lipidation upon autophagy induction. To generate a control mutant that does not translocate upon autophagy induction, site-directed mutagenesis was employed to mutate LC3 Gly120 to alanine. Constructs were transferred to pCDH-EF1-MCS (System Biosciences Inc.), a lentiviral vector containing the constitutive, moderately expressing EF1a promoter. 3rd generation HIV-based VSV-G pseudotyped lentiviral particles were generated using the pPACKH1 Lentivector Packaging System at System Biosciences.

Cell Seeding and Lentiviral Transduction

Cells in growth medium were seeded onto 8-well glass chamber slides for fixed cell imaging, or chambered cover glasses for live cell imaging. Seeding densities were selected to provide for 50-70% confluency after overnight culture. The day after seeding, medium was replaced with fresh growth medium.

High-titre lentiviral stock was diluted 1:40 with growth medium, and lentiviral volume was added to the seeded cells for the desired multiplicity of infection (MOI), the ratio of the number of infectious lentiviral particles to the number of cells being infected. Infected cells were incubated, lentivirus-containing medium was replaced with fresh growth medium, and cells were cultured again. For autophagy experiments, cells were either left in growth medium, or incubated in Earle's balanced salt solution (EBSS) containing a lysosomal inhibitor.

Live and Fixed Cell Imaging

For live cell visualisation, the chambered cover glass was placed in a temperature-controlled microscope stage insert. Imaging was initiated as rapidly as possible following addition of modulator. LC3-expressing cells were imaged in EBSS containing a lysosomal inhibitor. Live cell imaging was performed upon a Leica DMI6000B inverted wide-field fluorescent microscope.

Cells were fixed with 3.7% formaldehyde in Dulbecco's phosphate-buffered saline (DPBS) and rinsed with fluorescent staining buffer. For immuno-co-localisation studies, primary antibody in fluorescent staining buffer was added. Samples were rinsed with fluorescent staining buffer and incubated with fluorescent secondary antibody and DAPI in staining buffer. Samples were rinsed with fluorescent staining buffer and DPBS, and slides were coverslipped. Mounted specimens were imaged on inverted wide-field or Leica DMI4000B confocal fluorescent microscopes.

Analysis of GFP-LC3 localisation by flow cytometry

The LentiBrite™ Autophagosome Enrichment Kit (EMD Millipore) was employed for analysis of autophagosome formation in primary cells. Human umbilical vein endothelial cells (HUVEC) were incubated with lentivirus encoding TagGFP2-LC3 or TagGFP2-LC3G120A (control mutant) at an MOI of 40. After lentivirus removal, the cells were cultured again. Cells were either left in complete growth medium or incubated in EBSS containing a lysosomal inhibitor, and were detached with Accutase and permeabilised. U2OS cells stably expressing TagGFP2-LC3 were treated in parallel as a positive control. Samples were analysed on a Guava easyCyte™ 8HT flow cytometer. Data were analysed with the InCyte™ Software Module.

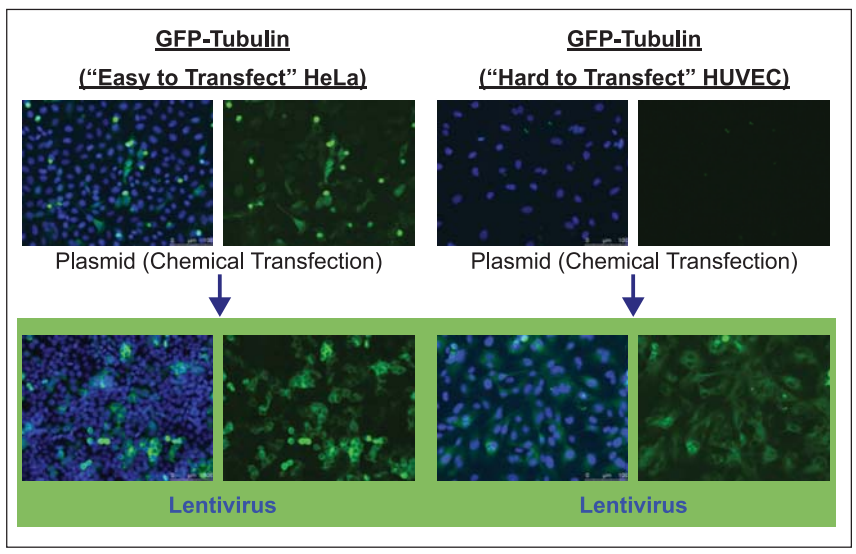

Figure 1. Plasmid vs. lentivirus transfection in easy- and hard-to-transfect cell types. HeLa cells and HUVECs were transfected with a TagGFP2-tubulin-encoding construct, either utilising plasmid DNA in conjunction with a lipid-based chemical transfection reagent, or using LentiBrite™ lentiviral particles. Images were obtained via wide-field fluorescent imaging with a 20X objective lens (blue = DAPI nuclear counterstain, green = GFP-tubulin). Lentiviral transduction resulted in higher transfection efficiency (particularly for HUVEC, for which plasmid transfection was unsuccessful) and GFP-tubulin signal of more uniform fluorescence intensity.

Results and Discussion

Plasmid vs. lentivirus transfection

The study demonstrated the improvement in transfection efficiency and homogeneity achieved by lentiviral transduction. In *Figure 1*, easily transfectable HeLa cells were transfected with GFP-labeled tubulin via plasmid (with a chemical transfection reagent) or lentivirus. Lentivirally-transduced cells demonstrated higher transfection efficiency (percentage of cells positive for signal, compared to total number of cells), as well as more homogeneous expression (compared to the range of high and low expressers in the plasmid-transfected population). For a typically "hard-to-transfect" primary cell type such as HUVECs, lentiviral transduction produced homogeneously bright signal in a significant proportion of cells, in contrast to plasmid transfection.

Specificity of localisation of lentivirally delivered GFP-LC3

Genetically-encoded biosensors for studying autophagy have been widely employed since the use of GFP-tagged LC3 to detect autophagosome formation was described [5]. However, transient transfection of plasmid DNA for GFP-LC3 expression has been criticised for causing artifactual, autophagy-independent punctae, and the preferred approach is to use cell lines stably expressing the FP-LC3 construct [6]. To determine whether lentiviral delivery of DNA encoding fluorescent proteins fused to LC3 avoids such artifacts, we produced lentivirus encoding TagGFP2-LC3 for transduction into a variety of cell types. Lentiviral delivery of fluorescent protein-tagged LC3 allows for accurate detection of autophagosome formation.

Figure 2 depicts matching localisation patterns between genetically-encoded fluorescent proteins and antibody-based immunofluorescence. HeLa cells were lentivirally transduced with GFP-LC3. GFP-LC3-expressing cells were left untreated or subjected to starvation conditions to induce autophagy. Both the fluorescent protein and anti-LC3 antibody displayed diffuse nuclear and cytoplasmic signal under fed conditions, and a punctate distribution following starvation.

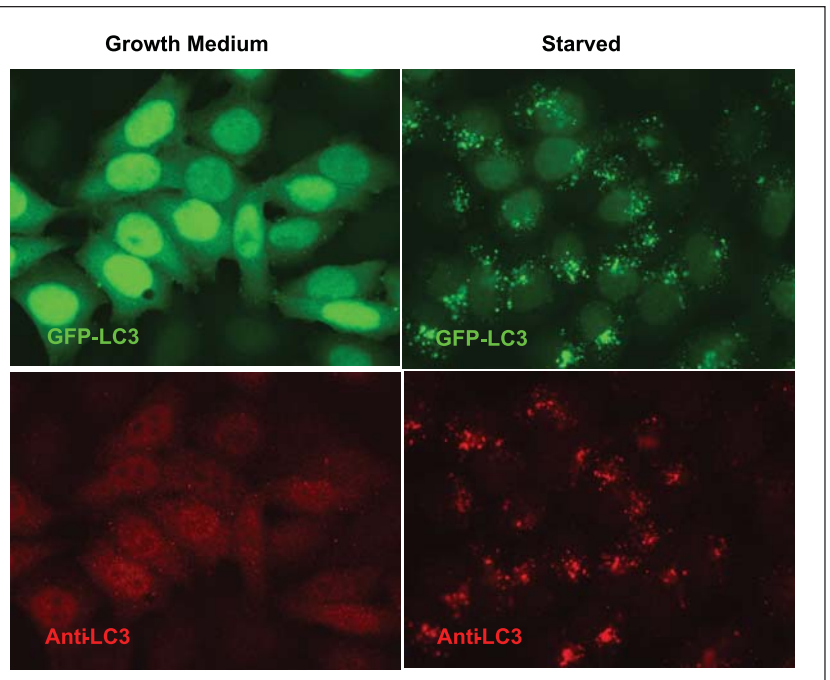

Figure 2. Fluorescent protein expression and co-localisation with fluorescent antibody staining. HeLa cells were transduced with TagGFP2-LC3, and 72 hrs later, either left in growth medium or starved for 4 hours in EBSS with a lysosomal inhibitor. Cells were subsequently fixed, immunostained, and imaged by wide-field microscopy. Starved, autophagic cells displayed punctate cytoplasmic LC3 distribution, in contrast to diffuse nuclear and cytoplasmic localisation under fed conditions. Fluorescently tagged protein co-localised with staining obtained with anti-LC3 antibody.

Still-frame captures of modulator-treated cells at various time-points are provided in *Figure 3*. Autophagy was induced in GFP-LC3-transduced HT-1080 cells via EBSS/lysosomal inhibitor starvation, resulting in accumulation of punctate LC3 in newly formed autophagosomes.

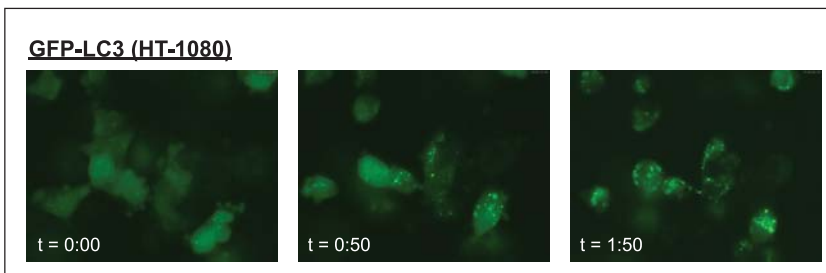

Figure 3. Live cell time-lapse imaging of lentivirally-transduced cells. HT-1080 cells were lentivirally transduced with TagGFP2-LC3, and imaged by oil-immersion wide-field microscopy in real-time. The cells were starved in the presence of a lysosome inhibitor, and imaging was immediately initiated, with images obtained every minute for 2 hours. Still-frame captures demonstrate formation of GFP-LC3-positive discrete cytoplasmic puntae. Full length video available at www.millipore.com/autophagyvideo.

For another assessment of the specificity of the GFP-LC3 biosensor, we employed two controls: a mutant LC3 resistant to lipidation, and an autophagy inhibitor. In *Figure 4*, cells were lentivirally-transduced with TagGFP2-LC3 or a TagGFP2-LC3 non-translocating control mutant. Transduced cells were starved in the presence or absence of 3-methyladenine, an inhibitor of PI3 kinase that blocks autophagosome formation. The mutant LC3 fusion protein did not translocate to a punctate cytoplasmic distribution upon starvation. Also, when starved in the presence of 3-methyladenine, both the wild-type and mutant LC3 fusion proteins displayed the diffuse distribution typical of fed cells.

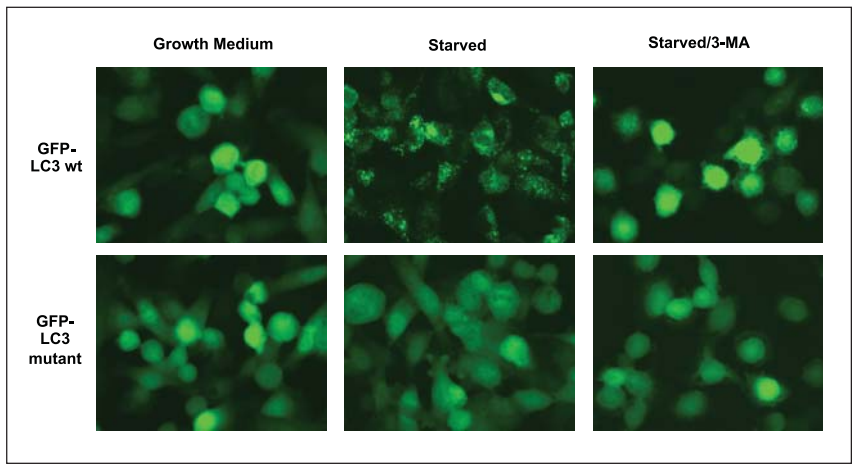

Figure 4. Localisation of GFP-tagged proteins following modulator treatment. HT-1080 cells were lentivirally-transduced with TagGFP2-LC3 wild-type (GFP-LC3 wt) or TagGFP2-LC3G120A (GFP-LC3 mutant) at MOI of 20. Transduced cells were either left in growth medium, or starved in EBSS with lysosome inhibitor in the presence or absence of 3-methyladenine (3-MA). Cells were fixed, mounted, and imaged by wide-field fluorescence microscopy. Cells transduced with wild-type GFP-LC3 no longer exhibited cytoplasmic puntae under starvation conditions in the presence of 3-methyladenine. In addition, cells transduced with a negative control mutant GFP-LC3 maintained diffuse nuclear and cytosolic distrbution under all conditions.

Analysis of autophagy by imaging and flow cytometry

Primary cell types, including HUVECs and human mesenchymal stem cells (HuMSC) are considered difficult-to-transfect for plasmid DNA-based chemical transfection. Lentiviral transduction is shown to efficiently induce fluorescent protein expression in these cells in *Figure 5*. The fluorescent protein fusions in the primary cells expressed diffusely when cultured in growth medium, and adopted a punctate distribution following starvation in the presence of a lysosome inhibitor.

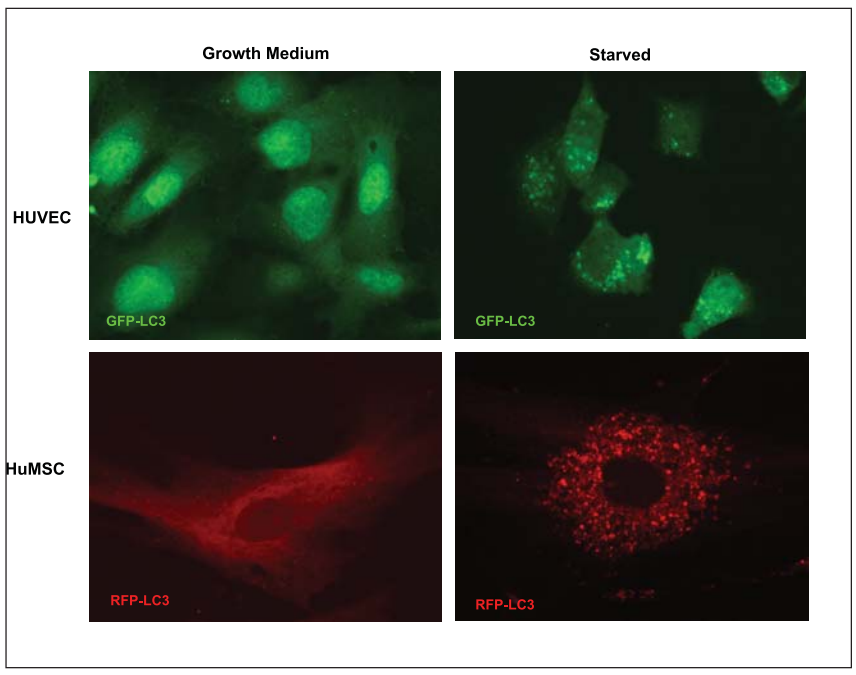

Figure 5. Lentiviral transduction enables analysis of autophagy in hard-to-transfect primary cell types. HUVEC and HuMSC were lentivirally transduced at an MOI of 40 with TagGFP2-LC3 or TagRFP-LC3, and fed or starved as in Figure 2. Cells were then fixed and imaged by wide-field microscopy. The transduced fluorescent proteins displayed diffuse distribution in growth media and a punctate distribution following starvation-induced autophagy.

To more accurately assess the extent of LC3 reporter redistribution in primary cells, we employed a flow cytometry assay in which the plasma membrane is selectively permeabilised such that free cytosolic fluorescent protein-tagged LC3 is released while autophagosome-bound LC3 fusion protein is retained. HUVECs were lentivirally transduced with TagGFP2-LC3 or TagGFP2-LC3G120A (control mutant). The cells were starved of amino acids or left untreated, then detached and either permeabilised or left intact.

Results from the flow cytometric analysis (*Figure 6*) indicate that, upon permeabilisation, the GFP-LC3 in starved cells is almost completely retained, but is greatly depleted in fed cells. This result is similar to the pattern observed in U2OS cells stably expressing TagGFP2-LC3.

In contrast, permeabilisation causes large reduction in TagGFP2-LC3G120A in both starved and fed cells. In U2OS cells transiently transfected with plasmid encoding TagGFP2-LC3, a broad distribution of fluorescence was observed, and the shift upon permeabilisation of fed cells was less pronounced.

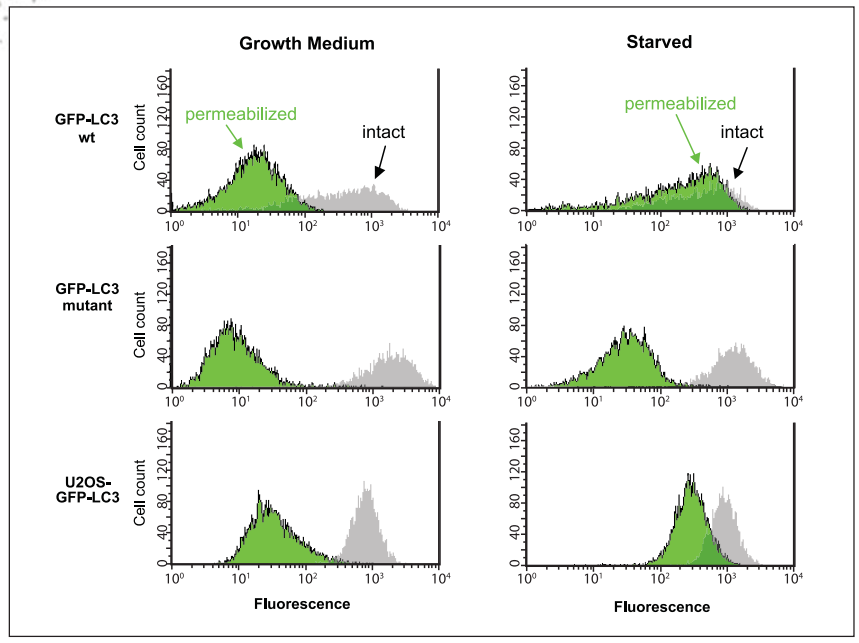

Figure 6. Analysis of GFP-LC3 localisation in HUVEC by flow cytometry. HUVECs were lentivirally transduced with TagGFP2-LC3 wild-type (GFP-LC3 wt, top row) or TagGFP2-LC3G120A control mutant (GFP-LC3 mutant, center row). U2OS cells stably expressing TagGFP2-LC3 wild-type were also analysed (U2OS-GFP-LC3, bottom row). Transduced cells were detached and either permeabilised to release free, cytosolic LC3 (green peaks) or left intact (gray peaks). After processing, the cells were analysed by flow cytometry on a Guava EasyCyte 8HT instrument. Upon permeabilisation, only TagGFP2-LC3 wild-type-expressing cells under starvation conditions display retention of the fusion protein, indicative of tight association of LC3 with autophagosomes.

Conclusion

The data presented here demonstrates the use of a GFP- or RFP-tagged autophagy marker, LC3, in fixed and live cell microscopy applications. The encoded proteins display co-localisation with antibody staining and appropriate redistribution upon treatment with known autophagy modulators. The lentiviral biosensors provide a ready-to-use solution for researchers seeking to fluorescently visualise the presence/absence or trafficking of a protein.

References

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Temperature Controlled Probe Stage used in Conjunction with Raman Microscopy

Market leaders in temperature controlled microscopy, **Linkam Scientific Instruments**, report on the work of Dr Sharath Sriram, a Research Fellow at RMIT University in Melbourne, Australia. He uses Linkam specialist temperature stages with micro-Raman spectroscopy systems to investigate temperature dependent spectral measurements.

Dr Sriram's interests in characterising piezoelectric thin films led him to choose temperature controlled stages from Linkam for use in combination with a Renishaw inVia Raman microscope. The piezoelectric effect is where an applied pressure generates an electrical charge in certain solid materials. This characteristic is useful for the production and detection of sound, generation of high voltages, generation of electron frequencies, and the ultrafine focusing of optical assemblies. This affect also forms the basis of scanning probe microscopy techniques.

The Groups main area of work has been the investigation of the reversal and pinning of Curie point transformation in thin film piezoelectrics. Using The HFS91-PB4 (HFS600) Linkam stage PSZT thin films were heated to 350°C and cooled at 10°C/min in situ with real-time collection of Raman spectra. This enabled the researchers to determine two main Raman peaks for the film at room temperature, ~575 and ~744cm-¹ (at which point the film had a rhombohedral structure). Controlled heating and cooling of the thin film causes peaks and intensity changes at the Curie point. This is indicative of a phase change occurring at the Curie point, where the film changes from a rhombohedral arrangement to a symmetrical cubic arrangement. This phase change coincides with loss of piezoelectric charge and piezoelectrical structure. With controlled cooling the cubic phase reverses back to the rhombohedral phase with minimum hysteresis, and piezoelectrical potential.

This Curie point transformation from cubic to rhombohedral can be disrupted by uncontrolled cooling, which results in locking in place the peak positions and intensities indicating a permanent phase change and the material remaining 'locked' in the cubic phase. This shows fast cooling permanently removes the piezoelectric charge within a material.

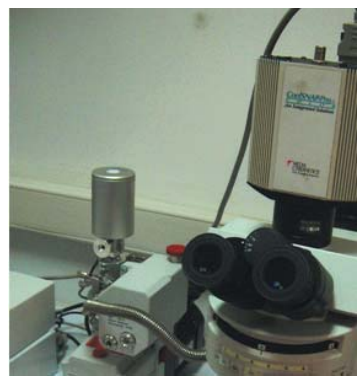
Future research will make use of the probe capability of the stage to apply an electrical bias and study Raman spectrum changes in various piezoelectric thin film samples.

Circle no. 204

Temperature Controlled Stage Used to Examine Atmospheric and Vacuum Frying of Starch Granules in Oil and Water

Linkam Scientific Instruments, have been chosen by Pontificia Universidad Católica de Chile to supply a THMS350V stage to understand the frying of starch in oil and water. At the Pontificia Universidad Católica de Chile, a research team headed by Pedro Bouchon PhD in the Chemical and Bioprocess Engineering Department are using a Linkam THMS350V stage to examine atmospheric and vacuum frying miniaturisation of starch granules in oil and water. The chemical and structural aspects of vacuum frying are still not very well understood and so further experimentation is required to understand the process. The goal of the group is to understand the role of different ingredients when they are processed under different conditions, studying the effect on micro and macro-structural properties, functionality, and the impact that these processed ingredients have on nutrition. The aim is to design and develop, based on scientific knowledge, new food matrices that fit new consumer demands for healthier, low fat snack products that also taste as good as the traditional ones.

The group are comparing the effect of vacuum and atmospheric frying using real-time hot-vacuum-stage microscopy. Isolated starch granules are examined for micro-structural changes during vacuum and heating in both oil and water. The Linkam THMS350V is mounted on an Olympus BX-61 light microscope and the samples are tested at different heating rates with different vacuum levels. Samples are totally immersed before frying, and real-time image capture is used to examine structural changes. Micro-structural changes are indicated by swelling and gelatinisation of the potato starch granules during heating at 15°C/min at atmospheric pressure. Granules start to swell at 64°C. As the temperature increases the granules start to lose their shape and at 100°C the granules begin to dehydrate due to water evaporation. The loss of birefringence of the granules indicates the onset of the gelatinisation process.



Pablo Cortes, a member of Pedro Bouchon's group, described how the Linkam stage has "allowed us to understand the development of the microstructure of potato starch granules in different conditions in real time. We have studied the gelatinisation process of isolated potato starch granules heated in excess water and embedded in a gluten and water matrix. This micro-structural approach has given us information of paramount importance to understand the frying and vacuum frying process." Heating-vacuum microscopy is an essential technique to begin to understand the complex process of vacuum frying which may lead to innovative ways to prepare our food.

Circle no. 205