# RAMAN ANALYTICS TO MONITOR INFLUENCE OF RESVERATROL ON LYMPHOMA CELLS

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**Abstract.** Resveratrol is a natural polyphenol with known antimicrobial, anti-inflammatory and anti-oxidative effects, which is often used as a nutritional supplement. Due to its multi-targeted effect in cells, it is also being investigated as supporting additive for therapy. In combination with general cancer drugs like Etoposide, it could support the effects of chemotherapy and / or lower its side effects. To observe the efficacy of these substances and to enable timely decisions in diagnostics and therapy, the changed metabolism of intact living cells must be monitored by a fast and simple work-flow. Surface-enhanced Raman Spectroscopy (SERS) is a suitable tool to collect information about the molecular composition of cells, especially regarding biomolecules like DNA, proteins or lipids. Multivariate statistics allows to extract this information about the cell status from the Raman spectra to assess the impact of the chemotherapy. Label-free sample preparation for the required enhancement of Raman signals is in SERS achieved through the addition of metallic nanostructures. Application of SERS for the differentiation of chemotherapeutically treated and untreated Hodgkin-Lymphoma (HL) cells was optimized. Various SERS substrates were investigated, an optimized Raman analysis method specific for HL cells was developed. For targeted differentiation and scientifically sound interpretation of the resulting Raman spectra, an automated, open-source data analysis work-flow was developed based on automated spectral selection and qualitative evaluation using PCA-LDA (principal component analysis and linear discriminant analysis). Etoposide/Resveratrol-treated HL cells were differentiated from untreated HL cells with high accuracy using this work-flow. The supporting role of Resveratrol can be further investigated by the successfully established reproducible SERS method and the fast data analysis.

**Keywords:** Resveratrol, cancer chemotherapy, SERS, multivariate statistics

## 1 INTRODUCTION

Resveratrol is a naturally occurring polyphenol found in different plants, including peanuts, various berries and grapes, where it acts as an antioxidant and antimicrobial agent. Red wine especially is known to contain high levels of Resveratrol. It is frequently studied for a potential

application in the treatment and prevention of a wide range of diseases, including cancer. Hodgkin lymphoma (HL) is a type of B-cell derived cancer. HL patients are cured with success rates of 65 – 90 % depending on the cancer status. However, radiation treatment and chemotherapy still carry considerable risks for patients, especially in later stages of the disease. [1] Fast and reliable methods are needed to improve the *in vitro* and *in vivo* cancer diagnostics. Raman spectra acquired by surface-enhanced Raman spectroscopy (SERS) contain information about the chemical constitution of the material. In case of cells the metabolites and constituents (proteins, DNA, lipids) and their modifications caused by the treatment can be detected. With SERS the change of signals (their number and intensity) due to the effects of chemotherapeutic substances can be recorded and interpreted in the biological context, regarding the detected biomolecules and their metabolism. [2]

The aim is the reduction of the sample preparation time, resulting in a simpler and faster work-flow for cancer therapy diagnostics. Employing the potential and know-how of bioinformatics and multivariate statistics we aimed on creating a robust evaluation tool enhancing the fitness of the entire analytical method, detecting hereby the effect of the combined Etoposide/Resveratrol treatment.

### 1.1 COMBINATION OF ETOPOSIDE AND RESVERATROL

Etoposide is a chemotherapeutic medication derived from podophyllotoxin which is used to treat a wide variety of cancers, including testicular cancer, lung cancer, lymphoma and leukemia. Its anti-cancer activity predominantly causes DNA damage and leads to apoptosis. This is based on the inhibition of the enzyme topoisomerase II, which is responsible for relaxing supercoiled DNA strands by introducing and re-ligating double strand breaks. Specifically, Etoposide inhibits the re-ligation of the broken DNA strand. Since cancer cells divide more rapidly and therefore replicate their DNA more often, this effect predominantly causes severe DNA damage which drives cancer cells into apoptosis. Growing area of interest for Resveratrol is its application as a synergistic agent in combination with general cancer therapeutics like Etoposide. On cellular and molecular level Resveratrol has an inhibiting impact on carcinogenesis in multiple targeting way, activating the expression of tumor suppressor proteins, and in high dosage, supporting the apoptotic pathways. [3]

### 1.2 SURFACE-ENHANCED RAMAN SPECTROSCOPY (SERS)

SERS is a powerful tool to investigate living intact cell cultures. The basic Raman micro-spectroscopy setup includes a monochromatic laser source, that irradiates the sample and excites local molecular vibrations. The bands in the spectra can be assigned to specific biomolecules and provide information about the general condition of the cell. Differences in the chemical composition of the observed sample can be collected within minutes. In Figure 1

the scheme of the SERS analysis is depicted. The main goal is bringing the sample in close contact with signal amplifying nanostructured materials to obtain reproducible results.

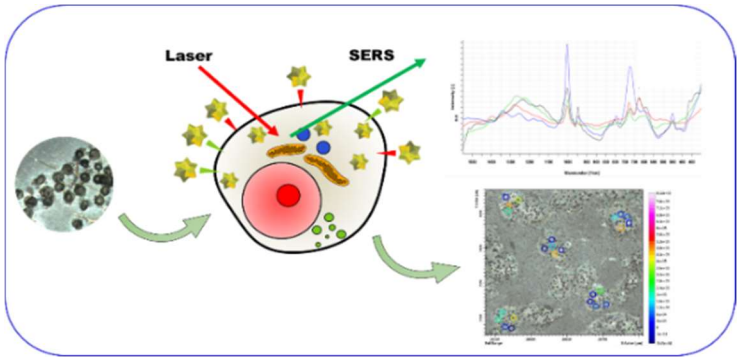


*Figure 1: Scheme of SERS Analysis.*

The signal enhancing colloidal metallic nanoparticles (NPs) can be prepared in various shapes (stars, pyramids, shares), materials (silver or gold) and a wide range of size (10 to 100 nm). This variety is achieved by application of several reducing agents (e.g. ethanolamine, citrate, sucrose) and a salt of the desired metal. Also decisive for the preparation is the ratio of the reagents used.

## 2 METHODS

The HL cells were cultivated according to established protocol, the treatment of the cells with Resveratrol and/or Etoposide was performed according chapter 2.3. Two crucial steps for reproducible SERS analytics were optimized: (A) the preparation of the colloidal metal NPs and their application on cells and (B) the development of data analysis work-flow to efficiently extract and use the complex information contained in Raman spectra.

### 2.1 SERS OPTIMIZED PARAMETERS

The optimized conditions were applied to determine the effect of Resveratrol as additive to a chemotherapeutic treatment with Etoposide on HL cells and to explore the resulting biochemical changes in the cells. The most important SERS parameters are listed in Table 1.

*Table 1: Final method used for the SERS analysis of Hodgkin Lymphoma cells.*

| Parameter | Optimized Value |
|---|---|
| **Nanoparticles** | Gold / Ascorbic acid & Sucrose reduced |
| **Laser Wavelength** | 785 nm |
| **Resolution** | 3-5 $cm^{-1}$ |
| **Integration time** | 4 s |
| **Co-additions** | 20 |

The 785 nm laser offers energy high enough to excite the vibrations, without destroying the cell material. Using a spectral selection tool [4] the quality of the sucrose reduced Gold-NP preparation was assessed as optimal based on the reproducibility of the measurement regarding the number of signals and their intensity [5].

### 2.2 DATA ANALYSIS WORK-FLOW

For data analysis two tools for a reliable, easy to use and fast unbiased evaluation of Raman-Spectra are available on Github [4]. The spectral selection tool mentioned above provides methods for baseline correction and different options for quality scoring aiming for the objective selection of spectra with highest quality possible. The second, qualitative evaluation tool includes visualization of Raman spectra, data pre-treatment and options for statistical predictive model building (based on PCA and/or on LDA). It allows an optimization for the selected data set, and interpretation of the results according to the biological importance. [5]

### 2.3 CHEMOTHERAPEUTIC TREATMENT OF HL CELLS

The effect of the chemotherapeutic Etoposide with and without Resveratrol on HL cells was determined in duplicates. HL cells (at $5x10^5$ cells/mL) were cultivated for 72h in 6 wells (scheme in Figure 2). Two of them were left untreated (C), two were treated with 5 µmol/L Resveratrol and in the last 24h of the cultivation 25 µg/mL Etoposide was added (RE). Two portions of the cells were treated with Etoposide only (E).

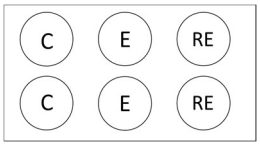


*Figure 2: Scheme of cell cultivation (C: Control; E: Etoposide; RE: Etoposide/Resveratrol)*

## 3 EVALUATION

### 3.1 EFFECTS OF RESVERATROL-ETOPOSIDE COMBINATION TREATMENT

Using the prepared colloidal gold NPs for SERS in combination with the qualitative evaluation tool, it was possible to differentiate between untreated and Etoposide-treated cells as well as between untreated and Resveratrol/Etoposide treated cells. In Figure 3 a-c the calculated LDA scores are visualized using boxplots where one dot corresponds to one measured spectrum. To assess the quality of the differentiation between the untreated control group (C) and the treated groups (E and RE) the accuracy of the predictive statistical PCA-LDA model was quoted. The untreated cells are significantly separated from both E and RE treated cells (values above 80% in accuracy). For differentiation between E and RE group, all calculated values are only slightly above 50 %, which indicates no differences detected here.

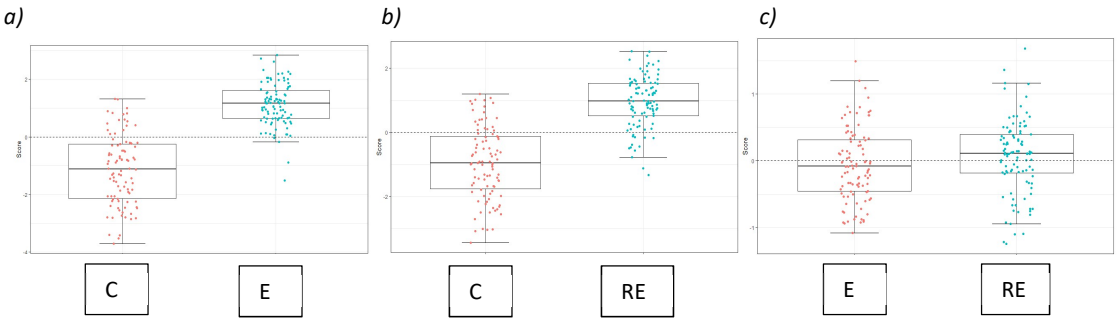


*Figure 3: LDA scores of the differentiation between the groups are plotted. a) C vs. E; b) C vs. RE; c) E vs. RE.*

## 4 CONCLUSION

The optimized SERS method clearly demonstrated an effect of etoposide on the cellular level of HL cells. The addition of the nutritional supplement Resveratrol to the Etoposide treated cells delivered the same result. Because no difference could be observed between the treatment with or without Resveratrol, the impact of the supplement itself is not detectable in this experimental set up yet. For future research the SERS spectra acquisition is fast and reproducible and the unbiased data analysis evaluation tool can be applied. The spectral changes will be assessed to specific signals of biomarker molecules and metabolites (nucleotides, amino acids, lipids, carotenoids, etc.), and varying the Resveratrol amount in biological replicates will help to investigate its effect on cell status.

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