

Formaldehyde Measurement in Biological Samples

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Formaldehyde (FA) is the simplest aldehyde present both in the environment and in living organisms. FA is an extremely reactive compound capable of protein crosslinking and DNA damage. However, FA is a product of normal cellular metabolism, and it plays an important role in many biochemical processes. Different methods initially used for non-biological objects have been adapted for biological samples. In addition, numerous approaches, including chemically-synthesized probes and genetically encoded FA-sensors for *in cellulo* and *in vivo* FA monitoring, were developed.

formaldehyde

aldehyde derivatization

fluorescent probes

aza-Cope rearrangement

formaldehyde sensor

1. Introduction

Formaldehyde (formula HCHO, abbreviated FA), is a simple, naturally occurring organic compound, is one of the first-row aldehydes and is the most poisonous of them [1,2]. It is a well-known fact that FA is a strong electrophile capable of reacting with various biological nucleophiles such as nucleic acids [3], and nucleophilic amino acids [4], thereby affecting the most important functions of living organisms. Nevertheless, FA is a normal component of the human organism itself, as physiological levels of FA are detected in exhaled air and blood. That fact became obvious with the development of methods by which FA could be assessed in biological samples [5]. Formaldehyde can enter the human body from outside—with polluted air or water, tobacco smoke [6,7], electronic cigarette vapour [8] and consumed food [9,10,11,12,13]. Moreover, there are numerous sources of endogenous FA as it is a (by-)product of different biochemical reactions in the organism performed by various enzyme systems, e.g., oxidative demethylation enzymes [14], semicarbazide-sensitive amine oxidases (SSAOs) [15,16,17], serine hydroxymethyltransferase [18], dimethylglycine dehydrogenase [19], lipid oxidation enzymes [20], P450 oxidase [21] and N-methyl group demethylase [22,23] (**Figure 1**). One of the main sources of FA in the human organism is exogenous and endogenous methanol that is oxidized by alcohol dehydrogenases, catalase and cytochrome P450 to FA [14]. Despite the continuous incoming flow of FA in the human organism in normal conditions, it is equal to the outgoing flow—FA concentration is maintained at low levels by different mechanisms of controlling its metabolism and clearance [14].

Formaldehyde formation

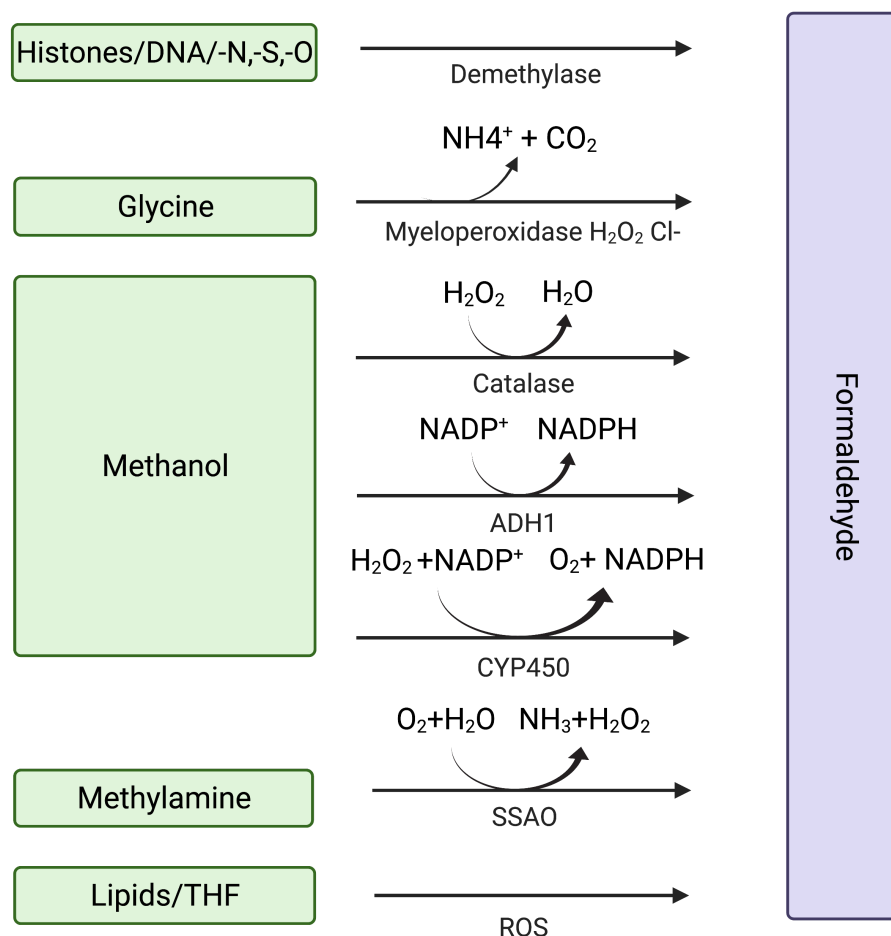


Figure 1. Sources of endogenous FA. Created with BioRender.com.

Nevertheless, in case of pathological conditions and malfunction of FA clearance systems, the increased levels of endogenous FA could lead to destructive and harmful processes. It plays a huge role in the origin and life of a cancer cell [25,26,27]. Also, fluctuations of endogenous FA concentration are characteristic of the numerous pathological conditions associated with cognitive impairment [30].

FA could be assumed to be not only a by-product of endogenous biochemical processes but to also have some important functions. This assumption has risen into the hypothesis that human endogenous FA can act as a potential anticancer metabolite [27]. Moreover, endogenous FA was demonstrated to be a regulatory molecule that can help memory formation [31][32,33].

The potential danger from exogenous FA was first discovered at the end of 19th–beginning of 20th century [34]. The first approach allowing FA concentration assessment was developed in the 1950s with the birth of gas chromatography. Methods for measuring FA were directed for its detection in waste water as well as in food and beverages [35], air and various types of alcoholic products [36]. For a long time, the scientific community did not

consider the existence and significance of endogenous FA. But recent advances in molecular biology, biochemistry and physiology have revealed its importance indicating that FA can serve as a molecular marker for many normal and pathological biochemical processes in a living organism. It became necessary to optimize the existing FA detection methods to make them applicable for biological samples. According to that demand, numerous novel techniques were developed for FA content assessment *in vitro*, *in cellulo* and *in vivo*.

A variety of approaches for FA measurement and monitoring that can be divided in to three main groups. The first group of methods includes gas and liquid chromatography and provide high sensitivity, selectivity, and reproducibility of measurements. However, these techniques are hardly applicable for FA assessment in real-time mode and in living cells and organisms. The second group comprises methods based on spectrophotometry detection of substances obtained in a reaction with FA. Some of these approaches could only be utilized for FA measurement in liquid samples (biological fluids, lysates, extracts etc.); but the others (mainly based on aza-Cope group rearrangement) could also be applied to monitor FA in cells and even living organisms. The third group includes genetically-encoded sensors that were developed for living cells and organisms. However, the variety of methods for FA assessment is not limited to those mentioned above: there are numerous other approaches for analysis of air samples using graphene- and metal oxide-based nanomaterial gas sensors [37,38,39,40,41], optical FA-sensors [42] and others that are out of the scope of this review.

The approaches that are used for FA measurement in liquid biological samples as well as in cells and organisms are summarized in the flowchart below (Figure 2).

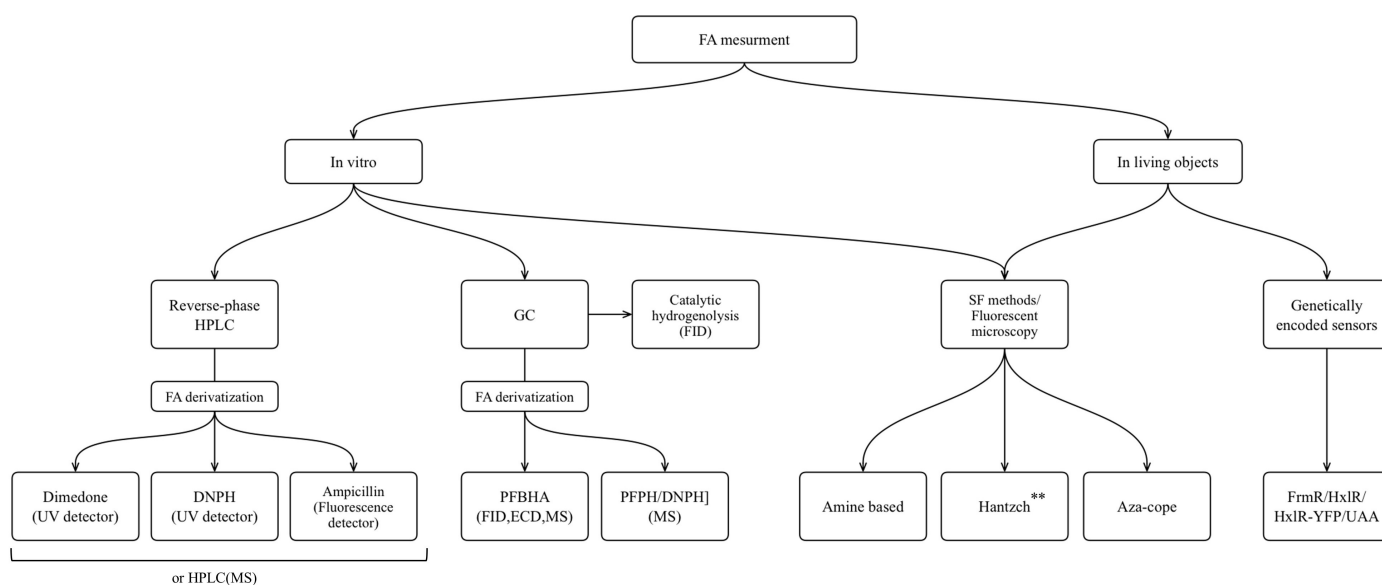


Figure 2. The approaches for FA measurement applicable for different biological samples: HPLC, high performance liquid chromatography; GC, gas chromatography; MS, mass spectrometry; FID, flame ionization detector; ECD, electron-capture detector; SF, spectrophotometry. **Only Hantzsch polymers that do not show cytotoxicity could be used for living objects.

2. In Vitro FA Measurement

The first objects in which it is necessary to measure the levels of endogenous FA are various biological fluids, such as blood, saliva, and urine as well as culture medium. Cell lysates and tissue extracts could also be assigned to this group of samples. As most of these samples contain proteins, the initial step of the sample preparation for chromatography is deproteinization that is usually performed by protein precipitation using trichloroacetic acid or acetonitrile. Measurement of FA in liquid samples does not need the invention of completely new methods, but rather, requires the use of known methods that are modified to achieve the best results.

2.1. Gas Chromatography

In early 1952, Martin and James carried out a variant of gas distribution chromatography (GC). Since that time, gas chromatography became one of the fastest growing methods in analytical chemistry. GC has many advantages over other measurement methods. Due to its high separation power, GC has found wide application in the food industry [43,44] and medicine [45,46]. No other method allows analysis of water samples or multicomponent liquid systems with hundreds of components in such a short time. Comparative simplicity of equipment management and ease of use are among the most important properties of gas chromatography. Gas chromatographs are relatively affordable, reliable, and allow an automated analysis process. High sensitivity of the chromatograph makes it possible to determine small amounts of organic compounds with great accuracy. Today, gas chromatography can reliably determine concentrations of 10^{-8} – 10^{-9} mg/mL [47]. The obtained data may be widened when GC is combined with various instrumental methods such as mass spectrometry (MS) and Fourier-transform infrared spectroscopy. GC–MS has become one of the most successful combined methods. By superposition of GC and MS, we get a tool combining the ability of GC to separate closely related molecules with the ability of MS to use data to identify and quantify separated substances. However, GC has its drawbacks, which can sometimes become critical during analysis. With GC, it is impossible to separate and analyze mixtures of non-volatile compounds, thermally unstable compounds or compounds dissociating in the analyzed solutions.

2.1.1. FA Derivatives' Detection with GC

How can researchers use the GC method to detect such a small metabolite as FA with a mass of only 30.026 g mol⁻¹? It is complicated and troublesome to catch this molecule without using special protocols of sample preparation. One of the common substances used for FA derivatization is O-(2,3,4,5,6-pentafluorobenzyl) hydroxylamine (PFBHA, CAS Registry No. 57981-02-9) or pentafluorophenylhydrazine (PFPH).

2.1.2. GC with Catalytic Hydrogenolysis Coupled with a Flame Ionization Detector (FID)

Although the GC analysis of FA derivatives detected with FID/ECD and especially MS described above provides high accuracy, it is costly and time-consuming. Jim Luong and colleagues developed a new GC technique: using a capillary column by which FA, acetaldehyde, and other components were separated from a gas-phase matrix, then the aldehydes were converted into methane and ethane, respectively, using a nickel-coated catalyst in a hydrogen atmosphere. The methane and ethane were then detected using an FID [52]. Further, the catalyst-based method

was upgraded and catalytic hydrogenolysis was performed directly in a 3D-printed FID jet consisting of an FID combined with a catalyst support for hydrogenolysis. The sensitivity of this approach reaches ppm level in the range of 0.5–300 ppm [53]. The main advantages of this method are the absence of the need for preliminary preparation, derivatization, and concentration of the sample. This method can be used safely for the FA measurement in biological fluids after proper separation of the aqueous phase (precipitation of proteins, centrifugation).

2.2. Liquid Chromatography

Liquid chromatography (LC) is a method for separation and analysis of complex mixtures of substances in which the mobile phase is liquid [54]. The LC method is applicable for the separation of a much wider range of substances than GC, since most of the substances are not volatile and are unstable at high temperatures. LC separation is usually performed at room temperature. High performance LC (HPLC) has long been used to work with systems of biological origin, but FA cannot be measured directly by LC due to the chemical and physical properties of FA. Moreover, the concentrations of endogenous FA in biological samples are very low. Thus, it is necessary to obtain more stable and less reactive derivatives as the products of irreversible reactions with FA. These FA derivatives could be analyzed using HPLC combined with different detectors (usually UV) or MS. The most widely used substance for FA derivatization is 2,4-dinitrophenylhydrazine (DNPH) [55]. DNPH–HPLC can be applied to virtually any non-biological sample including water [57,58], drug substance [59], food [60], leather [61], etc. This method also allows the measurement of FA in biological samples like tissue homogenates [62], urine [63], blood and serum [64,65,66]. Despite the wide use of this approach, it still requires elaborate sample preparation and special equipment, which is not always accessible. There are other substances for FA derivatization, for example, ampicillin (D-(2)-a-aminobenzylpenicillin) and dimedone [68].

To conclude, HPLC and GC are precise, selective and well-established methods for the detection of FA in various samples, including biological. However, they are only suitable for in vitro detection. Moreover, additional steps of sample preparation, including deproteinization and derivatization, are necessary. Therefore, if all these criteria are applicable to the particular research task and the corresponding equipment as well as a qualified operator is available, then chromatography represents one of the best choices for FA assessment.

3. From In Vitro to In Cellulo

3.1. Chromogenic or Fluorogenic FA Chemosensors

There is a group of methods used for FA concentration assessment based on the measurement of absorbency or fluorescence of the products obtained in the FA reactions with particular substances. Spectrophotometry allows measurement of chromogenic and fluorogenic substances, obtained as a result of such reactions. Most of these protocols are used for in vitro FA quantification as very convenient methods because they allow analysis of multiple samples in automatic mode (for example, microplate format); and only common devices found in molecular biology laboratories are needed including spectrophotometer, rather than specialist equipment such as HPLC or GC

devices. As data on the role and function of endogenous FA in living systems accumulated, and more and more information appeared around the participation of this molecule in various processes associated with diseases, a need arose for relatively simple and widely available methods for measuring FA in biological samples in vivo in real-time conditions. The benefit afforded by the high sensitivity of the GC and HPLC methods is largely offset by the need for expensive specialist equipment and a qualified operator. Moreover, such approaches cannot be exploited for in vivo FA monitoring. By contrast, fluorescent microscopy and special FA-sensing probes allow monitoring of FA levels in cell cultures and even animals. The main criteria for the substance to be suitable for FA monitoring in living systems are low toxicity, stability, cell-penetration ability, and selectivity against other aldehydes that are present in the cell. For in vivo monitoring, a near-infrared excitation/emission profile is also preferable. Below, the application of FA-sensors for in vitro application, and adjustment of these molecules or development of completely new ones to make them applicable for in vivo context are discussed.

3.1.1. β -Diketone Esters and Hantzsch Reaction

This reaction was known back in 1881 thanks to Arthur Rudolf Hantzsch [80]. Subsequently, this reaction found its use for measuring FA in biological samples in 1953, when Nash modified the reaction and used acetylacetone [69]. This allowed him to measure the FA content in suspensions of living bacteria for the first time. Since then, this colorimetric reaction could be carried out under milder conditions. The acetylacetone-based protocol is now mainly used for FA detection in non-biological samples such as textiles [81], cosmetics [82], beverages [83] etc. Nevertheless, a method is suitable for biological in vitro samples: FA content was assessed in rat hepatocyte lysates [84], human erythrocyte lysates [85], hemolysate [86] and mushroom extracts [87]. Moreover, a commercial kit for different biological samples was developed (Hach Company).

Nowadays, this reaction of β -diketone ester, an aldehyde and ammonia or an alkylamine is actively used for the spectrofluorometric determination of a wide range of non-fluorescent substances which contain a primary amino group, as well as for FA detection. The most common reagents used for FA assessment based on this reaction are: acetoacetanilide (AAA) [88,89] and Fluoral-P (4-amino-3-penten-2-one) [90].

3.1.2. Fluorescent FA-Sensors Based on Formimine, Hydrazine and 2-Aza-Cope Reactions

Recently excellent reviews by Xu et al. [91], Manna et al. [79] and Pan et al. [92] summarized advances on fluorescent probes and sensors, which were developed in the last decade and can be used to monitor FA in a variety of objects both in vitro and in vivo. The main reactions which are the basis for numerous FA-sensing probes for in vivo application are: formimine, hydrazine and 2-aza-Cope reactions. In general, these numerous probes contain a fluorophore moiety (BODIPY, 1,8-naphthalimide, rhodamine, tetraphenylethene, etc) functionalized with FA-reactive groups.

2-aza-Cope chemistry requires special attention, as this approach can be used to quantitatively measure FA in living objects [93]. The approach based on this rearrangement gave a basis for multiple tools for a qualitative and quantitative measurement of FA, which are not affected by the illumination of the sample and the change in the localization of the chromophore in living objects [95]. By now, more than 30 probes have been successfully tested

as FA-sensors in living systems [93]. It was shown that 2-aza-Cope rearrangement-based probes are a universal tool and can be applied to a wide variety of imaging platforms, such as multicolor and ratiometric fluorescence [96], positron emission tomography detection [97] and chemiluminescence [95]. This allows for real-time monitoring of endogenous FA levels in mammalian cells and in vivo in laboratory animals [93,97].

With the use of unique structures capable of entering 2-aza-Cope regrouping and the high reactivity of these analytes to FA, it is possible to work with a large number of different model organisms.

Further development of 2-aza-Cope reactions may in the future offer new approaches to the study of the role of FA in biological systems and even in humans.

4. Genetically-Encoded FA Biosensors

Recently, novel approaches based on the genetically encoded biosensors were developed. The main advantages of such sensors are: FA levels assessment in vivo; the convenient methods of detection based on the fluorescence measurement; and no dependence on expensive equipment. The invention of the first genetically-encoded FA biosensor was based on the study of pathways for FA detoxification in bacteria. To-date, only few genetically-encoded FA biosensors applicable for bacterial cells have been developed [98,99,100], and until recently there was no biosensor adapted for eukaryotic cells. However, based on the properties of the *Bacillus subtilis* HxIR transcription factor sensitive to FA [98,101], Zhu and colleagues developed a FA biosensor (FAsor) applicable for mammalian cells [102]. The following systems are in the basis of this sensor: (1) circularly permuted fluorescence proteins (cpFPs) the ability of which to emit fluorescence is highly sensitive and strongly depends on their conformation; and (2) the ability of HxIR to undergo conformational changes induced by FA [102]. By fusing cpYFP with two HxIR subunits, they obtained a sensor HxIR-cpYFP-HxIR designated FAsor that can translate FA-induced conformational changes into a change in fluorescence signal (**Figure 3**).

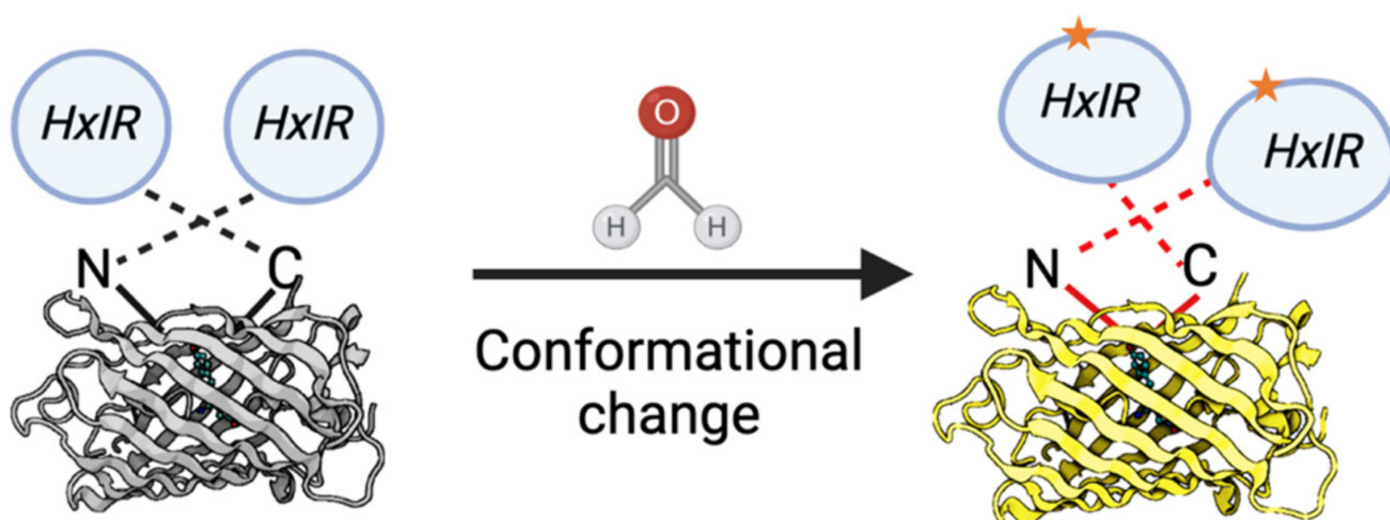


Figure 3. Schematic representation of the FA-sensor FAsor that contains cpYFP flanked with two copies of HxIR. FA induces “N-terminal helix-flipping” in each HxIR unit linking Cys11 and Lys13 residues on the HxIR α 1 helix

(crosslinked residues are marked with an asterisk). This event leads to a major conformational change in cpYFP module resulting in an increase in fluorescence.

5. Conclusions

FA is one of the products of cellular metabolism, and monitoring of FA levels can give the information about physiological and pathological changes in living systems. For detection of FA levels, characteristics of biological objects require the use of methods with high sensitivity, since endogenous FA concentrations and their fluctuations are in a micromolar range. In addition, these methods must meet several criteria: selectivity, reproducibility and resistance to interfering substances that may present in biological samples. Often, the amount of biological samples is limited, so this parameter should also be taken into account. The most precise and selective tools for the assessment of FA concentrations in liquid biological samples are GC–MS and HPLC–MS. However, their main disadvantages are the elaborate equipment required, and the complexity of the procedure and data interpretation. The alternative to chromatography is to use spectrophotometry-based methods. They are sensitive enough for detection of endogenous levels of FA, selective, easy to operate, suitable for microplate format and commercially available.

When it comes to measuring FA in living systems, additional requirements are added: low or negligible cytotoxicity and absence of side-effects on biological processes in cells are the essential features of such probes for monitoring FA in experimental animal organisms. Moreover, the probe emission should be close to the near-infrared range. The substance used for FA detection must be stable in the culture medium, inside the cell, and in the organism. Most probes for in vivo FA monitoring work on the basis of the aza-Cope rearrangement mechanism. The fundamental challenges in the innovative field of in vivo FA measurement relate to the lack of control over the concentration of the probes in a living organism due to physiological and biochemical processes. It is therefore necessary to assess the potential generalized effects of each novel probe and its “behavior” in a living system as this could complicate its usage in the research of the FA role in the organism. Currently, only short-time in vivo FA monitoring can be performed in a living organism because of the decrease of the probe concentration and its half-life.

Progress in the development of new probes for FA measurement in living systems promise to result in obtaining a new universal sensor, devoid of these drawbacks and meeting the required criteria in the near future. However, most of FA-sensing molecules developed for living systems are substances synthesized in-house and so are not available for wide use which impedes the application of this valuable tool for research.

Genetically-encoded FA-sensors have great potential as they are sensitive enough for endogenous FA level detection, could be targeted to different cellular compartments, have no cytotoxicity and do not require addition of chemical compounds to the cultured cells. However, one of the drawbacks is their sensitivity to methylglyoxal. Moreover, in vivo application of such sensors is not available yet. Thus, future efforts in upgrading of genetically-encoded sensors should include adjusted selectivity to FA and suitability for in vivo application.

A new generation of FA-sensing tools suitable for living objects is expected to be developed to meet the requirements mentioned above. The commercialization of novel probes thus making them available to the research community at large would greatly enhance research potential. researchers believe further progress in development of probes for FA monitoring in living objects could lead to breakthrough results and discoveries in the field of biochemistry of pathological conditions characterized by elevated levels of FA such as cancer and neurodegenerative disorders, providing new approaches for diagnostic and treatment.

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